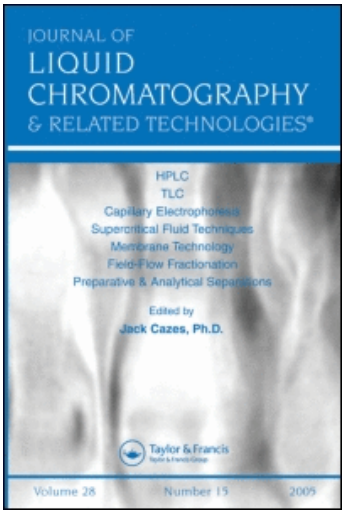

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Simultaneous Automated Determination of Catecholamines, Serotonin, and Their Metabolites in Brain Tissue by HPLC and Electrochemical Detection

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SIMULTANEOUS AUTOMATED DETERMINATION OF CATECHOLAMINES, SEROTONIN, AND THEIR METABOLITES IN BRAIN TISSUE BY HPLC AND ELECTROCHEMICAL DETECTION

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ABSTRACT

A robust procedure with minimal tissue sample pretreatment, based on reversed-phase, ion-pair high performance liquid chromatography (HPLC) combined with electrochemical detection has been developed for the automated and simultaneous determination of the biogenic amines norepinephrine (NE), epinephrine (E), dopamine (DA), 5-hydroxytryptamine (5-HT) and their metabolites 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), 3,4-dihydroxyacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindolacetic acid (HIAA) in as little as 0.5 mg brain tissue. The chromatographic conditions remain stable for at least 8 weeks. In particular small amounts of samples which do not allow reexamination are routinely automatically analysed without close supervision. The sample pretreatment comprises only homogenization of the tissue samples in the HPLC eluent and centrifugation. The supernatant can be directly injected into the HPLC system without further purification. Moreover, optimal concentrations of octane

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sulfonic acid (230 μM) and methanol (6%; v/v) in the recirculating eluent, a reversed-phase analytical column filled with 3 μm Nucleosil 100 C_{18} material, constant column temperature (22 $\pm$ 1°C) and an electrochemical detector with a dual electrode permitted baseline resolution without interference with the pharmacological dose of alpha-methyl-p-tyrosine frequently involved in catecholamine turnover studies as well as the determination of catecholamines, serotonin and their metabolites in femtomolar quantities.

INTRODUCTION

For the study of alterations in catecholaminergic activity in the brain two different methods are commonly applied. The first indirect method which determines the concentrations of biogenic amines assessed with and without prior blockade of tyrosinehydroxylase by the application of alpha-methyl-p-tyrosine (alpha-MT) allows the calculation of turnover rates.The second direct method lacking the use of inhibitors evaluates the ratios between monoamines and their metabolites.

To identify and quantitate the trace amines as norepinephrine (NE), epinephrine (E), dopamine (DA), 5-hydroxytryptamine (5-HT) in the biological samples, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has become the method of choice because of its high sensitivity and specificity.[1,2] The ability to evaluate additionally the aminergic metabolites as 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), 3,4-dihydroxyphenylacetic-acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindolacetic-acid (HIAA) makes the HPLC with ED attractive for a wide range of application including the more recent determination of aminergic activity with the aid of extracellular fluid withdrawn *in vivo* by push-pull perfusion[3,4] or microdialysis.[5] For baseline separation of all these compounds, however, time consuming chromatographic runs are necessary. Using manual injections the number of HPLC determinations is limited to a few samples per day. However, evaluation of neuronal activities as mentioned above demands the analysis of **many** samples. In order to shorten the time of analysis most methods fail to consider all aminergic compounds but are restricted to the determination of catecholamines or serotonin with their respective metabolites. An automated procedure may accelerate the time-consuming analytical process without loss of specificity and sensitivity. Methods described so far in the

literature indicate that automation of HPLC coupled with electrochemical detection is possible but these reports do not refer to the special problems associated with **automated** analysis.[6] Insufficient baseline separation is a major problem when analyzing metabolites like MHPG whose concentrations in the tissue samples are far below those of the parent amines or other compounds. HPLC resolution may deteriorate within a few hours and thus prevent automated determinations that continue during the night or weekend without close supervision by an operator. Moreover, re-examination of samples may be impossible if the amount of tissue is too small.

