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High-performance liquid chromatography with electrochemical detection for the determination of levodopa, catecholamines and their metabolites in rat brain dialysates

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ABSTRACT

A high-performance liquid chromatographic assay with electrochemical detection is described for the simultaneous determination of levodopa, 3-O-methyldopa, dopamine, dihydroxyphenylacetic acid, homovanillic acid, 3-methoxytyramine, noradrenaline, adrenaline, 3-methoxy-4-hydroxyphenylethylene glycol and 5-hydroxyindoleacetic acid in rat brain dialysates. Samples are obtained *in vivo* using the microdialysis technique. Microdialysis probes are placed in the brain area to be studied and neurochemicals are collected by perfusion of the probe with modified Ringer's solution. Direct injection of the dialysates allows rapid and reliable results to be obtained.

INTRODUCTION

Microdialysis permits the *in vivo* monitoring of drugs and endogenous compounds in the central nervous system (CNS) [1–6]. Most high-performance liquid chromatographic (HPLC) systems with electrochemical detection (ED) reported in the literature describe the analysis in dialysates of a restricted number of monoamines or metabolites [7–10].

In this study, we combined the microdialysis technique with an HPLC system with an amperometric detector for the simultaneous determination of levodopa (L-DOPA), 3-O-methyldopa (3-OMD), dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). With this method it is also possible to study levels of noradrenaline (NA), adrenaline (AD), 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), 5-hydroxyindoleacetic acid (5-HIAA) and 3-methoxytyramine (3-MT).

This system was then applied to a study in which rats with unilateral nigrostriatal lesions (hemi-Parkinson rats) were given 5 mg/kg L-DOPA intraperitoneally (i.p.). L-DOPA, 3-OMD, DA, DOPAC and HVA were monitored by placing microdialysis probes in the left and right caudate nucleus-putamen (NCP) and in left and right substantia nigra, the main origin of the dopaminergic innervation of the NCP. The system was also applied to the study of the *in vitro* characteristics of the microdialysis probes.

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EXPERIMENTAL

Materials

The HPLC system consisted of a Gilson (Villiers le Bel, France) Model 302 pump. The BAS-LC-4B electrochemical detector (Bioanalytical Systems, Indianapolis, IN, USA) was equipped with a thin-layer electrochemical cell fitted with a glassy carbon working electrode and an Ag/AgCl reference electrode. The cell volume was 1 μl. Separation was performed on a 250 mm × 4.6 mm I.D. reversed-phase analytical column (Ultrasphere ODS, particle size 5 μm; Beckman, San Ramon, CA, USA). A guard column (30 mm × 4 mm I.D.) was packed with μBondapak C_{18}/Corasil, particle size 37–50 μm (Waters Chromatography Division, Milford, MA, USA).

A refrigerated autoinjector (CMA 200; Carnegie Medicin, Stockholm, Sweden) equipped with a 100-μl Valco (Schenkon, Switzerland) injection loop was connected to the HPLC system. Integration of the chromatograms was performed with a Trio chromatographic computing integrator (Trivector, Sandy, UK).

Chemicals and reagents

L-DOPA and 3-OMD were supplied by Roche (Basle, Switzerland). L-DOPA and carbidopa for *in vivo* experiments were supplied by Merck, Sharp and Dohme Research Labs. (Rahway, NJ, USA), DA·HCl was purchased from UCB (Brussels, Belgium). 1-NA, 1-AD and HVA were supplied by Fluka (Buchs, Switzerland). MHPG, 3-MT · HCl, 5-HIAA and sodium 1-octanesulphonate were purchased from Janssen Chimica (Beerse, Belgium). DOPAC and 6-hydroxydopamine were supplied by Sigma (St. Louis, MO, USA). Stock solutions [0.01% (w/v), kept at 4°C] of the standards were prepared in 0.01 *M* HCl containing 0.1% (w/v) $Na_2S_2O_5$ and 0.01% (w/v) Na_2EDTA. Further dilutions were made in 0.5 *M* acetic acid. The perfusion fluid (modified Ringer's solution) contained 147 mequiv./l Na^+, 4 mequiv./l K^+, 2.2 mequiv./l Ca^{2+} and 156 mequiv./l Cl^-.

Chromatographic conditions

The mobile phase consisted of acetate–citrate buffer containing 0.1 *M* sodium acetate, 20 m*M* citric acid monohydrate, 1 m*M* octanesulphonic acid, 0.1 m*M* Na_2EDTA and 1 m*M* dibutylamine. The pH was adjusted to 2.87 with concentrated phosphoric acid. The flow-rate was set at 1 ml/min and the detector potential was +0.75 V *vs.* Ag/AgCl. The sensitivity was 0.5 nA full scale.

