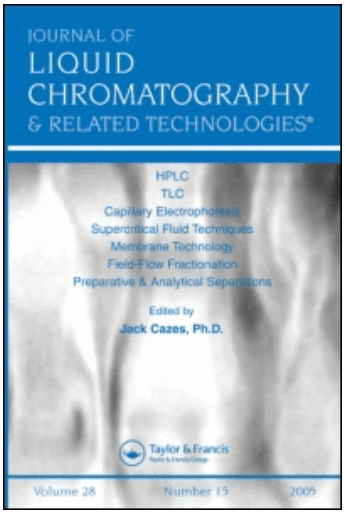

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Analysis of Trace Amounts of Catecholamines and Related Compounds in Brain Tissue: A Study Near the Detection Limit of Liquid Chromatography with Electrochemical Detection

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To cite this Article Westerink, Ben H. C.(1983) 'Analysis of Trace Amounts of Catecholamines and Related Compounds in Brain Tissue: A Study Near the Detection Limit of Liquid Chromatography with Electrochemical Detection', Journal of Liquid Chromatography & Related Technologies, 6: 12, 2337 — 2351
To link to this Article: DOI: 10.1080/01483918308064910
URL: http://dx.doi.org/10.1080/01483918308064910

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(12), 2337-2351 (1983)

ANALYSIS OF TRACE AMOUNTS OF CATECHOLAMINES AND RELATED COMPOUNDS IN BRAIN TISSUE: A STUDY NEAR THE DETECTION LIMIT OF LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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ABSTRACT

Analysis of catecholamines and related metabolites is often based on direct injection on the HPLC column of supernatants of centrifuged brain homogenates. In this study we have investigated to what extent direct injection techniques are also useful for analysis in the picogram-range. The use of Sephadex G 10 (which allows a rapid sample purification for 11 compounds) appeared an attractive alternative when direct injection techniques cannot be applied. Various aspects of the routine use of the electrochemical detector at a sensitive setting are discussed. Finally the identity of small peaks in the chromatogram is addressed.

INTRODUCTION

An increasing number of methods based on liquid chromatography and electrochemical detection (LC-ED) have been described for analysis of monoamines and related metabolites in brain tissue (1,2). These methods were mainly concerned with the assay of the neurotransmitters dopamine (DA), noradrenaline (NA), 5-hydroxytryptamine (5-HT) and their metabolites 3,4-dihydroxyphe-

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nylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindolacetic acid (5-HIAA) in various brain regions. Concentrations of these compounds are in the nanogram to microgram-range (0.2 - 12 µg/g; amounts of tissue available usually less than 50 mg). However, various other metabolites of DA and NA such as 3-methoxytyramine (3-MT), 3,4-dihydroxyphenylethyleneglycol (DOPEG), 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG), 3,4-dihydroxyphenylalanine (DOPA) and normetanephrine (NM) are found in the brain in the picogram-range (3-30 ng/g). In the same order of magnitude are the levels of DA, DOPAC and HVA in the so-called non-dopaminergic areas (brain regions with a relatively small dopaminergic innervation such as the frontal cortex, stem, hippocampus and cerebellum). Quantification of these amounts, which are two orders of magnitude less than the usually studied contents, require a careful analytical approach as these assays reach the detection limits of the routinely performed LC-ED techniques.

Various authors have described attractive methods based on direct injection on the HPLC column of the supernatant of centrifuged brain homogenates (3,4,5). These methods combine an easy sample handling with a high analytical recovery, and allow quantification of a large series of compounds (4-6) in a single chromatographic run. However, when this analytical approach is applicated to the analysis in the pg-range, interfering peaks in the chromatograms are to be expected. Purification of the brain samples is the classical answer to this analytical problem, and a number of purification procedures for monoamines and related metabolites are found in the literature (6,7,8).

In this study we have investigated to what extent direct injection techniques are also useful for analysis in the pg-range. When these techniques could not be used, pretreatment of samples on Sephadex G 10 was performed. This paper is also concerned with two aspects which require special care when the assay is in the pg-range: the routine use of an electrochemical detector at the highest sensitive setting, and the identity of small peaks in the chromatogram.