This paper describes that minimal changes of experimental conditions may disturb the quality of the analytical process and thus prohibit automatic analysis. The aim was to develop a robust, minimal sample pretreatment affording method which is suitable for the automated and simultaneous determination of NE, E, DA, 5-HT as well as MHPG, DOPAC, HVA and HIAA without interference with alpha-methyl-p-tyrosine in crude extracts of minute brain tissue samples.

MATERIALS AND METHODS

Chemicals

L-epinephrine hydrochloride, L-epinephrine barbitrate, 5-hydroxyindolacetic acid and alpha-methyl-p-tyrosine-methylester hydrochloride were purchased by Regis Chemical Company (Morton Grove, IL, USA), dopamine hydrochloride, homovanillic acid, 5-hydroxytryptamine and L-octanesulfonic acid sodium salt monohydrate were obtained from Fluka (Neu-Ulm, Germany), 3-methoxy-5-hydroxy-phenylglycol hemipiperazine, L-isoprenaline hydrochloride and 3,4-dihydroxyphenyl acetic acid from Sigma (Munich, Germany). Stock solutions of the reference compounds were prepared by dissolving 200 pmol/ml in the mobile phase (see below). Water and methanol were of HPLC grade (Baker, Groß-Gerau, Germany).

Animals

Rats of the strain Han: SPRD (Zentralinstitut für Versuchstierzucht, Hannover, Germany) were supplied with food and water *ad libitum*. The animals were kept at 21°C with 12 h light and 12 h dark.

Tissue Sample Extraction

The animals were killed by decapitation and the brains removed to dissect (0°C) distinct brain areas, the preoptic area (POA), mediobasal hypothalamus (MBH), prefrontal cortex (COR) and amygdala (AMY). Each tissue sample was homogenized using a 1-ml glass tube and a motor-driven PTFE pestle in 450 μl ice-cold mobile phase spiked with 36 pmol L-isoprenaline as internal standard. An aliquot was removed for protein determination using bovine serum albumin as internal standard. The protein concentration was determined in the homogenate using bovine serum albumine as internal standard.[7] The tissue homogenates were centrifuged (10 min at 10000 x *g*) and the supernatants filled into 1-ml vials (brown glass) with PTFE lined crimp caps (Chromatograpie Service, Eschweiler-Weisweiler, Germany). The samples were stored at -20°C until assayed. They could be stored for at least 3 months without significant losses of any compound of interest.

Chromatography

The chromatographic apparatus was equipped with a 2156 solvent conditioner (Pharmacia LKB, Bromma, Sweden), a 2150 HPLC pump (Pharmacia LKB), a 0.2 μm graphit filter element (ESA, Bedford, MA, USA), a 5020 Guard cell (ESA) to oxidize impurities in the mobile phase at +0.35 V, a SPH 125 automatic sample injector (Pharmacia LKB) equipped with a model 7010 Rheodyne valve and a 100 μl sample loop. Residual microparticles in the sample were removed by an inlet filter (Knauer, Berlin, Germany) placed between the injection valve and the analytical column. The separations were performed on a C_{18} reversed phase analytical column (250 x 4 mm I.D.) with

an integrated precolumn (5 x 4 mm) both filled with Nucleosil 100 (Macheray und Nagel, Düren, Germany) of either 5 μm or 3 μm particle size. Unless otherwise indicated the temperature was kept stable at 22 $\pm$ 1°C using a column oven (Gynkotek, Munich, Germany). Another 0.2 μm graphit filter element (ESA) was placed between the column and the electrochemical detector (Model 5100A, ESA). A dual coulometric-amperometric detector cell, type 5011 (ESA) was used. The applied potentials were +0.02 V at detector 1 and +0.35 V at detector 2. The signals from detector 2 were registered and integrated by a D2000 chromato-integrator (Merck-Hitachi, Darmstadt, Germany). Isocratic elution was performed at flow-rates of 0.6 or 1 ml/min, not exceeding a back pressure of 340 kg/cm^2. The mobile phase, pH 4.7, comprised of 0.1 M sodium acetate, 25 mM citric acid, and 134 mM ethylenediaminetetraacetic acid (EDTA), 6% (v/v) methanol and 230 μM octanesulfonic acid. Prior to use, the eluent was filtered through a 0.2 μm membrane filter (Millipore, Dreieich, Germany). The entire system was operated in an air conditioned room at 18°C.

