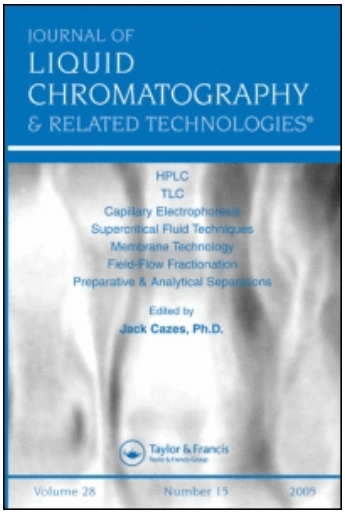

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A NEW TECHNIQUE FOR SIMULTANEOUS ASSAY OF BIOGENIC AMINES AND THEIR METABOLITES IN UNPURIFIED MOUSE BRAIN

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ABSTRACT

We developed a chromatographic system which separates catecholamines and indolamines as well as a large number of their metabolites (acids, aminoacids and glycols). Waters Radial Pak columns compressed radially to avoid formation of cavities in the packing, were used. These columns must be washed with methanol in order to obtain reproducible results when an ion-pairing mobile phase is employed. The conditioning of the columns was not affected by washing with triethylamine or variations in radial pressure. Good resolution of 13 samples was obtained in 28 minutes with a mobile phase composed of 0.1 M KH_2PO_4, 0.1 mM EDTA and 5 mM heptane sulfonic acid in water and $^2Me^4OH$ (85:15), pH = 3.6. Products were identified with the use of a Metrohm 641 electrochemical detector.

The technique was used for rapid, simultaneous determination of NA, DA, DOPAC, HVA, 5HT and 5HIAA in deproteinated mouse brain samples.

INTRODUCTION

Since pharmacological agents, especially psychotropic substances, may modify catecholamines, indolamines and their metabolites in the brain, it is essential to be able to measure these substances in order to detect variations due to treatment.

These substances can be selectively detected and quantitated at concentrations found in biological fluids using high performance liquid chromatography coupled to a sensitive detection

system, electrochemical for example (LC-ED). Most of the methods which have been described concern the separation and analysis of a limited number of compounds in purified extracts of biological tissues or fluids. Separation of a larger number of pure compounds was attempted with a Merck RP 8 column (1), and the performances of three other columns, a reverse phase Merck Lichrosorb RP 18 column and the Perking Elmer ODS-HC-Sil-X and ODS-Sil-X-1 ion exchange columns, have been compared (2). It has not yet been possible to separate and detect a large number compounds.

We have succeeded in separating a mixture of 13 pure catecholamine and indolamine derivatives using reverse phase Radial Pak A columns. These columns are used under radial compression to avoid cavity formation, permitting better separation of injected compounds. We have determined the conditions necessary for good compound resolution and reproducible results at concentrations of about 1 ng/µl.

The method was then applied to crude extracts of mouse brain without preliminary purification by alumine or ion exchange procedures which are time consuming and can be complicated. Catecholamines, indolamines and their metabolites could be assayed simultaneously in small regions of a single mouse brain containing less than nanogram of the compounds.

MATERIAL AND METHODS

We used a Waters 6000 A liquid chromatograph with a U 6 K injector, a Waters RCM-100 radial compression system, a Waters Radial Pak A column (C 18, 0.8 cm i.d. X 10 cm, 10 µm particle size), a Metrohm 641 electrochemical detector fitted with two vitrous carbon electrodes EA 286 - 1 and one reference Ag/AgCl EA 442 electrode. The potential used was + 0.8 V. The mobile phase consisted of 0.1 M KH_2PO_4 (Merck), 0.1 mM EDTA (Prolabo), 5 mM heptane sufonic acid (Waters Pic B7) in a mixture of demineralized water (Crouzat > 5 millions ohm cm^2/ cm) and MeOH (85:15). The pH was adjusted to 3.6 by addition of 0.1 M H_3PO_4 (Merck), filtered through a 0.45 µm Millipore filter and degassed in a Bransonic 220 ultrasonic cell, flow rate 1 ml/mn. Compounds tested were