METHODS

Reagents

Materials and their sources were as follows: dopamine.HCl, noradrenaline bitartrate, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylethyleneglycol, normetanephrine (Sigma), 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 5-hydroxyindolacetic acid (Fluka). All other chemicals were of analytical reagent grade and were purchased from E. Merck. All aqueous solutions were prepared from deionized water distilled in glass. Stock solutions of

the various compounds consisted of 100 µg/ml in 0.01 M formic acid and 0.1 mM EDTA and were stored in portions of approx. 1 ml in the freezer (-80°). Standard solutions were prepared every 2 weeks from a portion of the stock solutions after appropriate dilution in 0.01 M formic acid and 0.1 mM EDTA. Ascorbic acid (final concentration 0.01 mM) was added to the stock solution of 5-HIAA. The mobile phases used for the various assays are summarized in Table 1. The solutions were filtered before use (0.5 µm Millipore filter).

Animals, drug treatment and dissection

Male albino rats of 175-200 g weight (Wistar, C.D.L., Groningen, The Netherlands) were used. Pargyline.HCl was administered intraperitoneally. The animals were killed by decapitation. The brain structures dissected included the frontal cortex, striatum, hypothalamus, hippocampus, brain stem and cerebellum. The tissue samples were frozen on solid CO_2. Samples were kept at -80°C until assayed.

Isolation procedure on Sephadex G 10

Several columns (at least 40) can be handled in one run with the help of automated pipettes. Before use the columns were washed with 3.0 ml 0.02 M ammonia and 3.0 ml 0.01 M formic acid. Tissue samples (up to 150 mg) were homogenised in 1.0 ml 0.1 M perchloric acid. Following centrifugation (4000 g; 4°C) the supernatants were put on Sephadex G 10 columns (5 x 70 mm) prepared in long-size Pasteur pipettes. Two different routes of purification are possible (route I and route II).

Route I: After tissue extracts have passed through the columns, 1.0 ml 0.01 M formic acid and 1.5 ml 0.02 M ammonia (containing 0.01 mM ascorbic acid and 0.1 mM EDTA) were added. NA, DA, 3-MT, DOPAC, HVA and 5-HIAA are then eluted with 1.5 ml 0.02 M ammonia (containing 0.01 mM ascorbic acid and 0.1 mM EDTA). Because of instability of catecholamines in ammonia this fraction is collected in a vial to which 50 µl concentrated formic acid was added.

Route II: After tissue extracts have passed through the columns, 2.0 ml of a solution of 0.01 M formic acid and 0.1 mM EDTA was added. Tyrosine (TYR), DOPA, DA, NA, NM, 3-MT, DOPEG and MOPEG were then eluted with 1.0 ml of the formic acid solution followed by 1.5 ml of a phosphate solution (5 mM $Na_2HPO_4.2H_2O$ and 0.1 mM EDTA). DOPAC, HVA and 5-HIAA were subsequently eluted with 2.0 ml of a solution containing 0.02 M ammonia, 0.1 mM EDTA and 0.01 mM ascorbic acid. This fraction was collected in a vial to which 50 µl concentrated formic acid was added.

The columns are stored in 0.02 M ammonia. The isolation procedure is summarized in Fig. 1. The columns do not need regeneration; they are refilled after 6 months use.

Table 1.

Optimised chromatographic conditions for the various assays.

Compound	% methanol	type eluent	pH	potential setting (mV)
TYR	0	acetate/phosphate	5.8-6.0	850
DOPA	0	0.1 M TCA	3.1	550
DA, NA, DOPAC, 5-HIAA	15-20%	0.1 M TCA	3.9	550
HVA, 3-MT	15-20%	0.1 M TCA	3.9	700
free DOPEG, total DOPEG	0	citrate/phosphate	4.8-5.9	550
		or		
		acetate/phosphate	4.6-5.0	
free MOPEG, total MOPEG	0-3%	acetate/phosphate	4.6-5.0	750
NM	5	0.1 M TCA	3.4	850