RESULTS

Registration of hydrodynamic voltammograms for all compounds of interest revealed that an electrode potential of +0.02 V for detector 1 and of +0.35 V for detector 2 provided high sensitivity and low baseline noise. Substances with a catechol or 5-hydroxyindol structure generated detector signals at relatively low oxidation potentials while the 0-methylated derivatives, MHPG and HVA, required potentials of at least +0.25 V to obtain measurable detector signals (Fig. 1). In contrast, the signal of alpha-methyl-p-tyrosinemethylester was only measured at oxidation potentials above 0.35 V starting from 0.4 V.

Using column packages with 5 μm partical size serotonin and the catecholamines were well separated (Fig. 2). However, the separation of MHPG and DOPAC was unsatisfactory (Fig. 2). When injecting a standard mixture, both compounds were eluted in a single peak. This could neither be improved sufficiently by increasing, nor by decreasing the methanol content, or other constituents of the eluent (Fig.4), or by changing the column

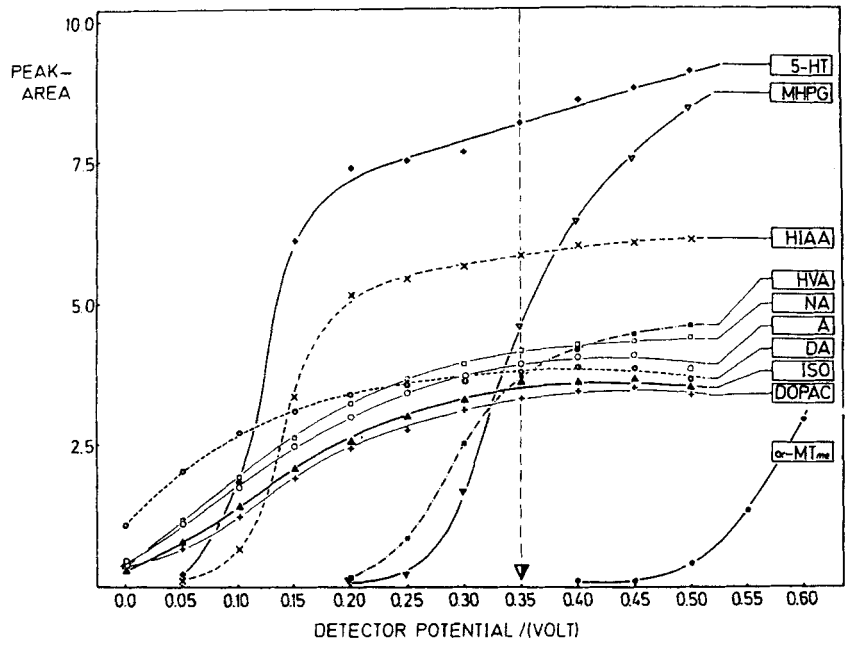

FIGURE 1: Hydrodynamic voltammograms of serotonin (5-HT) 3-methoxy-4-hydroxyphenylethyleneglycol (MHPG), 5-hydroxyindolacetic acid (HIAA), homovanillic acid (HVA), L-norepinephrine (NE), L-epinephrine (E), dopamine (DA), L-isoprenaline (ISO), 3,4-dihydroxyphenylacetic acid (DOPAC) and alpha-methyl-p-tyrosine-methylester (alpha-MT). The arrow indicates the oxidation potential selected for routine measurements.

temperature. Better separations than that shown in Fig. 2 were sometimes obtained using other batches of the 5 μm Nucleosil 100 material.

MHPG and DOPAC could be well separated. Batch to batch variations for columns filled with material of a particle size of 3 μm (Fig. 2) were not observed and baseline resolution of all compounds of interest could be obtained for more than 8 weeks.

Elution from the HPLC columns could be accelerated and the back pressure of the HPLC column decreased when increasing the temperature (Fig.

