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THE USE OF C_8-OCTYL COLUMNS FOR THE ANALYSIS OF CATECHOLAMINES BY ION-PAIR REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

The chromatographic behavior of norepinephrine (NE), epinephrine, dopamine, 3,4-dihydroxyphenylalanine, and 3,4-dihydroxyphenylacetic acid on octylsilane (C_8) reversed-phase high-performance liquid chromatography columns was observed under various mobile phase conditions including manipulations of pH, pairing ion and methanol concentrations. The optimum isocratic conditions permitting quantitative resolution of these substances in minimum time and with maximum detector response were determined. Employing a pH 3.0—3.2 mobile phase comprising an aqueous buffer solution containing 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, and 0.2 m*M* 1-octanesulfonate, admixed with a volume of methanol equal to 4% of the aqueous volume, the performance of the C_8 columns compares favorably to that of the more widely used C_{18} columns. The column eluates were monitored with an amperometric detector utilizing a glassy-carbon flow-cell electrode. The detector response for NE was 1.5—2.0 nA/ng and the baseline noise was as little as 0.002 nA thereby permitting quantitation of 5-pg levels or more in the injected samples. By coupling the liquid chromatographic system to a procedure which eliminates non-catechol contaminants from the neuronal and body fluid specimens by alumina adsorption of the catechols, a sensitive and dependable method was developed and employed for the determination of catechol levels in discrete regions of rat brain, cat spinal cord, and in human plasma.

INTRODUCTION

Ion-exchange [1—6] and reversed-phase [6—12] high-performance liquid chromatographic (HPLC) techniques have been used successfully to resolve the catecholamines and their related metabolites in extracts of tissues and in body fluids. Reversed-phase chromatography appears to be most useful in the analysis of mixtures of such substances which possess both acidic and basic organic functional groups [6]. The most widely used reversed-phase methods

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employ isocratic conditions with the mobile phase flowing over a bonded octadecylsilane solid phase such as found in the μBondapak C_{18} and the Li-Chrosorb RP-18 columns [8—12]. The selectivity and the resolution of these columns are enhanced by the use of a pairing-ion species such as 1-octanesulfonate (OSA) [12,13] and by the admixture of an organic modifier such as methanol in the mobile phase [12]. In such separations, an acidic pH proves optimal and modest pH adjustments produce significant changes in the retention of acidic substances [6,12].

One of the most significant advances in quantitation techniques available in liquid chromatography has been the development of the amperometric detector capable of monitoring column eluates and of detecting picogram quantities of oxidizable or reducible products [14]. Until recently, most amperometric detectors have utilized the carbon-paste flow-cell electrode which requires frequent standardization and repacking and whose performance deteriorates in the presence of admixed organic solvents [12].

Improvements in column and in detector technologies have produced high-performance bonded octylsilane (C_8) and octadecylsilane (C_{18}) solid phases which yield enhanced resolution, efficiency, and stability, and sensitive, low-maintenance, and durable glassy-carbon amperometric electrodes. We have utilized these components in the development of a sensitive and dependable method to determine the basal levels of norepinephrine (NE), epinephrine (EPI), dopamine (DA), 3,4-dihydroxyphenylalanine (DOPA), and 3,4-dihydroxyphenylacetic acid (DOPAC) in selected tissues and body fluids.

The optimum mobile phase conditions giving baseline resolution and maximum detector sensitivity with minimum processing time are described for the C_8 column (Ultrasphere-Octyl) and compared to conditions which yield similar results in the C_{18} column (Ultrasphere-I.P.). These parameters were determined from the systematic study of a range of mobile phase conditions which varied with respect to pairing-ion concentration, pH, and methanol admixture. Tissue samples and body fluids are prepared by a purification procedure utilizing alumina adsorption to eliminate non-catechol substances and to concentrate the products of interest.