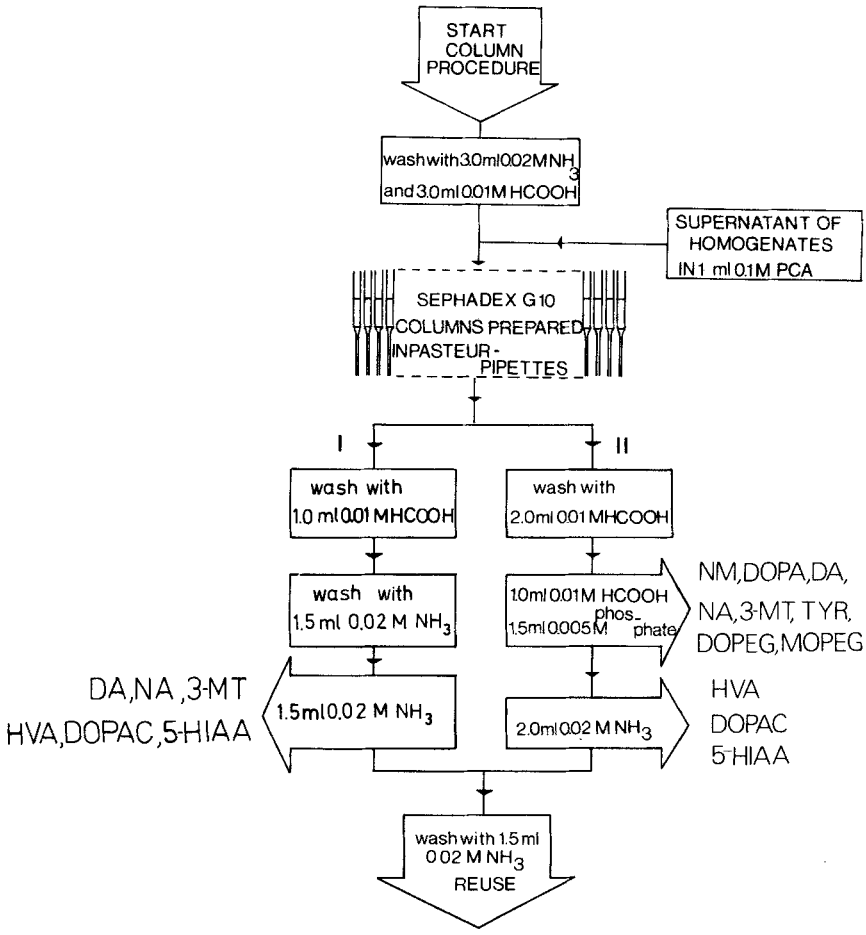

Fig. 1. Flow chart of the isolation procedures.

Chromatography

A Waters (model 6000A) liquid chromatograph was employed in conjunction with an electrochemical detector. A detector based on a rotating disc electrode was used (9). A potentiostat from Bioanalytical Systems, type LC-2A, has been used. The detector potentials for the various assays are summarized in Table 1. The column (150 x 4.6 mm i.d.) was packed with a slurry of Nucleosil 5 C 18 (Mackerey-Nagel, Düren, F.G.R.) reverse-phase material in methanol/carbon tetrachloride (20/80, v/v). The slur-

ry (10% w/v), degassed in an ultrasonic generator, was pumped into the chromatographic column at the highest possible flow rate. Columns were washed by passing 200 ml of methanol and further equilibrated with the mobile phase. Columns were refilled after 5-6 months of use. To maintain the integrity of the microparticulate column a precolumn (70 x 4.6 mm i.d.; filled with Nucleosil 5 C 18) was placed between the pump and the injection valve. The precolumn was packed according to the same procedure as the analytical column. Analyses were performed at a flow rate of 60 ml/h at room temperature. When supernatants of brain homogenates were directly injected into the chromatograph, a guard column filled with reverse phase material (Chrompack; 75 x 2.1 mm i.d.) was placed between the injection valve and the analytical column. Material was injected with a high-pressure injection valve (Rheodyne) fitted with a 50-500 µl sample loop. Concentrations in brain samples were calculated with the aid of (linear) calibration curves obtained after the injection of pure standards. Recoveries of the various compounds were determined by analysing spiked brain tissue. If the Sephadex eluates had to be preserved till the next day, they were stored at 4°C. The chromatographic conditions for the various assays have been described earlier (9,10,11,12) and are summarized in Table 1.