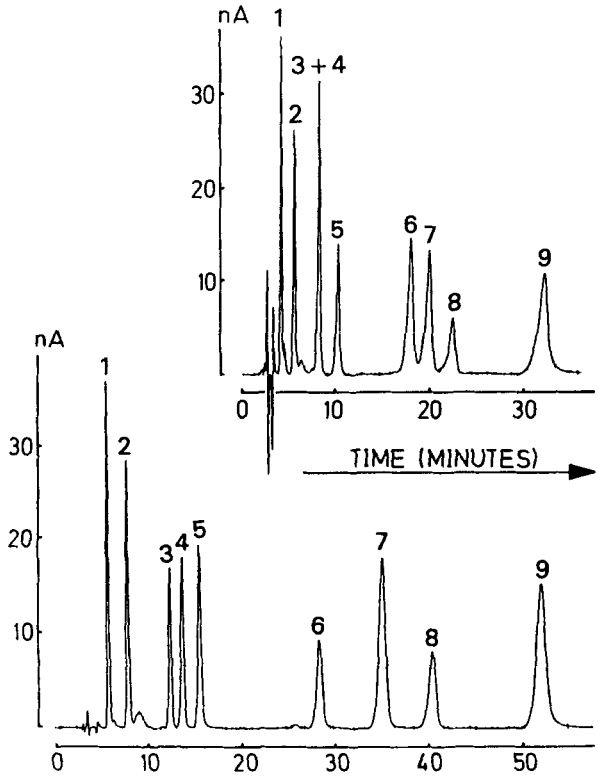

FIGURE 2: Representative chromatograms of standard solutions containing catecholamines, serotonin and metabolites (for abbreviations see legend to Fig. 1). Peaks: 1=NA; 2=A; 3=MHPG; 4=DOPAC; 5=DA; 6=ISO; 7=HIAA; 8=HVA; 9=5-HT. Separation was performed on a C_{18} column (250 x 4 mm I.D.) using Nucleosil 100 material of 5 μm (upper panel) or 3 μm (lower panel) particle size. The flow-rates were 1 ml/min (5 μm) or 0.7 ml/min (3 μm).

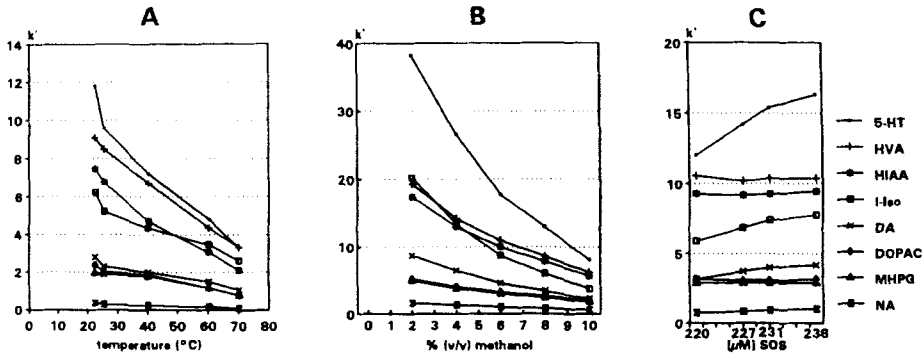

FIGURE 3: Effects of (A) column temperature or the content of (B) methanol or (C) octanesulfonic acid (SOS) in the eluent on the capacity factors of serotonin, catecholamines and their metabolites separated on a 5 µm C_{18} column. For abbreviations see legend to Fig. 1.

1a). These manipulations, however, impaired the resolution on both, 5 µm (Fig. 3a) and 3 µm (data not shown) stationary phases. A column temperature of 21 ± 1°C was considered optimal.

Minimal changes of the composition of the mobile phase, markedly interfered with the separation of the amines (Fig. 3b and c). An increase in the concentration of methanol led to a decrease in k'values for all compounds without affecting the order of elution except that of isoprenaline. Optimal resolution was achieved at a methanol concentration of 6% (v/v). Minimal changes in octanesulfonic acid concentrations in the eluent interfered with the retention of amines, especially that of serotonin without altering those of MHPG, DOPAC, HIAA or HVA. 230 µM of ion pair additive was found be optimal (Fig. 3c).