MATERIALS AND METHODS

Chemicals

The catecholamine standards, norepinephrine (NE), epinephrine (EPI), and dopamine (DA) were purchased from Sigma (St. Louis, MO, U.S.A.); the 3,4-dihydroxyphenylalanine (DOPA) and the 3,4-dihydroxyphenylacetic acid (DOPAC) were from Calbiochem (San Diego, CA, U.S.A.). The 1-octanesulfonic acid (OSA), sodium salt was purchased from Regis Chemical (Morton Grove, IL, U.S.A.). Glass-distilled methanol from Burdick & Jackson was purchased from Rainin Instrument (Woburn, MA, U.S.A.). All other chemicals were reagent grade from Baker (Phillipsburg, NJ, U.S.A.).

Mobile phase solutions

The aqueous solutions were prepared with glass-distilled deionized water. The buffer, prepared from concentrated solutions of 1.0 *M* NaH_2PO_4 (adjusted

to pH 2.8—2.9 with 1.0 *M* H_3PO_4) and 10% Na_2EDTA, contained 0.1 *M* phosphate and 0.1 m*M* EDTA. The crystalline pairing-ion reagent, 1-octanesulfonate (OSA) was added to the diluted buffer as prepared and the aqueous solution then was filtered (Millipore, 0.22 μm GS-type) and degassed under vacuum prior to the admixture of methanol. The proportion of aqueous buffer to methanol varied from 100:1 to 100:4 for the C_8 column and was as high as 100:8 for the C_{18} column. The pH of the mobile phase was approximately 3.0 and was adjusted with NaOH or H_3PO_4 to achieve optimum resolution of DOPA from NE and EPI.

Chromatography

The HPLC system consisted of the following components purchased from Rainin Instrument: Altex 420 Microprocessor Control System, Altex 100A pumps, Rheodyne 7210 and 7125 injector valves, Altex Ultrasphere-Octyl (C_8) and Ultrasphere-I.P. (C_{18}) columns (5 μm particle size; 250 × 4.6 mm), Kipp & Zonen BD 41 two-channel recorder. The amperometric detector was the Model LC-16 from Bioanalytical Systems (West Lafayette, IN, U.S.A.) and consisted of a TL-5 glassy-carbon electrode and an LC-3 controller. A vintage LC-2A detector also was used in some experiments. For maximum sensitivity the working electrode was operated at +0.9 V vs. a Ag/AgCl reference electrode.

Columns were operated at ambient temperatures and the flow-rate was 1.0 ml/min during experiments and was maintained at 0.1 ml/min when the columns were not in use. The amperometric detectors were left on as long as mobile phase flowed through the columns and flow-cells. This minimized the start-up time. Columns and detectors remained stable for extended periods of use, up to six weeks. At the end of a use period, the columns were washed free of mobile phase components with 400—500 ml of water and then were returned by means of a gradient to 70% methanol for storage. A few of the columns in use for over six months retain good efficiency and resolution despite prolonged exposure to aqueous solutions and acidic samples.

Standards

All stock catechol solutions (1.0 mg/ml free base) were prepared with 0.01 *N* HCl containing 0.1 m*M* EDTA. These solutions were stored in 100-μl aliquots at —20°C until used. Thawed aliquots were diluted with mobile phase solvent to yield working standards containing 10 or 50 ng/ml of each catechol. These working standards were prepared for each experiment and were kept on ice until used. The 20-μl injected standard mixture contained 0.2 or 1.0 ng of each catechol and the detector response was linear with concentrations from 0.1—20.0 ng for each species.