Microdialysis experiments

In vitro. Microdialysis probes (Carnegie Medicin, Stockholm, Sweden) of 0.52 mm O.D. and a membrane length of 2 or 3 mm were used. The molecular mass cut-off point was 20 000. The probes were connected to a microinjection pump (CMA 100; Carnegie Medicin) and perfused at a flow-rate of 2 μl/min. Dialysates were collected every 20 min. To prevent oxidation of the catechols, 80 μl of a mixture of 0.01 *M* HCl, 0.1% $Na_2S_2O_5$ and 0.01% Na_2EDTA was added to the dialysate. Volumes of 100 μl were injected into the chromatographic system.

In vitro calibration of the probes was carried out by placing the probe in a standard mixture of the compounds. Six or seven dialysates were collected, depending on the equilibration of the probe. The same procedure was used for standard solutions ranging from 2.5 to 10 ng per 100 μl to check linearity. The relative recovery was calculated by comparing the concentration found in the dialysate with that in the standard solution [11].

In vivo. Male albino Wistar rats (200 g) on a standard diet were used. Lesions were carried out by local injection of the neurotoxin 6-hydroxydopamine into the left substantia nigra. After two weeks, the rats were tested for rotational behaviour with 5 mg/kg of *d*-amphetamine sulphate i.p., to verify whether the dopaminergic neurons were sufficiently destroyed. After pretreatment with the amino acid decarboxylase inhibitor carbidopa (50 mg/kg orally for five days), the animals were anaesthetized with a mixture of ketamine (50 mg/kg) and diazepam (5 mg/kg) and placed on a stereotactic frame. A dialysis probe was implanted into the innervated and denervat-

ed striatum and also into the intact and lesioned substantia nigra.

Two hours after implantation of the probes, when basal levels were obtained, 5 mg/kg L-DOPA were administered (25 mg of L-DOPA dissolved in 250 μl of 1 *M* HCl; this was then diluted to 5 ml with 0.9% NaCl) and dialysates were collected for a further 4 h. Samples were analysed for L-DOPA, 3-OMD, DA, DOPAC and HVA. Controls received acidified 0.9% NaCl (w/v) i.p.

RESULTS AND DISCUSSION

A major advantage of the microdialysis technique is that no sample preparation is needed and that the samples are ready for injection into the chromatograph. When required, the internal standard dihydroxybenzylamine (DHBA) can be added to the dialysate before analysis; in our system DHBA is well separated from the compounds under study. However, in this single-step procedure, the use of an internal standard is not necessary and does not improve the method in terms of precision.

Previous determination of catecholamines, other neurotransmitters and metabolites, whether in plasma, urine or in brain homogenates, usually required selective extraction procedures before analysis [12–15]. This made it possible to develop HPLC systems for a specific and limited amount of compounds. To enable us to study the biotransformation of levodopa *in vivo* using microdialysis, we had to separate the compounds of interest from other neurotransmitters and metabolites that could be present in the dialysate.

Detection limits (signal-to-noise ratio = 3) for the various compounds vary between 30 and 240 fmol on-column and are given in Table I.

Fig. 1a shows that optimum separation was obtained for ten compounds.

A system suitability test was carried out by injecting a standard solution (2.5 ng/ml) of the compounds six times into the chromatographic system. Relative standard deviations were calculated for the capacity factors and peak areas. Linearity was also investigated over the concentration range 1–10 ng/ml. The results are shown in Table II.

We did not observe any changes in the voltammograms registered at pH values ranging from 2.8 to 4.2. The optimum oxidation potential was found to be 0.75 V.

The *in vitro* performances of the probes were studied by determination of the relative recoveries for the different compounds. It is important to control the parameters that govern both stability and diffusional properties of the analytes

TABLE I

LIMITS OF DETECTION (LOD) FOR THE VARIOUS COMPOUNDS EXPRESSED AS fmol ON-COLUMN

Compound	Capacity factor	LOD (fmol)
MHPG	1.9	181
NA	2.5	38
AD	3.3	50
L-DOPA	4.0	30
DOPAC	8.0	48
DA	9.0	42
3-OMD	9.9	240
5-HIAA	14.6	70
HVA	18.9	165
3-MT	21.2	190

TABLE II

SYSTEM SUITABILITY TEST FOR THE VARIOUS COMPOUNDS

Relative standard deviations (R.S.D.) of capacity factors (k') and peak areas for 2.5 ng/ml solutions are given. Linearity is expressed as the correlation coefficient in the range 1–10 ng/ml.

