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SIMULTANEOUS DETERMINATION OF DOPAMINE, DOPAC AND HOMOVANILLIC ACID

DIRECT INJECTION OF SUPERNATANTS FROM BRAIN TISSUE HOMOGENATES IN A LIQUID CHROMATOGRAPHY—ELECTROCHEMICAL DETECTION SYSTEM

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SUMMARY

A simple method based on high-performance liquid column chromatography with electrochemical detection is described for the simultaneous determination of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in discrete brain regions of rats. The supernatant of a tissue homogenate is injected directly onto a liquid chromatograph, thus omitting the commonly adopted adsorption step. Of the four different supports tested Nucleosil C_{18} (5 μm) was found superior with respect to chromatographic performance. The effects of pH, methanol and the ion-pairing agent hexyl sulfate on the retention were studied. The mobile phase used in the final studies consisted of citrate buffer pH 4.25—methanol (92:8, v/v) containing hexyl sulfate ($1.7 \cdot 10^{-3}$ *M*). Standard curves of dopamine, DOPAC and HVA were found linear up to about 600 pmol per injection for each compound. The precisions of the chromatographic step were (s_{rel} %): 0.72% (dopamine), 1.26% (DOPAC) and 2.69% (HVA).

INTRODUCTION

Dopamine (DA) is an important neurotransmitter in the brain, especially in the limbic system, striatum and hypothalamus. Several different types of centrally active drugs have potent effects on the various dopamine systems in the brain, producing marked changes in the metabolism and functional activity of DA [1]. Methods to measure the endogenous concentrations of DA and its metabolites in discrete brain regions are consequently of great importance in the analysis of DA neurotransmission. Micromethods for determination of catecholamines have been developed based on fluorescence [2], gas chromatography—mass spectrometry [3] and radio-enzymatic methods [4, 5]. An

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attractive alternative is the separation of catecholamines by high-performance liquid chromatography combined with electrochemical detection as developed by Kissinger et al. [6] and later applied by e.g. Maruyama and Kusaka [7] and Hefti [8].

The assay presented in this paper permits the simultaneous determination of DA and its acid metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). It employs liquid chromatography with electrochemical detection and involves a minimum of sample pretreatment, thus making the method highly suitable for routine analysis.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.); an injection valve, Model 7120 (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 20-μl loop; a thin-layer amperometric detector, Model LC-2A (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with graphite paste (CPO) as the working electrode operated at 0.60 V vs. an Ag—AgCl reference electrode unless otherwise stated; and a recorder, Model TE 200 (Tekman, Bicester, Great Britain). The supports were packed in glass-lined stainless steel columns (S.G.E., Melbourne, Australia).

A constant-pressure Haskel AO 15 gas amplifier pump was used for column packing.

Decapitation of rats was performed with a guillotine (Labora, Stockholm, Sweden) and the tissue was homogenized by sonification (Branson B 30 Sonifier, Branson Sonic Power, Danbury, CT, U.S.A.). The centrifuge and micro-test-tubes were from Eppendorf (Hamburg, G.F.R.), pH measurements were made with an Orion Ionalyzer Model 801A.

Chemicals and reagents

Five different commercially available supports were used for packing of the columns: LiChrosorb RP-8, 5 μm (Chrompack, Middelburg, The Netherlands), LiChrosorb RP-18, 5 μm (Merck, Darmstadt, G.F.R.), μBondapak C_{18}, 10 μm (Waters Assoc., Milford, MA, U.S.A.), SAS-Hypersil, 6 μm (Shandon Southern Products, Cheshire, Great Britain) and Nucleosil C_{18}, 5 μm (Macherey-Nagel, Düren, G.F.R.).

Methanol (May & Baker, Dagenham, Great Britain), citric acid, sodium chloride, perchloric acid (PCA) (Merck), sodium hydroxide (Eka, Bohus, Sweden), sodium bisulfite (Fisher, Fair Lawn, NJ, U.S.A.), were all of reagent grade. Hexyl sodium sulfate was obtained from Research Plus Laboratories (Denville, NJ, U.S.A.). Dopamine · HCl (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), *l*-epinephrine bitartrate, *l*-norepinephrine bitartrate, *dl*-metanephrine · HCl, *dl*-normetanephrine · HCl, 3-methoxytyramine · HCl, *l*-3,4-dihydroxyphenylalanine (*l*-DOPA); *dl*-2-methyl-3-(3,4-dihydroxyphenyl)alanine (α-methyl-DOPA), 5-hydroxytryptamine oxalate (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA) and bis(4-hydroxy-3-methoxyphenylglycol)piperazine (MOPEG) were purchased from Sigma (St. Louis, MO, U.S.A.), N-methyldopamine (epinine) was supplied by Serva (Heidelberg, G.F.R.) and haloperidol by Janssen Pharmaceutica (Beerse, Belgium). The water was deionized and then double quartz-distilled.