Tissue sample preparation

The catechol substances in tissue and in body fluid specimens were isolated by a modified alumina adsorption procedure employing batch elution to minimize final volumes [15]. Briefly, specimens were homogenized in 15% trichloroacetic acid and then centrifuged. The supernatant fluid was rapidly adjusted to pH 8.4—8.5 in the presence of EDTA and Tris buffer was poured

into screw-cap conical tubes containing 100 mg of activated alumina [16]. After thorough mixing, the fluid was removed and the alumina was washed with several volumes of water. The catechols were then eluted from the alumina with 0.3 ml of 0.8 *N* perchloric acid (PCA). Excess PCA was removed from the eluent by precipitation as the potassium salt formed on the addition of a small volume of concentrated KOH—K_2HPO_4 solution containing EDTA and OSA. An aliquot of methanol was added to the eluent, which then was similar in buffer and methanol composition to the mobile phase and which contained less than 0.1 *N* PCA. The final pH of the sample was 1.5—2.0 and it was kept on ice until injected into the HPLC system. These precautions were taken to safeguard the reversed-phase columns. The elimination of non-catechol substances permitted the operation of the detector at a potential of +0.9 V to yield maximum sensitivity. Tissue recoveries were determined in each experiment and generally ranged from 40% for DOPA and DOPAC to 60% for NE. Results were corrected for recoveries. The experimental procedures followed in the rat brain studies have been described previously [15].

RESULTS AND DISCUSSION

In order to establish the optimum mobile phase conditions required for the baseline resolution of NE, EPI, DOPA, DA and DOPAC on the C_8-Octyl columns, a systematic study of the influence of pH, OSA concentration, and methanol admixture on the retention of these catechol substances was conducted. The relationship of retention to OSA and methanol levels at pH 3.0 is shown in Fig. 1. At all pairing-ion concentrations, the admixture of methanol dramatically curtails the retention of the catechol compounds, compressing the elution profile for standard mixtures. The maximum methanol concentration is determined by the limit of resolution of NE, DOPA, and EPI and is approximately 4% for the C_8-Octyl columns. Increasing the concentration of the pairing-ion reagent increases the retention of the catecholamines and of DOPA but reduces that of DOPAC which lacks the cationic ammonium group. At the lowest OSA concentration examined (0.1 m*M*), quantitative resolution of the five catechols was achieved in the absence of methanol. Under these conditions, the DA and DOPAC peaks were quite broad and the peak heights minimal, the results of band broadening during the course of the extended elution time (approximately 1 h at ambient temperature and 1.0 ml/min flow-rate). The admixture of methanol effectively curtails band broadening and enhances peak height, thereby improving detector response (Fig. 2). The optimum conditions for achieving quantitative resolution, maximum sensitivity, and minimal elution time entail the use of 0.2 m*M* OSA and 4% methanol, at pH 3.0.

The retention of DOPA and of DOPAC is most sensitive to modest pH manipulations in this acidic range. Fig. 3 demonstrates the effect of pH on the retention of these catechol substances in the range 2.8—3.8. The catecholamines are unaffected by variations in this pH range while the DOPA retention is significantly altered below pH 3.4 and that of DOPAC above pH 3.4. Similar findings for DOPA [12] and DOPAC [17] have been reported previously in C_{18} reversed-phase columns. The operating pH of the mobile phase can be