Compound	R.S.D. (%)		Linearity
	k'	Peak area	
MHPG	1.61	2.08	0.9997
NA	1.76	2.07	0.9997
AD	1.54	3.07	0.9991
L-DOPA	2.11	2.29	0.9991
DOPAC	1.73	3.35	0.9991
DA	1.92	3.04	0.9983
3-OMD	2.23	4.85	0.9984
5-HIAA	2.24	4.49	0.9999
HVA	2.28	5.35	0.9987
3-MT	2.01	6.29	0.9963

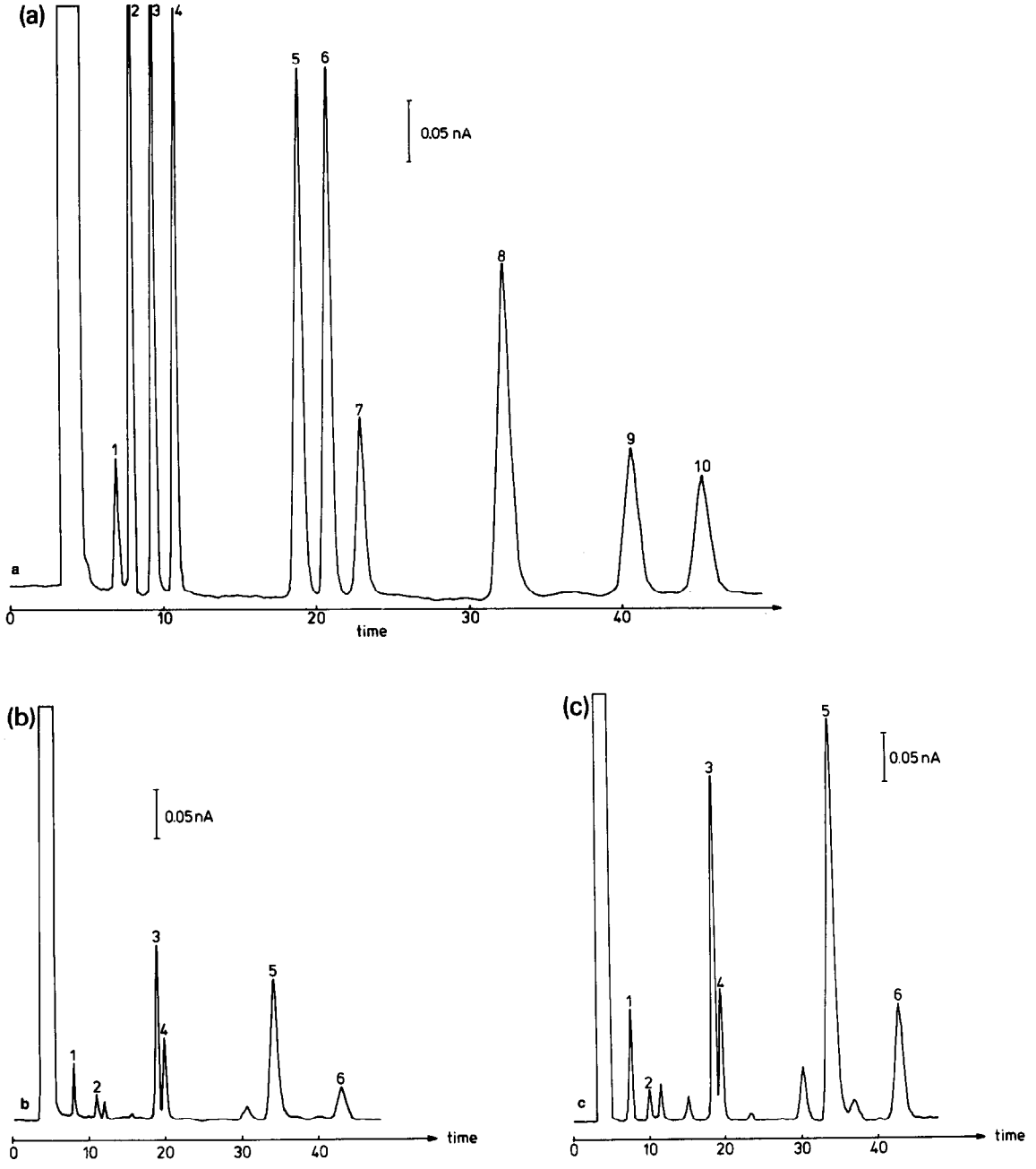

Fig. 1. (a) Chromatogram of a standard solution (500 pg on-column) (1000 pg on-column for 3-OMD). Peaks: 1 = MHPG; 2 = NA; 3 = AD; 4 = L-DOPA; 5 = DOPAC; 6 = DA; 7 = 3-OMD; 8 = 5-HIAA; 9 = HVA; 10 = 3-MT. (b) Chromatogram of dialysate from denervated striatum. Peaks: 1 = NAD; 2 = L-DOPA; 3 = DOPAC; 4 = DA; 5 = 5-HIAA; 6 = HVA. (c) Chromatogram of dialysate from intact substantia nigra. Peaks: 1 = NA; 2 = L-DOPA; 3 = DOPAC; 4 = DA; 5 = 5-HIAA; 6 = HVA.