DHMA : 3,4 - dihydroxymandylacetic acid (Aldrich) ; DHPG : 3,4 - dihydroxyphenylglycol (Aldrich) ; VMA : vanylmandelic acid (Aldrich) ; MHPG : 4 - hydroxy - 3 - methoxyphenylglycol (Aldrich) ; DOPA : 3 - (3,4 - dihydroxyphenyl) - alanine (Fluka) ; NA : noradrenaline (Aldrich) ; DHPMA : 3 - (3,4 -dihydroxyphenyl) - 2 - methylalanine (Aldrich) ; DBA : 3,4 - dihydroxybenzylamine, internal standard (Aldrich) ; DOPAC : 3,4 - dihydroxyphenylacetic acid (Sigma) ; DA : dopamine (Interchim) ; 5HIAA : 5 - hydroxyindole - 3 - acetic acid (Sigma) ; HVA : homovanillic acid (Aldrich) ; 5 HT : 5 - hydroxytryptamine (Sigma). They were dissolved at a concentration of 1 mg/10 ml water, then diluted 1/100 in the mobile phase (1 µl contains 1 ng). All concentrations were reported as the free base or acid.

Mice were sacrificed by decapitation at the same time of the morning to avoid circadian variations in the brain content. The brains were quickly removed from the skull and dissected on ice into different regions (cerebellum, brain-stem, hypothalamus, hippocampus, striatum and rest) according to the method of Glowinsky and Iversen (3) prior to freezing on dry ice. Weighed tissue samples were homogenized with an Ultra-turrax in 450 µl (900 for the rest) of 0.4 N perchloric acid containing 0.1 % EDTA and $Na_2S_2O_5$. Homogenates were centrifuged (Beckman J 21) at 8000 rpm and 4°C for 10 mn. The samples were adjusted to a pH of 5 by addition of 50 µl (100 µl for the rest) of 10 M potassium acetate. The samples were again centrifuged under the same conditions to eliminate $KClO_4$ and injected directly into the LC in the following amounts : 5 µl for striatum and rest, 10 µl for hypothalamus, 20 µl for hippocampus and brain stem, 50 µl for cerebellum.

The concentration of each compound was determined by compairing the respective peak heighs with those obtained with a standard mixture of compounds tested every six samples and calibrated with an internal standard. No deviation from linearity was detected over a range of 0.1 to 10 ng.

RESULTS

The aim of this study was to achieve separation of the cate-

chols and indoles most abundant in brain tissue using radially compressed columns. A phosphate buffer containing the ion pairing agent heptane sulfonic acid and MeOH was chosen for the mobile phase as a consequence of reports in the literature (4) as well as our own experience with Waters microbondapak C 18 columns. Results differed, however, depending on whether the columns were new or had already been exposed to solvents containing variable amounts of MeOH. DA retention, for example varied from 0 to 15 minutes. We therefore studied the conditioning of these columns necessary to obtain reproducible results with an ion pairing mobile phase.

Columns conditioning

MeOH : Our preliminary results suggested that the more the columns were washed with MeOH, the better they retained the samples. The following tests were, therefore, performed.

- two new columns were washed 1 hour with water, 1 hour with MeOH, 1 hour with water, then 20 hours with eluent, giving similar and acceptable results.
- a column with weak retention capacity, when washed abundantly with water, then 1 hour with MeOH, 1 hour with water and 20 hours with eluent, gave similar results.
- a new column treated with MeOH as above, tested with eluent, washed again with water followed by MeOH then water, and retested with eluent gave identical results in both tests.

Good reproducible resolution of the samples tested could therefore be obtained with both new or ineffective used columns after washing with MeOH. Columns from the same or different series gave similar results. In the presence of MeOH, the OH groups on the silica particles seem to become oriented like "bristles of a brush" on which the samples are retained.

Radial compression : In order to determine whether changes in radial pressure during conditioning alters the arrangement of the bonding phase, the pressure on three new dry columns was fixed at 0, 1/3 or 2/3 of the maximum, the latter being recommended by the

manufacturer. The three columns were washed as above with water and eluent. Radial pressure remained the same during washing, but pump pressure varied depending both on the solvent and the radial pressure.

