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Simultaneous high-performance liquid chromatographic determination of adenosine and dopamine in rat striatal tissue with combined ultraviolet absorbance and electrochemical detection

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Abstract

A method is described for the simultaneous determination of biogenic amines, adenosine and their metabolites in rat striatal tissue using high-performance liquid chromatography with ultraviolet spectrophotometric and electrochemical detection. Peaks in the chromatograms of striatal tissue extracts were identified by retention times and by on-line analysis of peak spectra for adenosine and its metabolites, and by comparing current ratios of the dual-electrode coulometric detector for monoamines and metabolites. The assay gives a linear response over the concentration range of 0.15–0.60 μg/ml for biogenic amines, 0.5–2.0 μg/ml for serotonin, 5–20 μg/ml for hypoxanthine, adenosine and N-methyladenosine, and 10–40 μg/ml for inosine. The limit of detection for striatal homogenates was 3.5 ng/g for monoamines, 9 ng/g for serotonine, 140 ng/g for hypoxanthine, 290 ng/g for inosine and 80 ng/g for adenosine. The recovery ranged from 88.5% for vanillylmandelic acid to 110.3% for dopamine. The method was used to measure biogenic amines, adenosine and related metabolites in rat striatal tissues.

1. Introduction

The endogenous nucleoside adenosine (Ado) acts as a neuromodulator in many areas of the central nervous system (CNS) [1,2]. In particular, it has been hypothesized that Ado tonically inhibits striatal dopaminergic neurotransmission [3–6].

Apart from modifications in receptor density and affinity, also the amounts of both dopamine (DA) and Ado may vary in brain in particular functional states. For example, a decrease in striatal DA levels has been reported in aged rats [7], while it has been shown that, in rodents, the brain levels of Ado rise following ischemia or hypoxia [8,9] as well as during seizures [10].

Although many chromatographic techniques are available for the measurement of specific classes of neurotransmitters, analysis of multiple classes generally involves separate tissue preparation protocols and separate analytical processing procedures [11–17].

A high-performance liquid chromatographic

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(HPLC) assay for simultaneous determination of Ado and DA in human urine has been reported [18]. However, no HPLC method exists for the simultaneous determination of Ado, its enzymatic breakdown products inosine (Ino), hypoxanthine (Hyp) and catecholamines, indoleamines and their major metabolites and precursors in brain tissues. The aim of the present study was to develop a rapid HPLC method for the simultaneous quantitation of biogenic amines, Ado and its metabolites in crude perchloric acid extracts of striatal tissue, using a photodiode-array detector (PDA) and an electrochemical detector (ED) connected in series.

2. Experimental

2.1. Chemicals and reagents

Ado, Ino, Hyp, N^6-methyladenosine (M-Ado), norepinephrine (NE), epinephrine (E), DA, homovanillic acid (HVA), vanillylmandelic acid (VMA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HIAA), 3,4-dihydroxybenzylamine (DHBA) and serotonin (5-HT) were purchased from Sigma (St. Louis, MO, USA). Analytical-reagent grade chemicals and HPLC-grade methanol were purchased from C. Erba (Milan, Italy).

2.2. Chromatographic system

The chromatographic apparatus consisted of a Series 4 liquid chromatograph (Perkin-Elmer, Norwalk, CT, USA), a pulse damper LP-21 (SSI, State College, PA, USA), a Rheodyne Model 7125 injection valve (Rheodyne, Berkeley, CA, USA) and a Coulochem II electrochemical detector (ESA, Belford, MA, USA) equipped with an analytical cell (Model 5011). The working parameters for the electrochemical detector (ED) were +0.35 V for the first electrode and −0.35 V for the second; these settings were found to provide optimal detection conditions. For ED data collection and calculations a Waters Maxima 820 Chromatography workstation was used. Single-wavelength detection using a Perkin-Elmer LC 90J UV detector set at 260 nm was used for most samples. Single-wavelength chromatograms were recorded using a Perkin-Elmer CC-12 computing integrator. When UV spectra of sample components were desired, a Waters Model 996 PDA was used in place of the single-wavelength detector. Peak spectra and purity parameters were obtained via Waters Millennium 2010 software.

Isocratic separation was achieved at ambient temperature using a Supelco LC-18 DB column (5 μm, 150 × 4.6 mm I.D., Supelco, Bellefonte, PA, USA). The mobile phase was acetate buffer (12 m*M* sodium acetate, 0.26 m*M* Na_2EDTA, 0.5 m*M* octane sulfonic acid sodium salt, pH 2.6)–methanol (86:14, v/v). The run time was 30 min with a flow-rate of 1.0 ml/min. The UV detector and the ED were connected in series.