RESULTS AND DISCUSSION

The necessity of Sephadex G 10 as purification step

HPLC of catecholamines and related compounds is usually carried out with alkylsulphonate-containing mobile phases and a reverse phase column (1,2,3,4,5). Alkylsulphonates give the reverse phase material cation-exchanging properties ("soap"-chromatography) and for this protonated catecholamines are strongly retarded on the HPLC column. Asmus and Freed (13) have described the use of trichloracetic acid (TCA) containing eluents as alternative to the alkylsulphonates. TCA is thought to form ion-pair complexes with protonated catecholamines and these ion-pairs are retarded on the reverse phase material. It is our experience that the use of TCA-solutions results in a somewhat better chromatographic separation of the studied compounds when compared with eluents based on alkylsulphonates. Moreover the noise level of the detector signal was more favourable in the case of TCA-solutions. During this study we have developed a TCA-containing eluent which could be used for simultaneous assay of 6 compounds. When the pH of the eluent was 3.9 ± 0.1, separation was achieved between NA, DOPAC, DA, 5-HIAA, HVA and 3-MT (Fig. 2). In Fig. 2[a] a chromatogram obtained after direct-injection of a homogenate of striatal tissue is depicted.

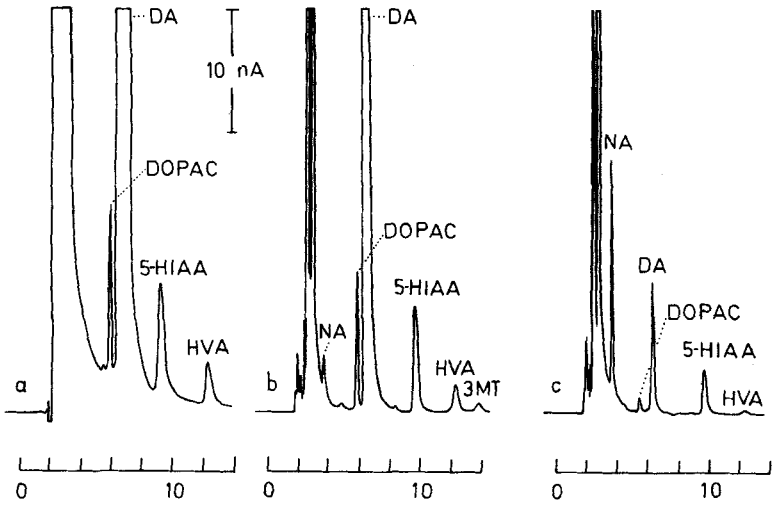

Fig. 2. (a) Chromatogram of 40 mg striatal tissue after direct injection of centrifuged homogenate. (b) Chromatogram of 48 mg striatal tissue after isolation on Sephadex G 10 (route I). (c) Chromatogram of 42 mg hypothalamic tissue after isolation on Sephadex G 10. Chromatographic conditions: mobile phase: 78% 0.1 M TCA, pH 3.9, 22% methanol; flow 1.0 ml/min; 100 µl sample loop; potential setting 750 mV.

It is clear from this figure that in case of striatal tissue the direct-injection method is acceptable for quantification of DOPAC, DA, 5-HIAA and 5-HT; but not for NA. The 5-HT and 5-HIAA content is in the same order of magnitude throughout the brain (Table 2), which means that for these compounds the direct injection technique is always the method of choice. However, in several areas of the brain the concentrations of DA, DOPAC and HVA are 20-100 times lower than in the striatum (Table 2). When these amounts are to be determined attenuation of the detector signal to 20-100 times that of Fig. 2 is necessary. Fig. 2 indicates that at such a sensitive detector setting the broad and tailing front of the chromatogram will seriously disturb the quantitation of DA, DOPAC and (to a less extent) HVA. The quality of the chromatogram was much improved when the brain samples were pretreated on Sephadex G 10 columns (Fig. 2b,c). Note that quantification of NA is now also possible. Only 5-HT cannot be determined as this compound is strongly bound to the Sephadex resin. The assays of the NA metabolites DOPEG and MOPEG are other examples of assays in which direct injections of homogenates cannot be used. Retardation on a reverse-phase column with TCA or alkylsulphonate is

Table 2.