Solvent \ Radial pres.	0	1/3	2/3
MeOH	70	70	90
Water	150	160	250
Eluent	280	310	380

Pump pressure in psi

When radial pressure was adjusted to its usual value, identical traces were obtained with the three columns. At the flow rate used (1 ml/mn), radial pressure did not affect column conditioning.

Triethylamine : In order to determine whether the retention of biogenic amines and their derivatives is affected by blocking unbonded silanol sites, the columns were washed with triethylamine. The following results were obtained :

- neither retention time or shape of peaks was affected by washing with Et_3N ;
- less washing with eluent seemed necessary to obtain reproducible results ;
- distorted peak symmetry could not be corrected with this procedure.

Mobile phase

Variations in pH : Varying the pH affected not only retention time, as seen in Figure 1, but also peak shape and symmetry. The values reported were obtained with the aqueous MeOH buffer. The pH was verified before every recording and corrected if necessary with 0.1 M H_3PO_4 or NaOH.

Retention time : Retention time increased as pH decreased ; the

longer the retention time, the greater the increase. The amino-acids DOPA and DHPMA, particularly the latter, were retained much longer.At pH > 3.4, VMA and DOPA were less well separated. Decreasing the pH improved separation but increased retention time for all the samples.

Peak shape : With some columns, an increase in pH changed the shape of the peaks obtained. After pH = 3.85 peaks became more and more asymmetrical. This may be explained by column wear(loss of C 18 chains).The pH must be low enough for stable undissociated ion pairs to form between the sample and the heptane sulfonic acid. Increasing the pH decreases the formation of ion pairs by protonation.

Percentage of MeOH : Increasing the percentage of MeOH in the eluent generally increased sample retention times. This is illustrated in Figure 2. With 10-15 % MeOH, DA and DOPAC were in close proximity. They changed positions with 15-20 % MeOH. Their retention times were identical with 17 % MeOH. At 10-15 % MeOH, DBA and DOPAC have similar elution times. Optimal separations could be obtained under the following conditions :

- glycols (MHPG, DHPG) and acids (DHMA, VMA, DOPA, DOPAC) were well separated with 10 % MeOH at a rate of 1 ml/mn ;
- all 13 samples could be separated with 15 % MeOH at a rate of 1 ml/mn (Figure 3) ;
- if only NA, DBA, DA and 5HT are to be quantified, as after Amberlite extraction, 15 % MeOH at 2 ml/min, rather than 20 % at 1 ml/mm, was optimal.

Column wear

Column wear results in increased pressure. Normal pressure with the proposed eluent was 400-700 psi. Above 1200 psi resolution decreases resulting in loss of separation between MHPG and NA, DA and DOPAC. DA-DOPAC separation can be partially recovered by washing with water, with MeOH for 5 hours, then again with water followed by eluent. The columns must be washed abundantly with water since MeOH precipitates heptane sulfonic acid. Two