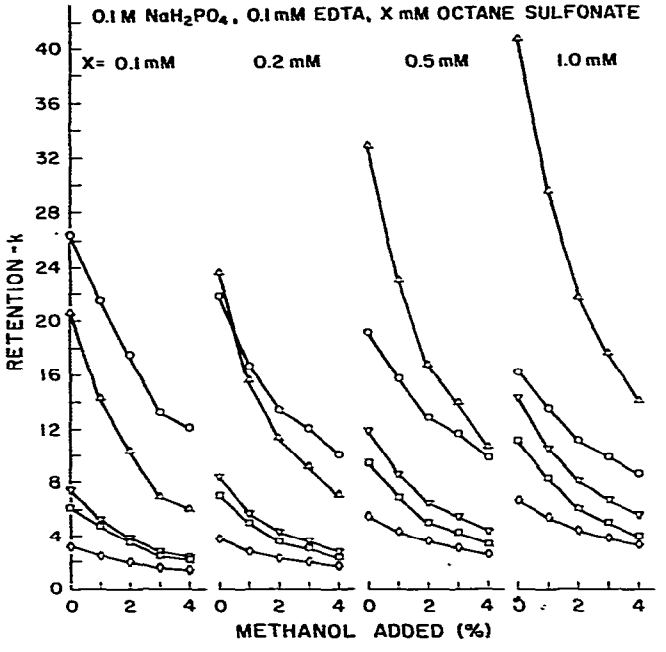

Fig. 1. Effect of OSA concentration and methanol admixture on retention of NE (◇), DOPA (□), EPI (▽), DA (△), DOPAC (○). Column: Ultrasphere-Octyl, 25 × 0.46 cm. Eluent: aqueous buffer, 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, and OSA (0.1, 0.2, 0.5, or 1.0 m*M*); methanol, 0—4% volume of aqueous buffer. pH 3.0. Flow-rate: 1.0 ml/min. Temperature: ambient. Detector: +0.9 V vs. Ag/AgCl reference electrode.

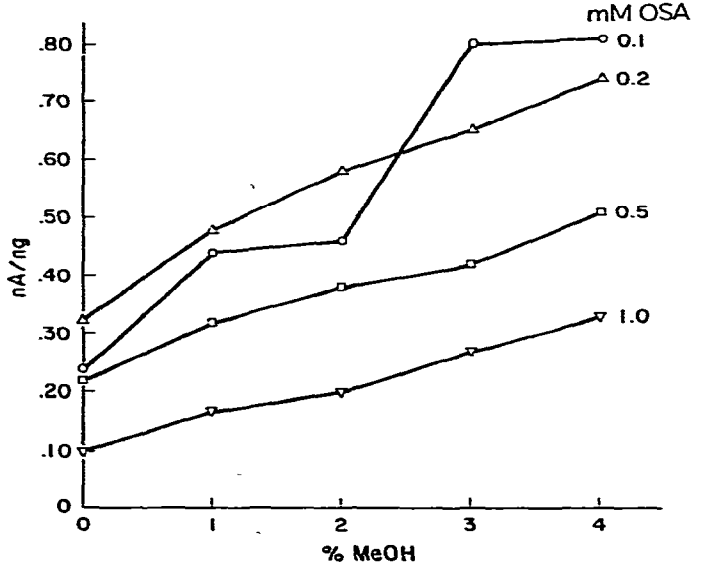

Fig. 2. Effect of OSA concentration and methanol admixture on detector response to DA. Mobile phase: 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, and OSA (0.1, 0.2, 0.5, 1.0 m*M*); methanol (0, 1, 2, 3, 4%): buffer. pH 3.0. Flow-rate: 1.0 ml/min. Temperature: ambient. Detector: +0.9 V vs. Ag/AgCl reference electrode. Column: Ultrasphere-Octyl.

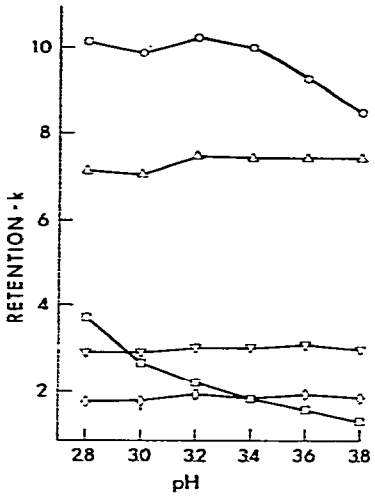

Fig. 3. Effect of pH on retention of NE (◇), DOPA (□), EPI (▽), DA (△), DOPAC (○). Mobile phase: 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, 0.2 m*M* OSA, and 4% methanol. Flow-rate: 1.0 ml/min. Temperature: ambient. pH adjusted with 1.0 *M* NaOH or 1.0 *M* H_3PO_4. Column: Ultrasphere-Octyl, 25 × 0.46 cm. Detector: +0.9 V vs. Ag/AgCl.