Concentrations of the various compounds in 6 brain areas of decapitated rats. The values are given in ng/g ± S.E.M. (n: 4-6), except for TYR which is given in µg/g.

Compound	Striatum	Hypothalamus	Frontal Cortex	Hippocampus	Stem	Cerebellum
TYR	12.1± 0.3	11.8± 0.5	10.8± 1.3	n.d.	n.d.	11.8± 0.9
DOPA	7.3± 0.4	7.7± 0.6	4.3± 0.2	n.d.	n.d.	3.6± 0.2
DA	9856 ±92	246 ±92	52 ± 3.1	4.6± 0.3	51 ±6	2.8± 0.2
DOPAC	873 ±42	35 ± 2.5	52 ± 3.3	3.0± 0.3	11.8±0.3	2.2± 0.2
HVA	1044 ±27	34 ± 4.5	67 ± 9.1	5.6± 0.3	20.0±1.8	8.0± 1.4
3-MT*	20.5± 0.9	< 1	< 1	< 1	< 1	< 1
NA	136 ±18	1450 ±58	237 ±58	352 ±61	n.d.	258 ±31
NM	4.3± 1.0	13.8± 2.6	8.0± 1.0	12.9± 1.3	7.9±1.0	12.6± 1.3
free DOPEG	7.2± 0.5	16.2± 3.5	n.d.	11.9± 1.1	10.7±1.3	15.0± 3.2
total DOPEG	25 ± 0.5	170 ± 2.8	n.d.	39 ± 2.5	55 ±5.4	49 ± 6.7
free MOPEG	11.3± 1.8	19.5± 3.8	n.d.	15.7± 2.1	38.7±4.0	45.9± 4.0
total MOPEG	61 ± 6.5	117 ±12.0	n.d.	78 ± 6.9	122 ±4.3	66 ± 1.4
5-HIAA	282 ±23	462 ±35	152 ±23	272 ±23	n.d.	42 ± 4

*: rats killed by micro-wave.

n.d. = not determined.

not possible with alcoholic derivatives such as DOPEG or MOPEG. That pretreatment of samples on Sephadex G 10 is required for quantification of DOPEG is illustrated in Fig. 3[a] and 3[b]. Determination of free DOPEG was achieved with a phosphate-acetate buffer (Table 1; manuscript in preparation). The need of sample purification was also established for the determination of DOPEG sulphate, free MOPEG, MOPEG sulphate, DOPA and NM (results not shown).

The procedure of purification of samples on Sephadex G 10 columns is depicted in Fig. 1. Two different routes are possible. Route I, which was developed during this study, concentrates DA, NA, 3-MT, tyrosine (TYR), DOPAC, HVA and 5-HIAA in a relatively small fraction of 1.5 ml. DOPEG and MOPEG are not quantitatively recovered in this fraction, whereas chromatographic separation of NM, DOPA and interfering compounds was not complete. Route II has been described in an earlier study (9). Purification via this route is more complete, as the method affords two fractions. The first fraction contains the catecholamines, DOPEG and MOPEG. Endogenous levels of DOPA and NM can also be quantified from this fraction according to the appropriate chromatographic conditions (Table 1). The acidic metabolites are now found in a second