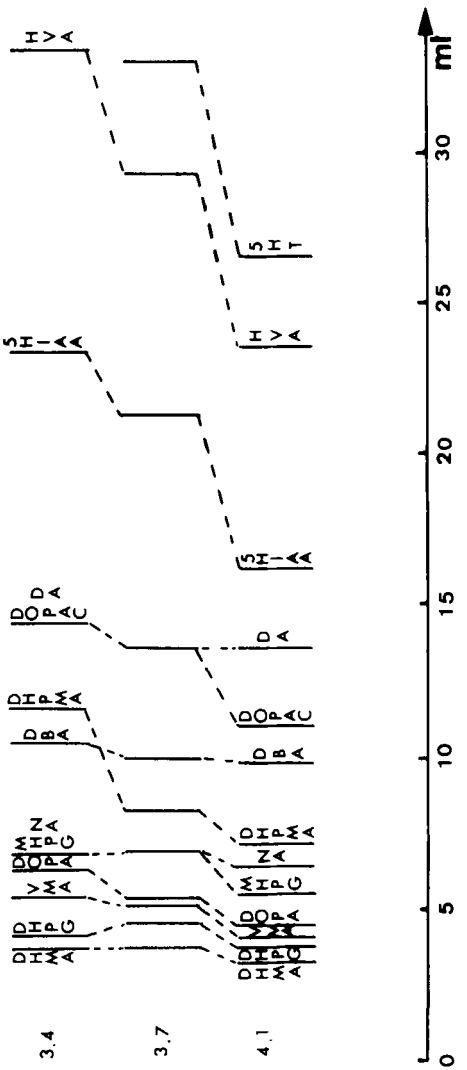


FIGURE 1 : Effect of pH on Retention Times. Radial Pak A, 0.1 M KH_2PO_4, 0.1 mM EDTA, 5 mM heptane sulfonic acid and 15 % MeOH ; flow rate 1 ml/mn.

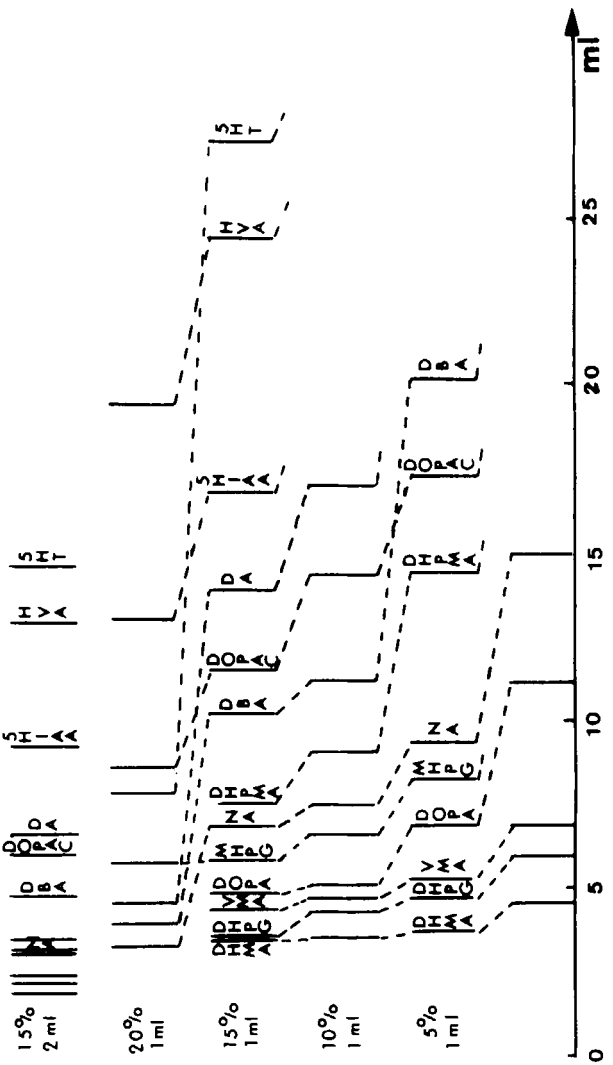


FIGURE 2 : Effect of MeOH and Elution Rate on Retention Times. Radial Pak A, 0.1 M KH_2PO_4, 0.1 mM EDTA, 5 mM heptane sulfonic acid, pH = 3.6.