Chromatographic technique

The columns were packed by the upward slurry packing technique as described by Bristow et al. [9], with methyl isobutyl ketone as the slurry medium and dichloromethane as the follower. All stainless steel column parts were cleaned by treating successively with dichloromethane, acetone, nitric acid (5 *M*), acetone and dichloromethane again in an ultrasonic bath for 5 min. Glass-lined columns were washed with ethanol. The slurry concentration was approximately 3% (w/v) and the applied pressure 350—400 bar. After packing the support was purified by pumping about 100 ml methanol at a flow-rate of 2—3 ml/min through the column. Column performance was tested according to Bristow and Knox [10].

Mobile phases were degassed in vacuum for 10 min, and deaerated by bubbling through oxygen-free nitrogen for 10 min. Column equilibrium was achieved within 1—2 h at a flow-rate of 0.8 ml/min.

Capacity ratios were calculated relative to the first peak in the composition disturbance, and peak symmetries were measured at a distance from the baseline of 10% of the peak height. All chromatographic values are the means of duplicate or triplicate determinations.

Assay of DA, DOPAC and HVA in rat brain regions

The rat brain was removed within 30 sec after decapitation and dissected on ice. The regions were immediately cooled with dry ice and stored at —70°C until analyzed. For analysis the pieces of tissue (4—50 mg) were weighed in conical 1.5-ml test-tubes and a solution (300—500 μl) consisting of 0.1 *M* perchloric acid, $4 \cdot 10^{-5}$ *M* sodium bisulfite (PCA solution) and the internal standard epinine ($2.5 \cdot 10^{-6}$ *M*) was pipetted into the tubes. The mixture was sonicated at 200 W for about 5 sec while kept on ice, and the homogenate was centrifuged for 2—3 min at 8000 *g*. An aliquot of the supernatant was injected onto the chromatographic column (100 × 3 mm glass-lined stainless steel column packed with Nucleosil C_{18}, 5 μm). Mobile phase was citrate buffer pH 4.25 (ionic strength = 0.1)—methanol (92:8, v/v) containing hexyl sulfate ($1.7 \cdot 10^{-3}$ *M*). The sensitivity setting of the detector was optimized for each peak of interest. Typical settings for a sample of striatum were 10 nA/V (DOPAC), 50 nA/V (DA), 20 nA/V (epinine) and 2 nA/V (HVA).

Quantitations were performed from standard curves of peak height ratios relative to the internal standard against concentrations. The compounds were dissolved directly in pure PCA solution since, as demonstrated below, standard curves obtained from spiked homogenates from the biological material were co-linear with those obtained from such solutions. The standard curves were reproducible from day to day, thus making further simplification of the quantitation procedure possible. A large batch of standard solutions containing DA, DOPAC, HVA and epinine, was divided into small portions, and stored together with samples from a completed study. During analysis standard solutions were injected intermittently (on the average every sixth) with the samples and the concentrations (c_i) of DA and metabolites were calculated based on the response of this standard solution (either a mean value or a value from a neighbour standard sample, if a drift in the response of the standards

is noted during the analysis). Calculations are made according to following formula:

$$c_i = \frac{R_i \times (E - 0.7W)}{S \times W \times I} \quad (1)$$

where R_i = ratio of peak heights, DA or metabolite to internal standard; E = volume of added homogenate solution (μl); W = weight of tissue (mg), 70% of the tissue weight is assumed to be water; S = calculated peak height ratio of 1 pmol of DA, or metabolite, respectively and epinine; I = injected volume (μl).