Washing of the analytical column with methanol which is frequently recommended before using the final eluent or discontinuation of the flow for several hours highly affected the quality of amine separation (Fig. 4). When two 3-h washes in absolute methanol preceded HPLC separation of a standard mixture the retention of DA, ISO and 5-HT on the analytical column decreased while the sequence of elution of acetic or neutral amine metabolites was not

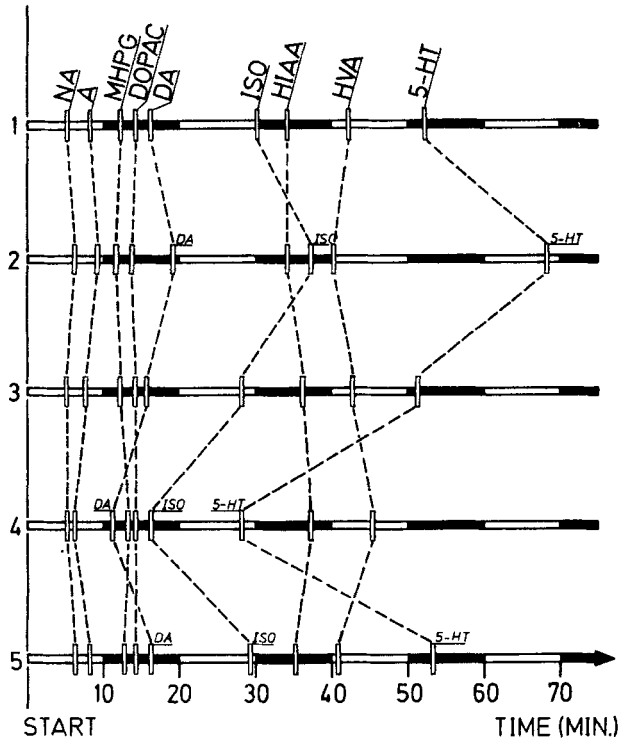

FIGURE 4: Alterations in the sequence of elution of catecholamines, serotonin and their metabolites before and after sequential manipulations. The open bars on the x-axis indicate the position of peaks and the run time of the different compounds eluted during the HPLC run. For each chromatographic separation the identical standard solution was used. Axis

(1) derived from the original chromatogram,
(2) pattern obtained after a 12-h stagnation of the flow of the HPLC-system and re-chromatography,
(3) pattern registered after a 3-h washing period using 100 % (v/v) methanol before re-chromatography,
(4) pattern obtained after re-chromatography following additional 3 h washing period with 100% (v/v) methanol and
(5) sequence of elution after another stagnation of the system for 12 h.

Before re-chromatography following the methanol washes or flow stagnation (2 to 3 and 3 to 4) the column was equilibrated for 1 h with eluent.