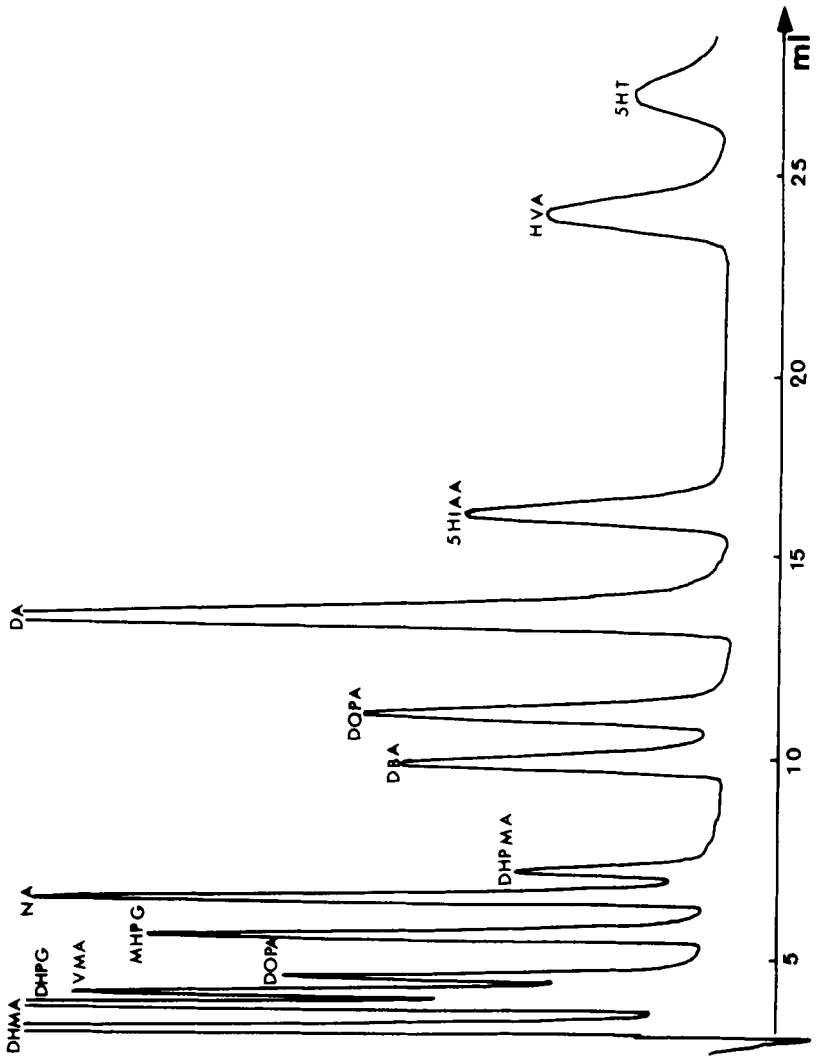
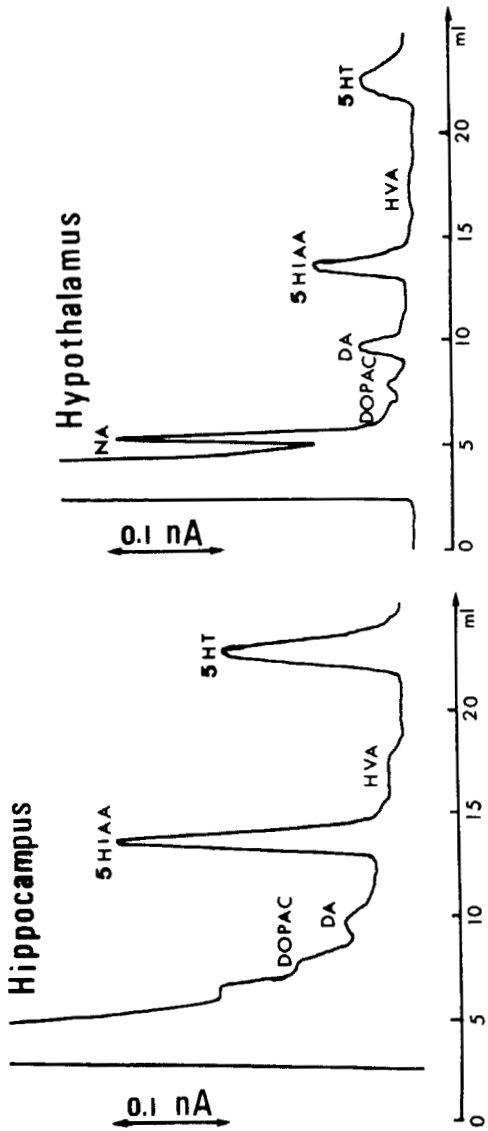


FIGURE 3 : Chromatograms of a standard solution containing 13 catecholamines, Indolamines and their Metabolites. Radial Pak A, 0.1 M KH_2PO_4, 0.1 mM EDTA, 5 mM heptane sulfonic acid and 15 % MeOH, pH= 3.6 flow rate 1 ml/mn. + 0.8 V electrode potential vs Ag/AgCl, sensitivity 1 nA.