RESULTS AND DISCUSSION

Choice of chromatographic support

Commerical supports may vary in chromatographic performance for different kinds of compounds [11—13]. Commercially available supports were tested (in preliminary studies), in order to find an acceptable material for the compounds of interest. 5-HT was chosen as the test compound (Table I),

TABLE I

CHROMATOGRAPHIC DATA FOR SOME COMMERCIAL SUPPORTS

Test compound: 5-OH-tryptamine. A. Mobile phase: phosphate buffer pH 6.50; flow-rate: 0.8 ml/min; column length: 100 mm. B. Mobile phase: citrate buffer pH 4.50; flow-rate: 0.8 ml/min; column length; 150 mm.

Support	k'	h^*	N	Asymmetry
A. Lichrosorb RP-8, 5 μm	10.3	127	157	2.1
Lichrosorb RP-18, 5 μm	12.8	87	230	1.8
μBondapak C_{18}, 10 μm	8.2	6.7	1500	1.0
SAS-Hypersil, 6 μm	8.1	90	185	2.4
B. μBondapak C_{18}, 10 μm	14.6	5.9	2525	1.06
Nucleosil C_{18}, 5 μm	28.3	5.0	6050	1.25

$^*h = \frac{\text{HETP}}{\text{dp}}$.

since amines often tend to give low chromatographic performance. Three of the supports gave very low efficiencies and severely tailing peaks, and only μBondapak C_{18} gave an acceptable performance. Later Nucleosil C_{18} was found to give a good reduced efficiency (h) and although the peak symmetry was slightly inferior to that obtained on μBondapak C_{18}, Nucleosil was chosen for further studies because of a much better absolute efficiency, which mainly depends on the smaller particle size (HETP is 25 μm compared to 59 μm).

Influence of pH on chromatographic performance

The retention of protolytic compounds in reversed-phase systems with neat aqueous mobile phases is governed by hydrophobic forces, pK_a values and adsorption of the ionized compounds as ion-pairs with buffer components [14, 15]. The dependence of the capacity ratios on pH (Fig. 1) shows on the whole the expected performance. The retention of the two acids (DOPAC

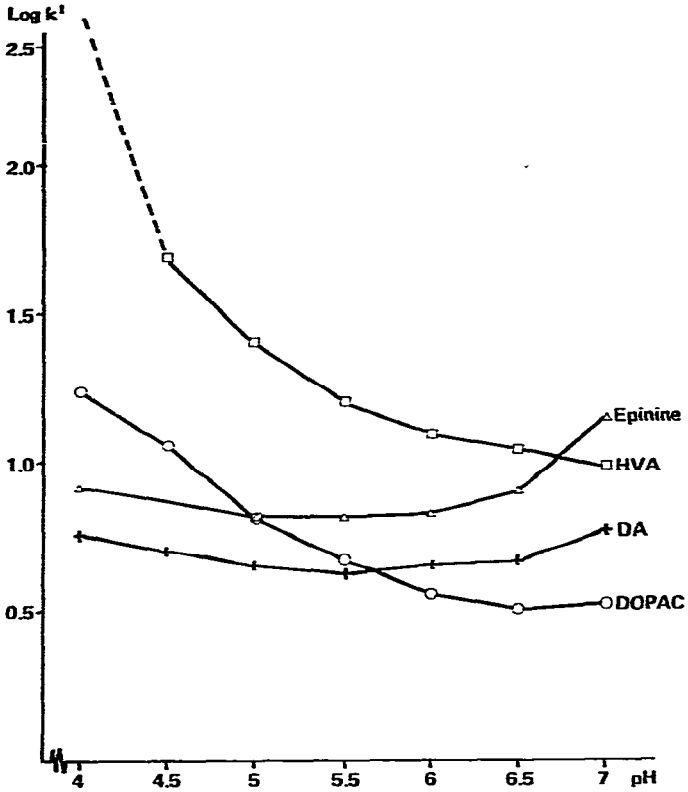

Fig. 1. The effects of pH on the capacity ratios. Support: Nucleosil C_{18} (5 μm). Mobile phase: citrate buffer (ionic strength 0.1).

and HVA) decreased with increasing pH, and the amines tended to be more strongly retained at higher pH. The increasing retention for the amines at pH < 5 is probably a consequence of ion-pair formation with the monovalent citrate anion (pK_2 for citric acid = 4.8). Large selectivity changes occurred with pH; DOPAC, for example, was eluted as third at pH 4 but elutes first at pH 7.

Peak symmetries (Fig. 2) were acceptable at low pH but increased with increasing pH, slightly for the acids but rather drastically for the amines. For further studies a low pH (4.25) was consequently chosen.