adjusted to achieve a fine tuning of any column, as circumstances require, and since the column performance rapidly reflects this influence, such manipulation can be effected shortly prior to use. As shown in Fig. 3 the baseline resolution of DOPA from NE and EPI is achieved under several pH conditions. At pH values below 2.9, DOPA emerges after EPI, while at pH values above 3.4, it emerges ahead of NE in which case it may overlap with minimally-retained solvent front substances. We chose the pH range 3.0—3.2 in which DOPA emerges between NE and EPI since no contaminant peaks were observed in this region in the tissue and body fluid samples examined. In the experiment involving the DOPA-decarboxylase inhibitor, seryltrihydroxybenzylhydrazine (Roche No. Ro 4-4602), a large peak was seen in the chromatograms after the EPI peak position.

Thus, the standard conditions under which the C_8-Octyl columns are operated include a mobile phase containing 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, 0.2 m*M* OSA, and 4% volume of admixed methanol, pH 3.0—3.2, a flow-rate of 1.0 ml/min, ambient temperature, and amperometric detectors set at a working electrode potential of +0.9 V vs. Ag/AgCl reference electrodes. Under these conditions, the detection sensitivity permits the quantitation of as little as 5 pg of NE in the injected (20 μl) sample. The detector response for NE is 1.5—2.0 nA/ng and the baseline noise is less than 0.002 nA (LC-16 detector). The column efficiency as measured by a theoretical plate calculation has shown values of *N* ranging from 7000—12,000 for these 250-cm columns, using the DA peak retention time and width at half-height ratio.

Fig. 4 compares the elution profiles of catechol mixtures separated by the C_8-Octyl column with that achieved by a C_{18} Ultrasphere-I.P. column. The greater retention affinity of the C_{18} column required the admixture of a larger volume of methanol and produced a virtually congruent chromatogram. Owing

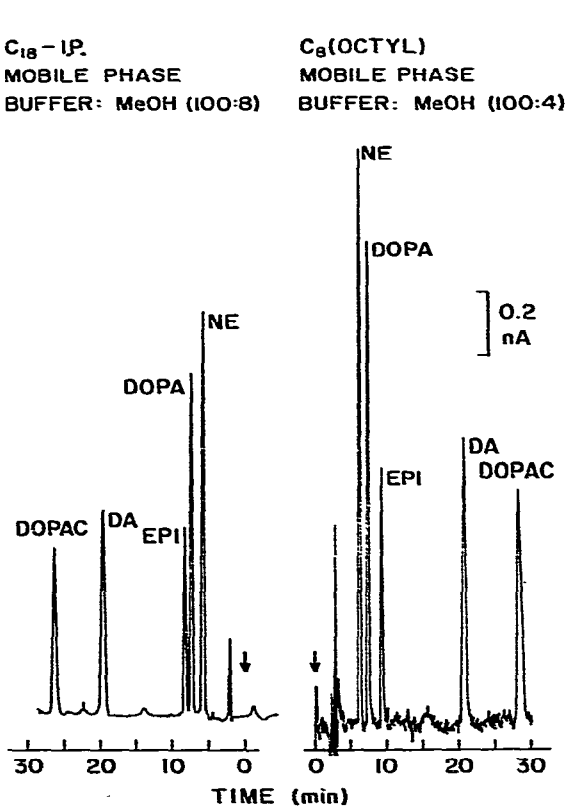

Fig. 4. Chromatograms of NE, DOPA, EPI, DA, DOPAC separations effected by C_8 Ultrasphere-Octyl and C_{18} Ultrasphere-I.P. columns. Mobile phase: pH 3.0; aqueous buffer (0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, and 0.2 m*M* OSA)—methanol, 100:8 for C_{18}-I.P. and 100:4 for C_8-Octyl. Detectors: LC-16 for C_{18}-I.P. and LC-2A for C_8-Octyl; both +0.9 V vs. Ag/AgCl. Columns: 25 × 0.46 cm. Flow-rate: 1.0 ml/min. Temperature: ambient. Sample loop: 20 µl. Standard mixture: 1.0 ng/20 µl, each catechol.

to the reversed output polarities of the detectors the injection points are juxtaposed and the elution directions opposite thus giving a mirror-like comparison. The aqueous buffer conditions of the mobile phase are identical. The pH optimum of the C_{18}-I.P. column (pH 3.2—3.25) is slightly higher than that required to position DOPA exactly between NE and EPI in the C_8-Octyl columns.