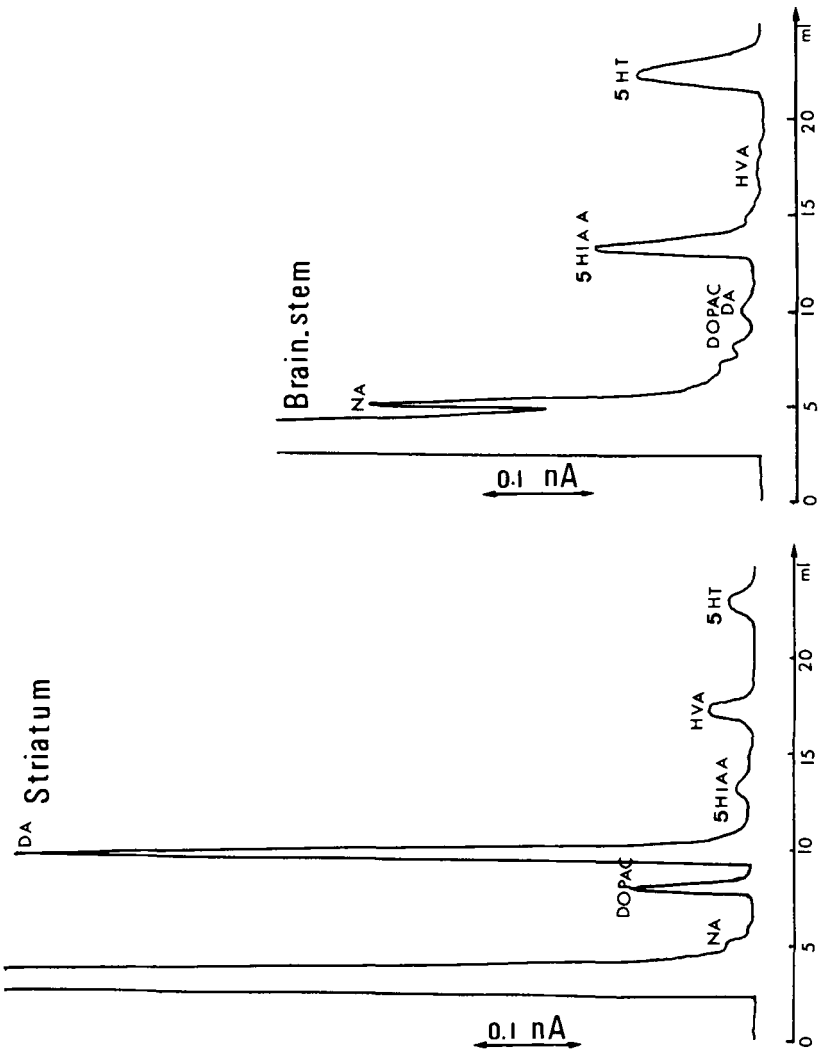


FIGURE 4 : Chromatograms of Tissue Extracts from Mouse Brain. Radial Pak A, 0.1 M KH_2PO_4, 0.1 mM EDTA, 5 mM heptane sulfonic acid and 15 % MeOH, pH = 4.1, flow rate 1.2 ml/mn, + 0.8 V electrode potential vs Ag/AgCl, sensitivity 1 nA.

factors contribute to loss of column efficacy with time :
1) MeOH partially eliminates organic substances which are strongly retained by the column and interfere with the normal equilibrium between the two chromatographic phases ;
2) hydrolysis of octadecyl molecules from the surface of the packing material and physical loss of the material, both of which are irreversible.