Addition of methanol and ion-pairing agent

The rather long retention times with neat aqueous buffer solutions were decreased by the addition of methanol (Fig. 3) with the elution order unchanged and about the same selectivities. Early eluting endogenous peaks will then, however, interfere with DA and epinine. Addition of hexyl sulfate eliminated this problem by increasing the retention times for the amines by an ion-pair effect. Unexpectedly the retention of the acids increased slightly by this addition — a decrease by competition of sites on the support between the two negatively charged species was the expected result [16] as seems to happen with neat aqueous buffer (see Fig. 3) as the mobile phase.

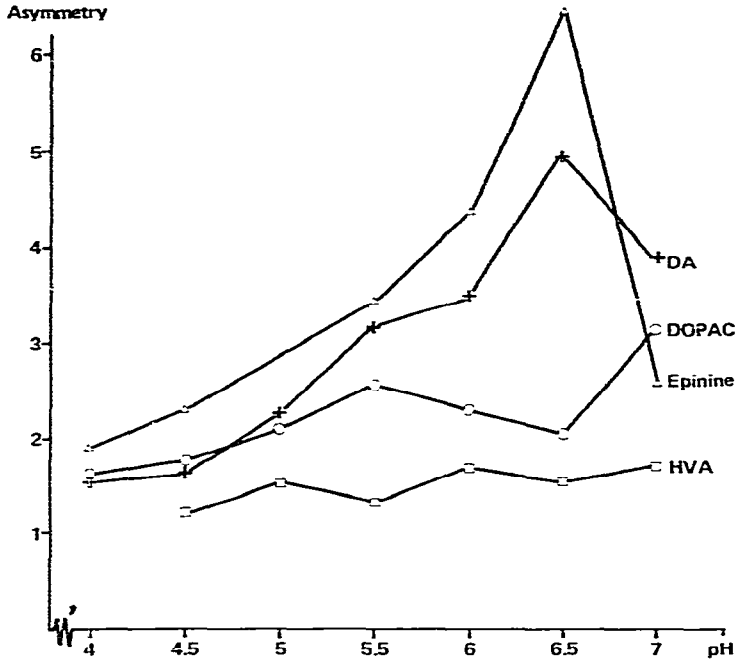

Fig. 2. Effects of pH on peak symmetry. Mobile phase: citrate buffer (ionic strength 0.1). Support: Nucleosil C_{18}, 5 μm. Symmetry = peak asymmetry (back/front) at 10% of top height.

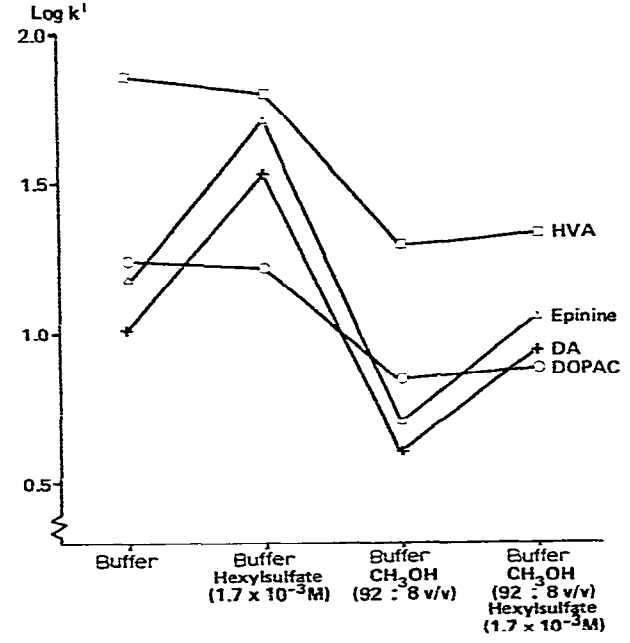

Fig. 3. Effects of methanol and hexyl sulfate on capacity ratios. Support: Nucleosil C_{18}, 5 μm.

Chromatographic selectivity

Capacity ratios of some related compounds which may be present in brain tissue show that no interference may be expected for DA, DOPAC and HVA. Metanephrine elutes just before the internal standard, epinine, but its concentration in brain regions is very low and coupled with low electrochemical response at used potential a significant interference is unlikely and has hitherto not been observed. Some selected selectivity features of the chromatographic system (Table II) demonstrate its capability to separate for example secondary and primary amines, aromatic methoxy- and hydroxy-substituted compounds.