TABLE III

IN VITRO CALIBRATION OF 2- AND 3-mm PROBES

Linearity is expressed as the correlation coefficient in the range 2.5–10 ng per 100 μl.

Compound	2-mm probe		3-mm probe	
	Relative recovery[a] (%)	Linearity	Relative recovery[a] (%)	Linearity
MHPG	14.7 ± 1.1	0.9993	18.6 ± 1.9	0.9977
NA	13.3 ± 1.2	0.9946	17.9 ± 0.6	1.0000
AD	14.2 ± 0.8	0.9994	18.3 ± 1.1	0.9996
L-DOPA	14.7 ± 1.1	0.9983	18.7 ± 1.1	0.9999
DOPAC	20.1 ± 1.4	0.9946	24.2 ± 2.8	0.9976
DA	13.6 ± 0.9	0.9923	17.3 ± 1.9	0.9903
3-OMD	14.3 ± 1.2	0.9982	18.4 ± 1.2	0.9995
5-HIAA	19.4 ± 1.0	0.9971	23.4 ± 1.2	0.9991
HVA	20.2 ± 1.9	0.9968	24.9 ± 1.9	0.9873
3-MT	15.6 ± 1.2	0.9919	21.1 ± 1.9	0.9977

[a] Mean ± S.D. (n = 20).

TABLE IV

BASAL AND PEAK VALUES OF L-DOPA, DA, DOPAC AND HVA IN DIALYSATES FROM VARIOUS BRAIN REGIONS AFTER ADMINISTRATION OF 5 mg/kg L-DOPA i.p.

Values are expressed as pmol per 40 μl ± standard error of the mean (number of animals n = 6).

Region[a]	Basal	Peak
L-DOPA		
IST	<LOD	0.23 ± 0.09
DST	<LOD	0.24 ± 0.13
ISN	<LOD	0.11 ± 0.04
LSN	<LOD	0.17 ± 0.04
DA		
IST	0.46 ± 0.11	0.92 ± 0.27
DST	0.18 ± 0.12	0.53 ± 0.33
ISN	0.15 ± 0.05	0.31 ± 0.07
LSN	0.05 ± 0.02	0.08 ± 0.01
DOPAC		
IST	42.5 ± 5.4	66.8 ± 7.80
DST	4.60 ± 1.94	5.96 ± 2.06
ISN	2.63 ± 0.40	4.82 ± 0.48
LSN	0.57 ± 0.12	2.74 ± 0.57
HVA		
IST	15.81 ± 2.73	24.00 ± 2.42
DST	3.27 ± 0.73	5.93 ± 1.54
ISN	3.67 ± 0.51	6.14 ± 0.25
LSN	1.81 ± 0.30	3.36 ± 0.65

[a] IST = intact striatum; DST = denervated striatum; ISN = intact substantia nigra; LSN = lesioned substantia nigra.

across the dialysis membrane, such as temperature and pH [16]. In our study, relative recovery was determined at 25°C with a constant pH of the perfusion fluid. All dialysates were collected in vials containing an antioxidant mixture (see Experimental). *In vitro* recoveries are given in Table III. The relative recovery is higher for the 3-mm than for the 2-mm probe, because of the larger surface area available for exchange of molecules between standard solution and perfusion fluid. The relative recovery is higher for acidic than for basic compounds. The *in vitro* data should not be used to calibrate recovery *in vivo*, mainly because the complex transport characteristics of tissue are not well approximated by a homogeneous perfusion solution. This is a serious problem in studies where absolute concentrations in tissue must be measured, rather than relative changes in concentration.

The described HPLC system was also applied to the analysis of dialysates from intact and denervated striatum and substantia nigra before and after administration of 5 mg/kg L-DOPA i.p. Basal levels and peak levels of L-DOPA, DA, DOPAC and HVA are given in Table IV. No 3-OMD was detected.

Basal values (expressed as pmol per 40 μl of dialysate) are in agreement with previous studies

[17–19]. Using this set-up we are able to study the availability of exogenously administered L-DOPA in different brain areas and its effect on dopamine release and metabolisation.

Fig. 1b and c show chromatograms of dialysates from striatum and substantia nigra, illustrating that other neurotransmitters (*e.g.*, NA) or metabolites (*e.g.*, 5-HIAA) can also be monitored.

CONCLUSION

The technique described allows adequate separation and sensitivity for monitoring levodopa, catecholamines and their metabolites in rat brain dialysates.

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