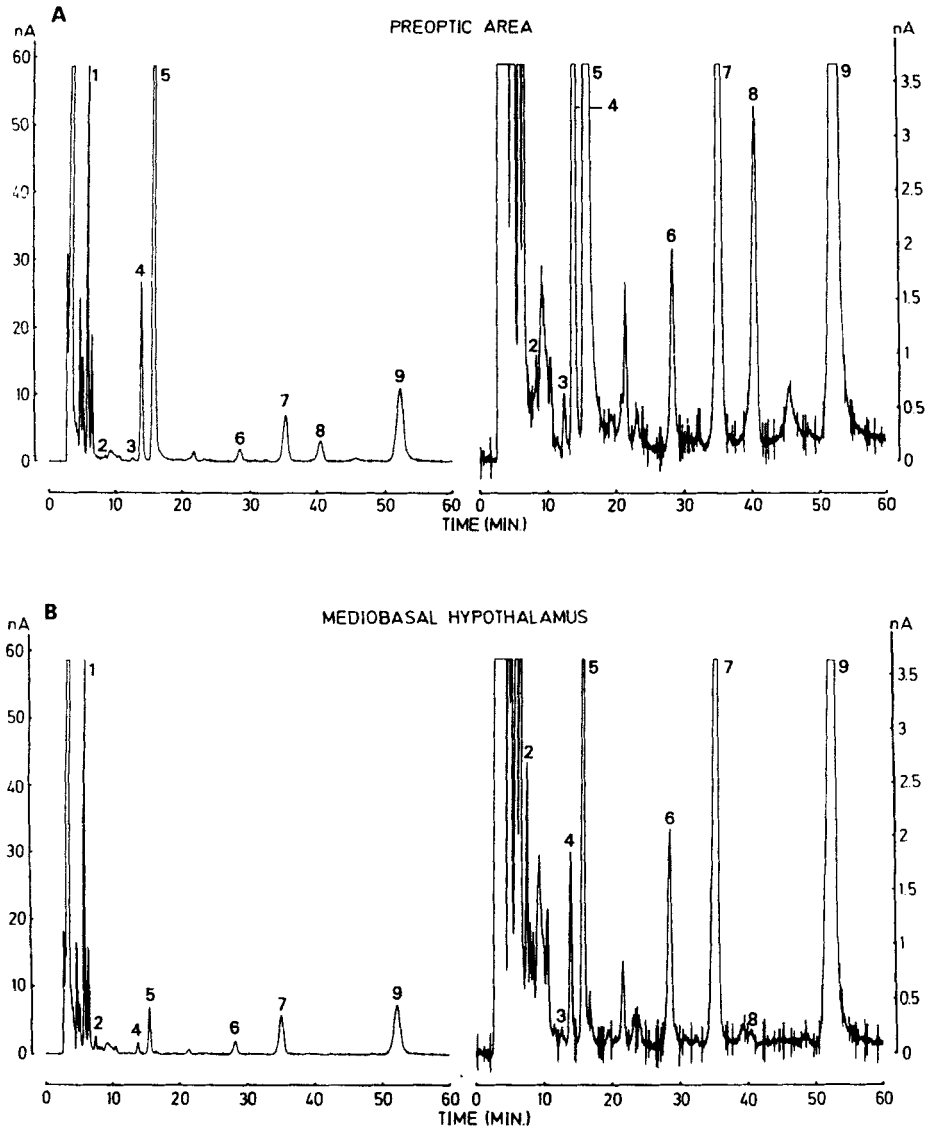

FIGURE 5: Representative chromatograms of rat brain tissue samples obtained from 4 microdissected brain areas, (A) preoptic area (POA), (B) mediobasal hypothalamus (MBH), (C) corticomedial amygdala (AMY) and (D) cerebral cortex (COR) of a female rat. For numbering of peaks see legend to Fig. 2.