TABLE II

SELECTIVITY OF SELECTED STRUCTURES

Compounds	Selectivity factor
Secondary/primary amine	1.43 ± 0.14 (n = 3)
aromatic OCH_3/OH	2.61 ± 0.47 (n = 4)
aliphatic CH_3/H	1.52
aliphatic H/COOH	3.86
aliphatic NH_3^+/OH	1.20

Selectivity

Typical chromatograms of samples from rat striatum run at two different detector potential settings, 0.60 and 0.80 V, respectively (Fig. 4) show that some compounds give a much higher response at the high potential — HVA, five times; 3-methoxytyramine, ten times; while that of DA, DOPAC and epinine increases only moderately. A drawback of the high potential may be a reduced selectivity as indicated by the larger front (Fig. 4 B) which interferes slightly with the DOPAC peak, and the appearance of additional peaks. For most studies a potential setting of 0.60 V was therefore chosen, but when very low concentrations are expected the higher potential may be used for selected compounds.

Quantitative determinations

Studies on other compounds [17, 18] have indicated an advantage mainly regarding precisions in quantitative determinations by incorporating an internal standard in the method. Epinine, the secondary N-methylamine analogue of DA, that was selected as the internal standard in the present method is here expected mainly to compensate for variations in the performance of the column and working electrode during a working day.

In a possible metabolic pathway for DA conversion to epinephrine, epinine, may be formed as an intermediary compound [19]. The absence of epinine was, however, established in four samples of rat striatum — this region was chosen because of its high content of DA. The detection limit for epinine under the chromatographic conditions used was < 100 fmol, which compared to the amount of epinine used as internal standard, 50 pmol, means that the amount of endogenous epinine was < 0.2% of the added amount. It is improbable that other brain regions contain significantly higher amounts of the compound.

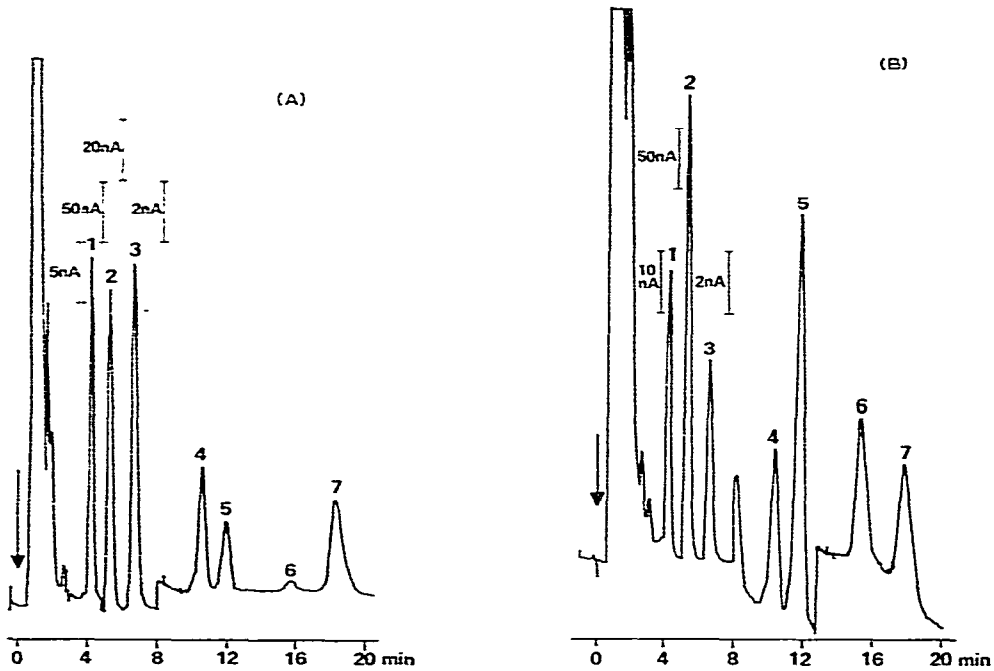

Fig. 4. Chromatograms of rat striata; effect of detector potential, (A) 0.60 V; (B) 0.80 V. Support: Nucleosil C_{18}, 5 μm; mobile phase: citrate buffer pH 4.25 containing methanol (8%) and hexyl sulfate ($1.7 \cdot 10^{-3}$ *M*). Peaks: 1, DOPAC (7.5 pmol); 2, DA (99 pmol); 3, epinine (50 pmol); 4, 5-HIAA; 5, HVA (7.8 pmol); 6, 3-methoxytyramine and 7, 5-HT.