2.3. Standard solutions

Stock solutions of M-ado (internal standard, I.S.), Ado, Ino and Ipo (1, 2, 1 and 0.5 mg/ml were prepared in 0.01 *M* perchloric acid–methanol (20:80, v/v). Stock solutions of DHBA (I.S.), VMA, NE, E, DOPAC, DA, 5-HIAA, HVA (100 μg/ml) and 5-HT (300 μg/ml) were prepared in 0.01 *M* perchloric acid–methanol (20:80, v/v). All stock solutions were stored at −20°C and no significant degradation occurred over a period of 60 days. The standard mixture was freshly prepared by combining 15 μl of each monoamine solution and 50 μl of Ado, Ino, Ipo and M-Ado solution and diluting with the mobile phase to 5 ml.

A standard stock solution of the two I.S. (DHBA 0.3 μg/ml and M-Ado 10 μg/ml) was prepared in 0.2 *M* perchloric acid and used for tissue extraction.

2.4. Tissue preparation

Adult male Wistar rats (250–280 g) were used. Under slight ether anestesia, the animals were killed by decapitation. The brains were rapidly dissected, the striata were removed and placed on dry ice. The tissue was homogenized in 10 volumes of 0.2 *M* perchloric acid containing both internal standards. The homogenate was cen-

trifuged (12 000 *g*, 4°C, 10 min), and the resulting supernatant was analyzed by HPLC.

3. Results and discussion

The specific and sensitive method reported here for the simultaneous determination of Ado, its metabolites and some biogenic amines in striatal tissue employs HPLC with two different detection systems (ED and UV detection) connected in series. ED was performed with a coulometric detector with a high-sensitivity analytical cell containing dual working electrodes operated in the redox mode.

Fig. 1 shows chromatograms of a standard mixture containing VMA, NE, E, DHBA (I.S.), DOPAC, DA, 5-HIAA, HVA, 5-HT (ED) and Hyp, Ino, M-Ado (I.S.) and Ado (UV). All compounds studied were well resolved. Fig. 2 shows chromatograms of an extract of rat striatal tissue.

Peaks of VMA, NE, E, DOPAC, DA, 5-HIAA, HVA and Hyp, Ino, Ado were identical to those in Fig. 1. The purity of the compounds separated and detected by ED was determined from recordings of the ratios of the detector responses (oxidation current/reduction current) [19,20]. The peak purity of electrochemically inert substances was confirmed via on-line analysis of the peak spectra. The Millennium PDA software determines if a peak is spectrally homogeneous by comparing the peak apex spectrum against a spectrum of a standard.

To demonstrate the utility of the present method, we performed a quantitative determination of all compounds under study in rat striatal tissue. The concentrations of the monoamines, adenosine and relative metabolites are given in Table 1. The concentrations of the monoamines and their major metabolites, as well as those of Ado, are comparable with those reported in the literature [21,22].