The equilibration of the C_8-Octyl columns with the mobile phase has been studied and the results are shown in Fig. 5. Early attempts to place columns into use after a few hours of mobile phase equilibration failed since the retention times of the eluted peaks were not constant. In this experiment, the columns were pre-equilibrated with mobile phase containing a water—methanol mixture similar to that in the complete mobile phase (100:4). At a given time, the columns were set into mobile phase and chromatograms were obtained of the separation of a standard mixture of the catechols every hour for 5 h. When the elution times of the peaks were found to be changing after this length of time, the columns were allowed to equilibrate overnight and the separation was repeated the next day. By then, the retention times remained constant. Fig. 5

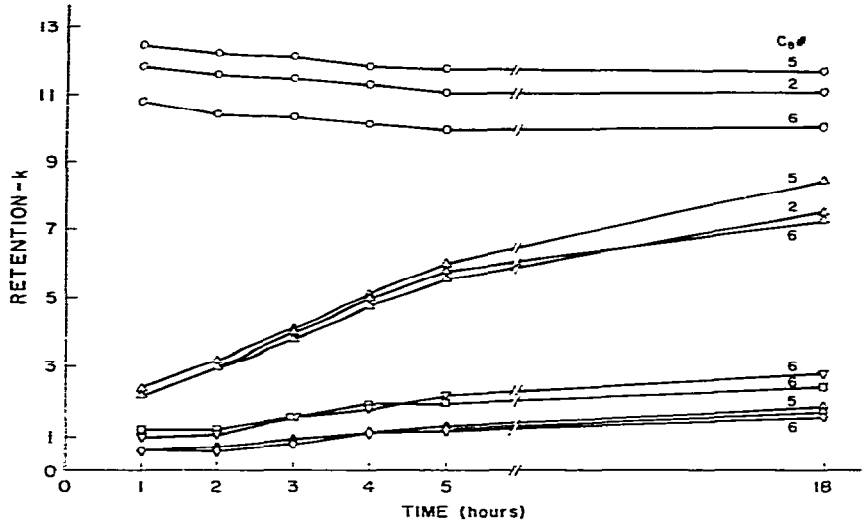

Fig. 5. Time course for equilibration of C_8-Octyl columns. Mobile phase: standard conditions, pH 3.0. Flow-rate: 1.0 ml/min. Temperature: ambient. ◇, NE; □, DOPA; ▽, EPI; △, DA; ○, DOPAC.

indicates that the retention times of all catechols except DOPAC increase with time. This suggests that the rate-limiting step in the equilibration is the binding of the pairing-ion OSA to the reversed-phase packing. This is consistent with the findings of Crombeen et al. [18] who observed changing retention values of these and other substances as a function of column loading with a pairing-ion reagent. Their study suggested that a preliminary column loading with the pairing-ion reagent followed by replenishing with a minimum steady-state level furnished by the mobile phase might be an alternative approach to take to achieve equilibrium. Based on these results, it appears that the pairing-ion species binds to the reversed-phase medium and creates a dynamic cation-exchange system [18].

The results of these studies provide a sound basis for selecting the mobile phase conditions required to achieve quantitative separation of mixtures of basic, zwitterionic, and acidic catechol substances. For instance, the difficulty of separating NE from other minimally-retained solutes can be overcome by increasing the OSA concentration. The attending increase in the retention time of DA can be largely offset by increasing the methanol volume admixed which diminishes the retention time of DA and DOPAC proportionally more than that of NE, DOPA, or EPI. The DOPA also can be shifted to positions before, between, or after NE and EPI by making minor pH changes to accommodate different situations.