Standard curves of DA, DOPAC and HVA with and without a striatum homogenate were co-linear in all three cases which is illustrated in Table III. Consequently it is not necessary to make standard additions to the rat brain homogenate, but the quantitations can be performed from a simple standard curve made directly in the PCA solution. In practice the standard curves cover a more limited range than those demonstrated here, corresponding more closely to expected endogenous levels. The standard curves were linear up to about 600 pmol per injection for each compound. The amount injected from the

TABLE III

STANDARD CURVES, WITH AND WITHOUT STRIATUM — LINEAR REGRESSION ANALYSIS

Compound	Slope and confidence limits ($P = 0.05$)	Intercept	Correlation coefficient
DA			
with striatum	0.1364 (0.1355—0.1373)	7.87	1.0000
without striatum	0.1330 (0.1301—0.1358)	0.175	0.9998
DOPAC			
with striatum	0.1371 (0.1357—0.1385)	0.911	1.0000
without striatum	0.1343 (0.1309—0.1378)	0.056	0.9998
HVA			
with striatum	0.03230 (0.03139—0.03321)	0.146	0.9997
without striatum	0.03245 (0.03162—0.03328)	0.026	0.9998

actual brain material normally never exceeds 100 pmol. The limit of detection may vary somewhat from day to day depending on the condition of the chromatographic system and may also differ between different times. This measure of sensitivity is difficult to estimate when the sample contains relatively large amounts of DA and metabolites. In the frontal cortex where the levels are low, the detection limits were approximately 50 fmol (DA and DOPAC) and 150 fmol (HVA).

The precision of the chromatographic step as determined by making 10 consecutive injections of the same sample, a pooled striata homogenate from 10 rats were (s_{rel}%): 0.72% (DA), 1.26% (DOPAC) and 2.69% (HVA).

In the present method the supernatant of the homogenate is directly injected onto the column, thus eliminating the commonly adopted step of adsorption on alumina. This is time-saving and also eliminates a possible source of error and a loss in sensitivity, since several studies on catecholamines (e.g. refs. 20, 21) have reported unsatisfactory recoveries from such adsorptions. As an alternative to adsorption on alumina the extraction of impurities with butanol and heptane has been utilized [22], but the recoveries of DA and 5-HT were only 50%; low recoveries (31—89%) of acid metabolites of DA by extraction with diethyl ether have also recently been reported [8].

A drawback of the direct injection of biological material is a shortened lifetime of the column, especially the column top [23]; a remedy may be the insertion of a pre-column [20, 24]. On average the columns used have been found satisfactory for about 500 injections after which the resolution between DOPAC and DA as well as between HVA and 5-HIAA deteriorates.

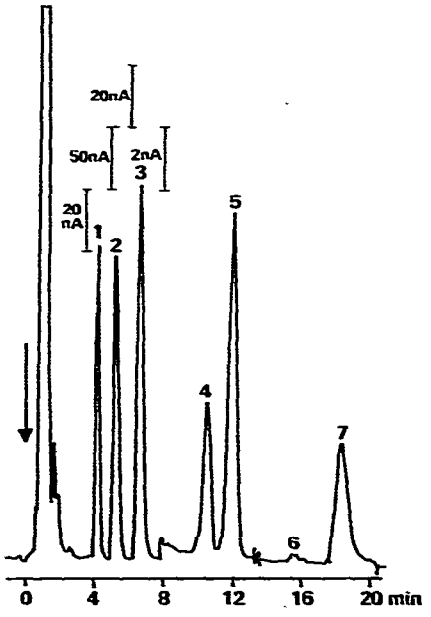

Fig. 5. Chromatograms of striata from rats 2 h after treatment with haloperidol (5 μmol/kg body weight intraperitoneally). Chromatographic conditions: see Fig. 4; potential, 0.60 V. Peaks: 1, DOPAC (22 pmol); 2, DA (67 pmol); 3, epinine (50 pmol); 4, 5-HIAA; 5, HVA (22 pmol); 6, 3-methoxytyramine and 7, 5-HT.