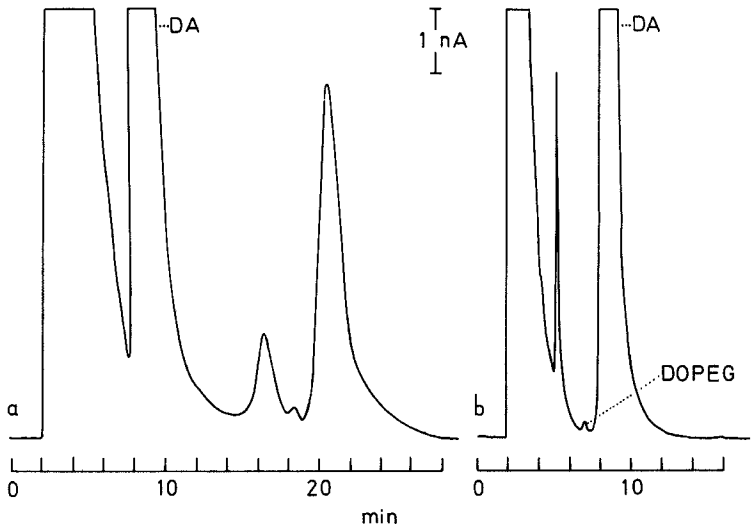

Fig. 3. (a) Chromatogram of 55 mg striatal tissue after direct injection of centrifuged homogenate. (b) Chromatogram of 60 mg striatal tissue after isolation on Sephadex G 10 (route II). Chromatographic conditions: mobile phase citrate/phosphate buffer 0.35 M, pH 5.8; 50 µl sample loop; flow 1.0 ml/min; potential setting 550 mV.

fraction. Route II, which is somewhat more time-consuming than route I, results in a better purification of brain samples. The analytical recoveries of both routes were between 80 and 90%. Internal standards were not used. From 18 rat brains we have analyzed the bilateral striatal structures with the direct injection method (left striatum) and with the method including Sephadex G 10 (route I)(right striatum). The results (µg/g ± S.E.M., n = 18), were, DA: 9.7 ± 0.4 (left) and 9.7 ± 0.3 (right); DOPAC: 0.57 ± 0.03 (left) and 0.55 ± 0.02 (right); HVA: 0.73 ± 0.03 (left) and 0.68 ± 0.03 (right). From these data it is concluded that both methods yield similar recoveries.

In summary, there are several conditions when direct injection techniques cannot be used. The use of small Sephadex G 10 columns is then an attractive alternative. The eluates of the Sephadex procedure consist of relatively clean solutions and allow the detection of small amounts of compounds with minimal interference of other biological substances. Purification on Sephadex G 10 is possible for a total number of 11 compounds. This versatile application is an advantage to currently used purification methods such as alumina pretreatment, ion-exchange chromatography, or solvent-solvent extraction (6,7,8), as the latter methods are restricted to catechol-containing compounds. As the columns can be used during many months and for a variety of assays, they are very suitable for routine determinations. Handling of the columns with the help of automated pipettes takes only some seconds per column. New Sephadex G 10 material may contain some compounds that interfere with the chromatographic separation. These compounds disappear after excessive washing of the resin or during routine use of the columns.

Routine use of the electrochemical detector

In our laboratory the HPLC is carried out with a rotating-disk electrochemical detector. The rotating-disk detector prosesses some advantages over the widely used thin-layer cells that are of importance for determination of compounds in the pg-range (9). The rotation of the electrode results in an increased sensitivity of the detection (by 250-600%, depending of the compound). The sensitivity of the detector is in the range of 3-6 nA/ng for the compounds in this study (50-200 µl sample loop; retention time less than 10 min). The most sensitive setting during routine use was 2 nA/V. If we assume 1% of the full scale value as detection limit, it means that 0.01 x 2 nA = 20 pA can be detected. 20 pA corresponds to 3-7 pg per injection. When a 200 µl sample loop is used and assuming that the compounds are eluted in 1.5 ml, it can be calculated that $\frac{1.5}{0.2}$ x (3-7) = about 25-50 pg/sample is the detection limit of the method during routine use. The di-

lution of the sample during the Sephadex G 10 procedure is a limitation of the method. However, several assays (HVA, 5-HIAA, 3-MT) permit the use of a 500 µl sample loop with virtually no peak broadening.

When pg amounts are to be detected, special attention should be given to the following aspects:

--When various mobile phases were compared, the signal-to-noise levels varied as follows: phosphate/citrate buffer = phosphate/acetate buffer > trichloracetic acid containing eluent > alkylsulphonate containing eluent. In general the noise level increased with a decreasing pH value. It was often noticed that a mobile phase of pH < 4 resulted in an unstable baseline. These baseline fluctuations (in which the pump-pulsation was sometimes recognized) could be overcome by the addition of EDTA to the mobile phase (final concentration: 10^{-4} M). This indicates formation of Fe^{2+} in the HPLC equipment, probably caused by the relatively low pH of the buffer.