Assay of crude Mouse Extracts

Three solutions were tested for extraction of the compounds and precipitation of proteins. Proteins precipitated slowly and tailing peaks appeared on the traces with ethyl alcohol. $ZnSO_4$ could not be used because it was detected electrochemically with an RT identical to that of NA. Protein precipitation and compound extraction was best with $HClO_4$ (5). To increase the pH of 0.4 N perchloric acid (pH < 1), CH_3COOK was added. This substance is highly soluble in water minimizing dilution of the extracts and induces a buffer zone (around 5). The $KClO_4$ formed is practically insoluble and can be eliminated by centrifugation.

Oxidative metal ions were complexed with 0.1 % EDTA to protect the catecholamines from oxidation. Ascorbic acid could not be used as a reducing agent because it appeared with our mobile phase, as a large peak which overlapped low RT compounds ; $Na_2S_2O_5$ was used instead since it is eluted as a narrow peak in the void volume of the column.

NA, DA and its metabolites DOPAC and HVA, 5-HT and its metabolite 5-HIAA were readily detected in regions of mouse brain. Figure 4 shows typical chromatograms. Products with RT's lower than that of NA were masked by endogenous substances such as ascorbic acid.

Recordings were obtained quickly and easily (about 20 minutes per sample) and required only a limited number of preparatory steps (deproteination) which avoids loss in sensitivity due to unsatisfactory recovery during adsorption procedures. The results in Figure 4, however, were obtained with a column used daily for six months.

TABLE 1
Regional Levels (ng/g) of NA, DOPAC, DA, 5HIAA, HVA and 5HT in Mouse Brain

	NA	DOPAC	DA	5HIAA	HVA	5HT
Cerebellum				68±1	10±1	68±5
Brain-stem	374±28	17±2	26±3	480±26	45±9	422±23
Hypothalamus	1425±86	92±12	302±32	416±35	170±15	717±57
Hippocampus	-	-	-	233±8		203±4
Striatum	-	775±52	6952±530	238±18	767±74	292±34
Rest	107±7	108±10	684±34	201±14	177±26	231±26

Catechol and indole assays in regions of mouse brain have previously been hampered by the small size of the samples requiring pooling of regions from several brains to measure substances present in low concentrations. Typical results for several regions of mouse brain are shown in Table 1. Only one comparable report of NA, DA and 5-HT levels in mouse brain has been found in the literature (6). Our values are in agreement with the values reported by these authors as well as with those reported recently in rat brain by investigators using LC-ED.

CONCLUSION

Using radial Pak A columns and an ion pairing eluent, 13 compounds, including catecholamines, serotonin and their metabolites, could be separated. This study demonstrates the importance of MeOH in column conditioning when an ion pairing mobile phase is used. Retention times can be adjusted to the needs of a particular assay by varying the pH or the amount of MeOH present. Resolution decreases with column life and can only partially be recovered with MeOH conditioning.

Six compounds could be assayed directly in deproteinated extracts of mouse brain regions in about 20 minutes.

ACKNOWLEDGEMENTS

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REFERENCES

(2) Scratchley, G.A., Masoud, A.N., Stohs, J.J. and Wingard, D. W., High-performance Liquid Chromatographic Separation and Detection of Catecholamines and Related Compounds, J. Chromatogr., 169, 313, 1979.

(3) Glowinski, J. and Iversen, L.L. Regional Studies of Catecholamines in the Rat Brain I The Disposition of {^{3}H} Norepinephrine, {^{3}H} Dopamine and {^{3}H} DOPA in various Regions of the Brain, J. Neurochem., 13, 655, 1966.

(4) Wagner, J., Palfreyman, M. and Zraika, M., Determination of Determination of Dopamine, DOPAC and Homovanillic Acid. Direct Injection of Supernatants from Brain Tissue Homogenates in a Liquid Chromatography Electrochemical Detection System, J. Chromatogr., 221, 237, 1980.

(6) Christensen, M.D. and Leroy Blank, C. The Determination of Neurochemicals in Tissue Samples at Subpicomole Levels, Biological/Biomedical Applications of Liquid Chromatography II, HawK GL, ed., Marcel Decker, New-York, 1979, p.133.