The general method developed from these studies has been applied to the determination of the catecholamines and their related catechols in several neuronal tissues and in plasma. Fig. 6 shows chromatograms of extracts of cat spinal cord tissues which were spiked with DOPA as an internal standard. The amount of gray matter used in the assay was 17 mg while 53 mg of white matter was analyzed. The only endogenous catecholamine detected in sig-

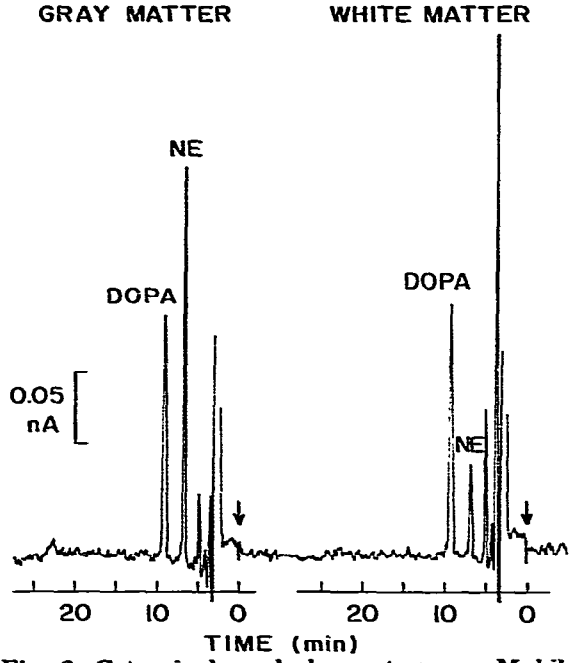

Fig. 6. Cat spinal cord chromatograms. Mobile phase: standard conditions, pH 3.0. Tissue specimens: 17 mg of gray matter; 53 mg of white matter. DOPA added as internal standard. NE content: gray matter, 330 ng/g; white matter, 24 ng/g.

nificant amounts was NE (330 ng/g in gray matter and 24 ng/g in white matter).

The chromatogram of human plasma is shown in Fig. 7. One milliliter of plasma was used for the analysis and NE, DOPA and DOPAC were easily detected in the 20-μl sample injected. Recent studies using a larger sample loop (100 μl) have facilitated the quantitation of EPI (not shown). The challenge in this assay is the resolution of NE from the solvent front components. In this case, this was achieved by increasing the OSA concentration to 0.5 m*M*. We have found that the decline in column performance is signalled by the loss of such resolution even with the use of elevated OSA concentrations. The C_{18}-I.P. columns exhibit greater NE retention and permit its resolution at 0.2 m*M* OSA [19]. The plasma NE level in this sample corresponds to a concentration of 4.4 nmole/l which is within the range observed by Hallman et al. [1] for normal human plasma samples.

The steady-state levels of NE, DA, and DOPAC for various regions of rat brain are shown in Table I. To date, the most extensive use of this method in our laboratory has been for the determination of these substances and of DOPA in discrete regions of rat brain under a variety of drug-altered conditions [15]. Fig. 8 shows one such experiment in which DOPA accumulation was studied in animals treated with a DOPA-decarboxylase inhibitor (Ro 4-4602). The chromatograms are of prefrontal cortex samples. This study measured the DOPA accumulation and catecholamine concentrations in rat corpus striatum, olfactory tubercle, as well as in prefrontal cortex [15]. The C_8-Octyl columns are admirably suited to such investigations.