--External sources of electrical noise can seriously disturb the baseline of the detector, especially at a sensitive setting. This problem is usually overcome by placing electronic filters between the HPLC equipment and the mains supply.

--The various parts of the HPLC and detector equipment should be earthed properly. A Faraday cage around the detector should be used when the analysis is in the pg-range.

--An unfavourable signal-to-noise level is not always solved by repacking the carbon paste electrode. A carbon electrode can be used during several weeks/months. A high residual current and an instable baseline are indications that the electrode needs repacking (when other sources of baseline noise are excluded).

It should be noted that these precautions do not prevent the fact that often an inexplicable day-to-day fluctuation in the amount of electrical noise was noticed.

Identification of small amounts in chromatograms

Determination of compounds near the detection limit usually means quantification of one of the smallest peaks in the chromatogram. It is therefore of utmost importance to have sufficient certainty about the identity of the peak to be calculated. Apart from the typical chromatographic behaviour, there are in general three possible ways to confirm the identity of these compounds.

a) Determinations of a voltammogram. Chromatograms of both a purified brain extract and a standard solution are recorded at various oxidation potentials. The studied compound and the authentic one should have similar electrochemical behaviour. In Fig. 4 the electrochemical behaviour of endogenous DOPA,

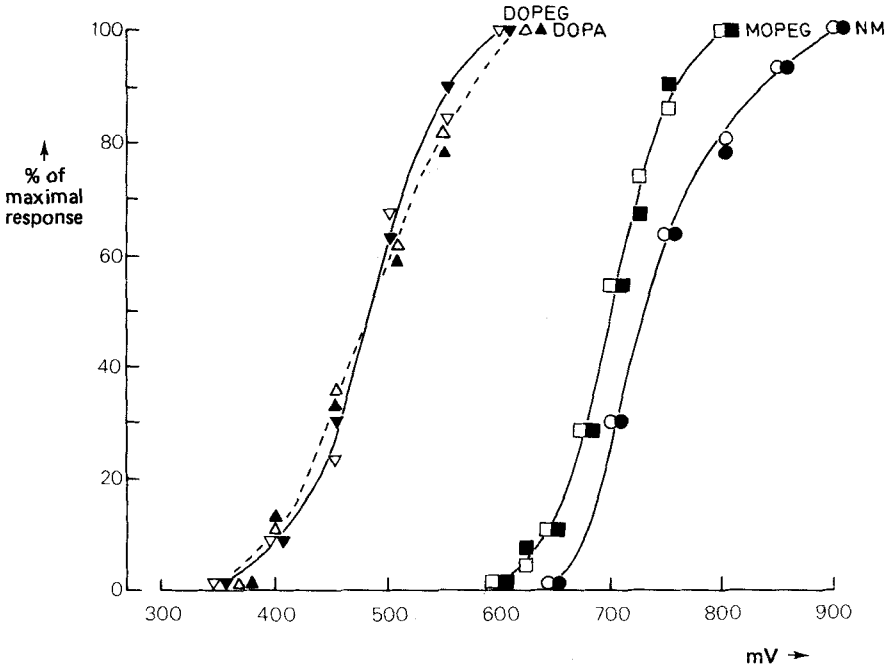

Fig. 4. Influence of the electrode potential setting on the response of authentic DOPA, DOPEG, MOPEG and NM (open symbols) and on the compounds isolated from brain tissue (closed symbols).

free DOPEG, free MOPEG and NM isolated from the brain tissue, is compared with corresponding standard solutions. The similarity of the voltammograms supports the specificity of the assay.

b) Catecholamines and related amino acids are retarded on Sephadex G 10 columns as ion-pair complexes with perchlorate (9). If brain samples are homogenised in 0.1 M HCl instead of perchloric acid, the former compounds do not bind to the Sephadex resin and disappear from the chromatograms (acids such as HVA, 5-HIAA and DOPAC do not require perchlorate and these compounds remain in the chromatogram). This method has been used in our laboratory to support the identity of endogenous DOPA, NM and 3-MT in brain samples.

c) The most elegant way to contribute to the identity of a compound is the use of selective enzyme inhibitors. Inhibition of the enzymatic reaction can remove certain metabolites from the brain (and subsequently from the chromatograms). Concentrations of oxidative deaminated metabolites such as DOPEG, MOPEG,

Table 3.