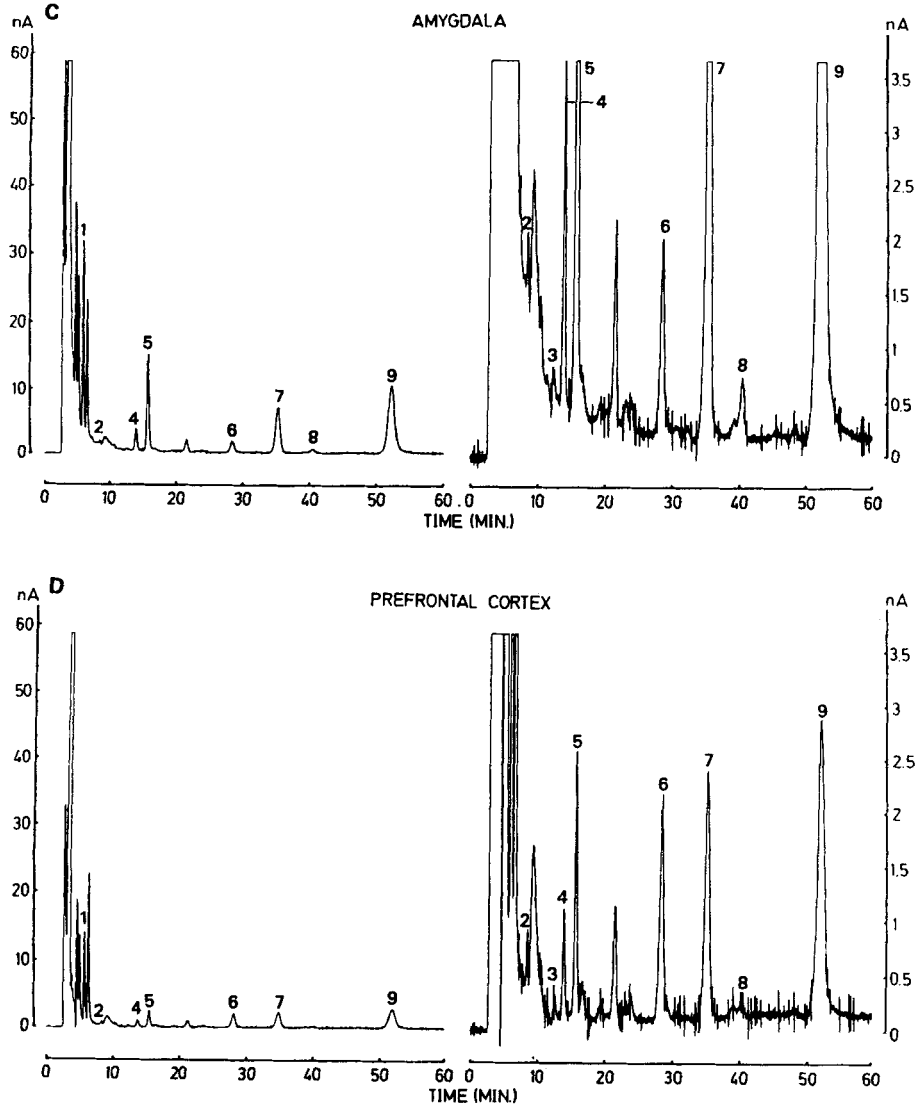

FIGURE 5 (continued)

Table 1

Concentration of Catecholamines, Serotonin and their major Metabolites in distinct Brain Areas of female Rats Values given (pmol/mg protein) are means ± S.D., n = 8.

	Brain area		
	COR	MBH	POA
Norepinephrine	31.5 ± 0.81	115.0 ± 3.0	89.1 ± 31.7
Epinephrine	n.d.	2.94 ± 1.27	1.4 ± 0.58
Methoxyhydroxy-phenylethylene-glycol	1.25 ± 0.5	2.29 ± 0.88	3.2 ± 2.6
Dihydroxyphenyl-acetic acid	1.3 ± 0.27	5.16 ± 5.4	43.5 ± 16.0
Dopamine	3.7 ± 1.39	24.8 ± 5.4	254.8 ± 35.8
Hydroxyindol-acetic acid	6.17 ± 1.3	18.64 ± 1.85	21.8 ± 8.8
Homovanillic acid	n.d.	10.74 ± 0.28	19.7 ± 10.1
Serotonin	7.9 ± 2.2	32.11 ± 4.47	46.7 ± 18.4

COR = Cerebral cortex, MBH = mediobasal hypothalamus, POA = preoptic area.

affected significantly. On the other hand, intermediate discontinuation of the eluent for flow for 12 h without other manipulations resulted in an increase in elution time for DA, ISO and 5-HT (Fig. 4). The altered retention times of the amines remained constant for 4 to 6 weeks after the manipulations.

The assay linearity was checked by linear regression analyses of six independent calibration curves derived from standard solutions containing 0.5 to 50 pmol/100 μl. Linear regression analysis indicated no significant deviation from linearity for any of the compounds. Calculating the peak areas relative to that of ISO as internal standard, the correlation coefficients were computed as ranging between 0.9979 for HVA and 0.9997 for NA. The curve intercepts were calculated as -1.64 and 11,6 for NA, -1.7 and 11.6 for A, -1.42. and 11.6 for DA, -1.74 and 11.6 for 5-HT, -1.65 and 11.6 for MHPG, -1.66 and 11.6 for DOPAC, -1.74 and 11.6 for HVA -1.84 and 11.6 for HIAA. The detection limits were in the range of 25 fmol/100 μl for all compounds. Coefficients of variation (CV)

calculated from 12 consecutive injections of standard solution containing 20 pmol/100 μl resulted in CV ranging from 3 to 4.6.

When using the chromatographic conditions described above optimal baseline separation and sensitive detection on the dual electrode electrochemical detector, which remained stable for at least 8 weeks after equilibration of the HPLC system, it was possible to detect and quantitate all compounds of interest in as little as 0.5 mg brain tissue wet weight. Moreover, minimal sample pretreatment was sufficient to extract the amines and their metabolites. When microdissected brain areas such as the preoptic area, mediobasal hypothalamus or part of the frontal cortex were homogenized in about 10 to 30 volumes of the eluent NE, E, MHPG, DOPAC, DA, HIAA, HVA and 5-HT were measurable (Fig. 5). Automated analysis of a series of crude brain tissue extracts revealed concentrations ranging between 1.2 (MHPG) and 255 (DA) pmol/mg protein (Table 1).