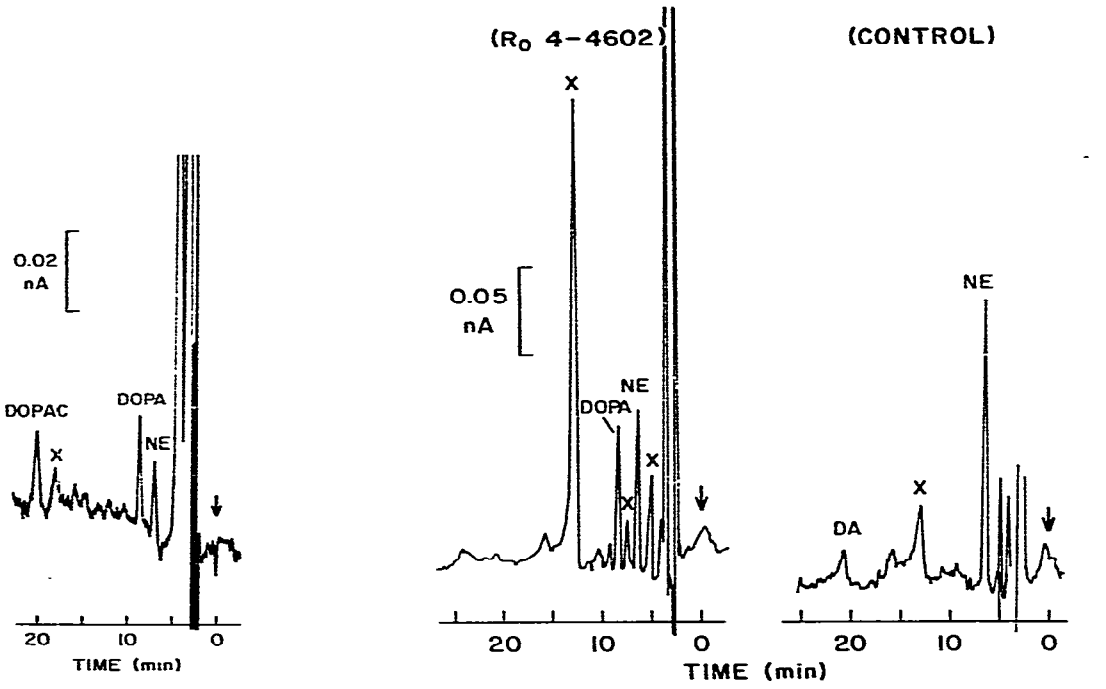

Fig. 7. Human plasma chromatogram. Mobile phase: 0.1 m*M* NaH_2PO_4, 0.1 m*M* EDTA, 0.5 m*M* OSA, and 4% methanol, pH 3.0. Flow-rate, 1.0 ml/min. Temperature: ambient.

Fig. 8. Effect of DOPA-decarboxylase inhibition on chromatogram of rat prefrontal cortex. Mobile phase: standard conditions, pH 3.0. Flow-rate: 1.0 ml/min. Temperature: ambient.

TABLE I

REGIONAL VALUES FOR RAT BRAIN NE, DA AND DOPAC

Rat brain regions differing greatly in catecholamine content have been studied using this method. Values indicate the mean ± the standard error of the mean, expressed as ng per g wet weight tissue. Brain regions were dissected bilaterally and pooled before determinations. Two DA-rich areas, the corpus striatum and olfactory tubercle are shown. NE, DA, and DOPAC values for a small section of prefrontal cortex receiving a DA projection [15] are also included. The cerebellum and hippocampus receive a noradrenergic (but not dopaminergic) innervation, and the DA and DOPAC levels in these tissues are consistent with the role of DA as a precursor to NE in noradrenergic nerve terminals.

Brain area	Weight (mg)	N	NE (ng/g)	DA (ng/g)	DOPAC (ng/g)
Corpus striatum	70 ± 4	7	94 ± 6	7129 ± 166	988 ± 38
Olfactory tubercle	21 ± 1	7	287 ± 10	6820 ± 123	1093 ± 46
Prefrontal cortex	21 ± 1	7	310 ± 17	89 ± 11	47 ± 4
Hippocampus	120 ± 4	5	239 ± 12	12 ± 1	4 ± 1
Cerebellum	242 ± 5	5	171 ± 9	6 ± 1	2 ± 1

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