Effect of monoamine oxidase inhibition (75 mg/kg pargyline, i.p.) on the levels of the oxidative desaminated metabolites of DA and NA in the striatum of rats. Values are given in ng/g ± S.E.M. (n).

Compound	time of pre-treatment with pargyline (h)	controls	pargyline treated
DOPAC	1	873 ±42 (5)	22 ± 5.9 (3)
HVA	1	1044 ±27 (5)	141 ±14.6 (3)
free DOPEG	3	7.2± 0.5 (6)	< 5
total DOPEG	3	25.0± 0.5 (4)	< 5
free MOPEG	3	11.3± 1.8 (4)	< 5
total MOPEG	3	61 ± 6.5 (6)	23.8± 4.2 (3)

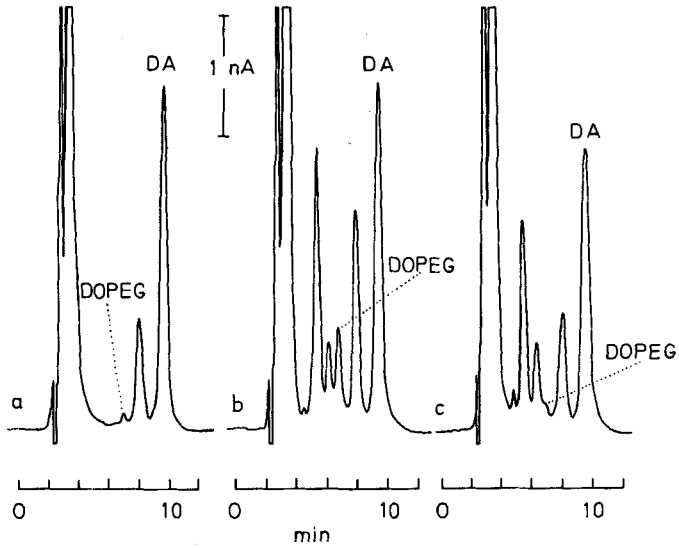

Fig. 5. (a) Chromatogram of 64 mg hypothalamic tissue (50% of the sample). (b) Chromatogram of the remaining 50% of the same sample after thermic hydrolysis (10', 100°C). (c) Chromatogram of 40 mg hypothalamic tissue after thermic hydrolysis (10', 100°C). The rat was pretreated with pargyline.HCl (100 mg/kg) for 3 hours. Chromatographic conditions: isolation on Sephadex G 10 (route II), HPLC conditions see legend to Fig. 3.

DOPAC; HVA and 5-HIAA are considerably reduced after monoamine oxidase inhibition (Table 3). Fig. 5[b] shows the chromatogram of a brain extract after thermic hydrolysis. The hydrolysis induced a pronounced rise in the "DOPEG" peak (probably caused by hydrolysis of DOPEG-sulphate), but the chromatogram does not seem very reliable as various compounds eluted with retention times near that of DOPEG. Pretreatment of the rat with a monoamine oxidase inhibitor (pargyline.HCl; 100 mg/kg), virtually removed "DOPEG" from the chromatogram, thereby confirming its identity (Fig. 5[c]).

Other pharmacological tools which can be used for identification of compounds are tropolone and reserpine. The catechol-O-methyl transferase inhibitor tropolone induces a selective and pronounced decrease (> 90%) in brain concentrations of methylated metabolites such as 3-MT and HVA. Reserpine is known to deplete (> 90%) catecholamine stores in nervous tissue.

ACKNOWLEDGEMETS

The author wishes to thank Mrs. Aaf Westerbrink for valuable technical assistance, and Mrs. Nelly Teeuwen for help in preparing the manuscript.

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