DISCUSSION

The presented minimal sample pretreatment affording HPLC technique proved to be robust enabling automated, simultaneous determination of NE, DA and 5-HT and their metabolites DOPAC, HVA and HIAA but nevertheless the critically low concentration of MHPG within a single chromatographic run using minute brain tissue samples. Moreover, our procedure excludes alpha-MT, exogenously applied in turnover studies, as a disturbing factor. The method described here is the outcome of the fine-tuning of parameters that determine the quality of HPLC separations and sensitive electrochemical detection. Optimal conditions were elaborated for the setting of oxidations potentials, the concentration of ion-pairing reagent and organic modifier in the mobile phase, for the particle size of the stationary phase and for the column temperature.

Mobile Phase

As expected, methanol enhanced the elution of all compounds whereas octanesulfonic acid increased the retention of amines only. In addition to the

findings reported by others[8] it was shown that even micromolar changes in the concentration of the ion pairing reagent markedly affected the elution.

Stationary Phase

The major improvement in resolution was achieved by introducing a column filled with material of 3 μm particle diameter instead of 5 μm sized material commonly applied by others.[6,9,10] Using the small particle size the plate number increased from 5,250 to 11,000 for 5-HT. In contrast to 5 μm materials batch to batch variabilities of the 3 μm particle sized Nucleosil packages were not critical. In case of suboptimal resolution changes of the octansulfonic acid content was sufficient to optimize the separation and to avoid interferences.

Temperature

Changes in temperature had different effects upon the different compounds under investigation. The introduction of a column oven was essential to achieve the maintenance of a constant temperature which was a prerequisite for the reproducibility of elution patterns. The resolution of MHPG and DOPAC increased with decreasing temperature which enhanced the back pressure due to the increased vicosity of the eluent. To avoid excessive back pressure the flow was limited not to exceed 340 kg/m^2.

Most surprisingly, manipulation such as washing of the column with absolute methanol or intermediate discontinuation of the eluent flow for several hours had marked effects on the elution of amines especially dopamine and serotonin. Such manipulations are recommended either to wash out impurities or to prepare columns prior to their application or to minimize abrasion of the HPLC system. The alterations in amine retention were reproducible when using Nucleosil-100 material. It seems difficult to give reasonable explanations for the underlying chemical mechanisms. Moreover, we do not know at the moment whether the phenomena are restricted to Nucleosil material. Since alterations after methanol washing or shutting down

the flow were restricted to amines it seems likely that residual acidic groups on the silica material might have been involved.

Handling

In addition to the robust chromatographic conditions the simplicity of the described HPLC technique is pertinent avoiding time consuming preextraction. Following simple deproteinization by homogenizing the brain tissue samples in the eluent including ISO as internal standard the supernatants could be directly injected into the HPLC-system leading to high recovery rates ranging from 88.2 to 99.4%.[6,11] In additional advantage, the levels of compounds under investigation remained constant for four months at -20°C. A variety of similar techniques useful for determination of catecholamine, serotonin and metabolite concentrations in brain tissue samples has been described in the literature. None of these methods, however, meets all the requirements for automated and simultaneous determinations of NE, E, DA, 5-HT and their metabolites MHPG, DOPAC, HIAA and HVA in a single HPLC run. Many of the described procedures have the disadvantage of working at oxidation potentials measuring the signal of alpha-MT, too[9-12], or exhibit a reduced resolution thus preventing a reliable quantification of compounds such as MHPG which are eluted too close to the void volume.[10,12] To overcome the problems related to the inability to detect the presence of co-eluting substances adequately, two different eluents or an extensive extraction procedure for brain tissue samples proved to be necessary.[9,13-16] In some cases HPLC equipment was supplemented with either an additional analytical cell[17] or a second analytical column[18] leading to an increased maintenance rate which is in contrast to the non-stop function of the apparatus for a large number of automated determinations during routine use.

In conclusion, the application of 3 μm stationary phase material combined with the conditions described here, allows automated and simultaneous determination of NA, DA, 5-HT and their metabolites MHPG, DOPAC, 5-HIAA and HVA in femtomol quantities even in the presence of large amounts of alpha-MT without frequent interventions by the operators for more than 2 months. Our method is as accurate, precise and reproducible as any published

procedure and offers the advantage of a minimal sample pretreatment and an automated operation which may not be restricted to the quantification of neurotransmitter and/or metabolite concentrations from brain homogenates but also useful for other body fluids such as extracellular fluids lately withdrawn *in vivo* by push pull perfusions[19] or microdialysis.

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