TABLE IV

CAPACITY RATIOS OF SELECTED MONOAMINES AND METABOLITES

Mobile phase: citrate buffer pH 4.25, containing hexyl sulfate ($1.7 \cdot 10^{-3}$ *M*) and methanol (8%); support: Nucleosil C_{18}, 5 μm.

Compound	k'
DA	8.9
DOPAC	7.7
HVA	22.0
Epinine	11.6
DOPA	2.3
Norepinephrine	3.1
α-Methyl-DOPA	3.5
Epinephrine	4.4
MOPEG	5.4
Normetanephrine	6.5
Metanephrine	10.3
5-HIAA	18.8
3-Methoxytyramine	27.8
5-HT	31.8

The method is used for screening of potential neuroleptic drugs. As an example it is demonstrated (Fig. 5) that haloperidol increases the rat striatum levels of DOPAC and HVA significantly (cf. Fig. 4).

The concentrations of DA, DOPAC and HVA have been routinely determined in the following regions of the rat brain: corpus striatum, nucleus accumbens, tuberculum olfactorium, substantia nigra and frontal cortex. In analysis of mesencephalon, however, DOPAC could not be determined due to the interference of an adjacent peak.

The studies presented here have thus led to the development of a simple method for the simultaneous determination of DA, DOPAC and HVA in small samples of brain tissue. However, since several other compounds of importance in psychiatric disorders are well resolved in the chromatographic system (see Table IV), it has a greater unexplored potential for studies in neurochemistry; 5-HIAA, 5-HT and 3-methoxytyramine, for example, appear as resolved peaks with this bioanalytical method.

REFERENCES

1 K. Fuxe, S.-O. Ögren, B. Fredholm, L. Agnati, T. Hökfelt and M. Perez de la Mora, in J. de Ajuriaguerra and R. Tissot (Editors), Rhinencéphale Neurotransmetteurs et Psychoses, Symposium Bel-Air V, Georg & Cie., Geneva, 1977, p. 253.
2 B.H.C. Westerink and J. Korf, J. Neurochem., 29 (1977) 697.
3 S. Takahashi, M. Yoshioka, S. Yoshiue and Z. Tamura, J. Chromatogr., 145 (1978) 1.
4 A. Argiolas and F. Fadda, Experientia, 34 (1978) 739.
5 L.L. Zschaeck and V.D. Ramirez, J. Neural Transm., 39 (1976) 291.
6 P.T. Kissinger, C. Refshauge, R. Dreiling and R.N. Adams, Anal. Lett., 6 (1973) 465.
7 Y. Maruyama and M. Kusaka, Life Sci., 23 (1978) 1603.
8 F. Hefti, Life Sci., 25 (1979) 775.
9 P.A. Bristow, P.N. Brittain, C.M. Riley and B.F. Williamson, J. Chromatogr., 131 (1977) 57.

10 P.A. Bristow and J.H. Knox, Chromatographia, 10 (1977) 279.
11 D. Westerlund and A. Theodorsen, J. Chromatogr., 144 (1977) 27.
12 D. Westerlund and E. Erixson, in preparation.
13 A. Sokolowski and K.-G. Wahlund, J. Chromatogr., 189 (1980) 299.
14 C. Horváth, W. Melander and I. Molnár, Anal. Chem., 49 (1977) 142.
15 B. Henke and D. Westerlund, J. Chromatogr., 187 (1980) 189.
16 A. Tilly-Melin, A. Ljungcrantz and G. Schill, J. Chromatogr., 185 (1979) 225.
17 D. Westerlund, A. Theodorsen and Y. Jaksch, J. Liquid Chromatogr., 2 (1979) 969.
18 D. Westerlund and E. Erixson, J. Chromatogr., 185 (1979) 593.
19 B.L. Goodwin, C.R.J. Ruthven, G.S. King, M. Sandler and B.G.S. Lesk, Xenobiotica, 8 (1978) 629.
20 C.R. Freed and P.A. Asmus, J. Neurochem., 32 (1979) 163.
21 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren, E. Rosengren and L.-E. Edholm, J. Chromatogr., 162 (1979) 7.
22 S. Sasa and C. LeRoy Blank, Anal. Chem., 49 (1977) 354.
23 D. Westerlund, J. Carlqvist and A. Theodorsen, Acta Pharm. Suecica, 16 (1979) 187.
24 H.P.M. van Vliet, Th.C. Bootsman, R.W. Frei and U.A.Th. Brinkman, J. Chromatogr., 185 (1979) 483.