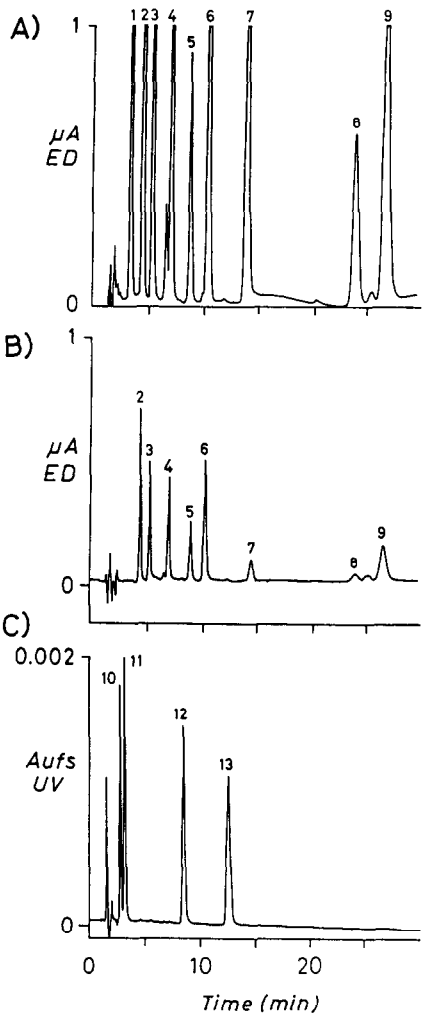

Fig. 1. Chromatograms showing ED (A and B) and UV (C) responses after injection of 10 μl of a standard solution containing 3 ng each of VMA (1), NE (2), E (3), DHBA (4), DOPAC (5), DA (6), 5-HIAA (7) and HVA (8), 10 ng of 5-HT (9), 100 ng of Hyp (10), 200 ng of Ino (11), 100 ng of Ado (12) and M-Ado (13). Chromatographic conditions: column, Supelco LC-18 DB, 5 μm (150 × 4.6 mm I.D.); mobile phase, acetate buffer (12 m*M* sodium acetate, 0.26 m*M* Na_2EDTA, 0.5 m*M* octane sulfonic acid sodium salt, pH 2.6)–methanol (86:14, v/v); flow-rate, 1.0 ml/min. Detection UV at 260 nm. The potential applied to ED was +0.35 V at the first electrode (A) and −0.35 V at the second (B).

3.1. Recovery and precision

The experimental recoveries of the analytes extracted from striatal tissue were assessed by comparing the area under the peaks obtained from standard stock solutions with the peak areas from a homogenized striatal tissue pool to which known amount of analytes were added. The mean values of the data obtained from five

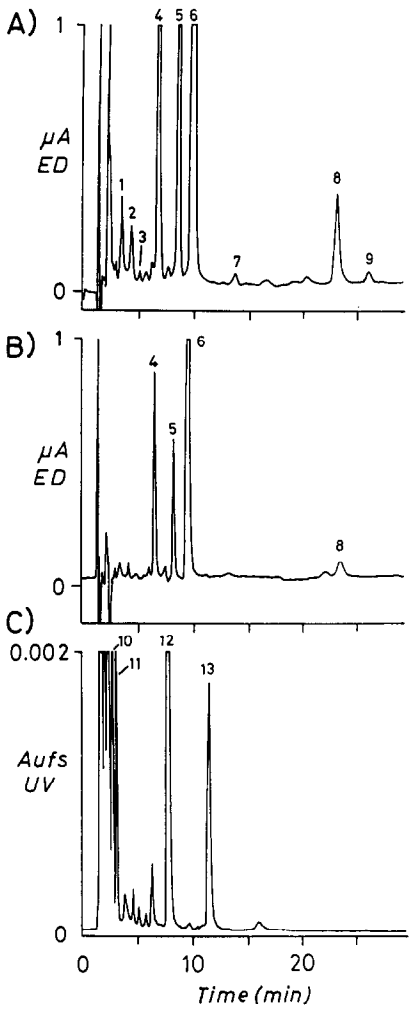

Fig. 2. Chromatograms obtained by direct injection of 10 μl of perchloric acid extract of rat striatum tissue containing 0.5 ng of NE (2), 0.2 ng of E (3), 1.9 ng of DOPAC (4), 11.2 ng of DA (6), 0.40 ng of 5-HIAA (7), 1.2 ng of HVA (8), 0.6 ng of 5-HT (9), 80 ng of Hyp (10), 150 ng of Ino (11) and 0.8 μg of Ado (12). Chromatographic conditions as described in Fig. 1.

replicates gave coefficients of variation (C.V.) ranging from 1.6 to 7.6% over the whole concentration range studied. The concentrations were as follows: 0.15–0.6 μg/ml for catecholamines and metabolites, 0.50–2.0 μg/ml for 5-HT, 5–20 μg/ml for Hyp, Ado and M-Ado and 10–40 μg/ml for Ino. The recoveries of the eleven substances studied ranged from a minimum of 88.5% for VMA to a maximum of 110.3% for DA.

Table 1
Striatal concentrations of monoamines, adenosine and relative metabolites

Compound	Concentration (μg/g)
VMA	0.52 ± 0.10
NE	0.28 ± 0.05
E	0.03 ± 0.01
DOPAC	1.39 ± 0.36
DA	8.09 ± 2.10
5-HIIA	0.48 ± 0.12
HVA	0.93 ± 0.24
5-HT	0.48 ± 0.17
Hyp	12.28 ± 5.63
Ino	19.30 ± 4.52
Ado	171.28 ± 57.78

The results represent mean ± S.D. from 10 animals. The amounts are expressed in μg/g wet tissue.

3.2. Detection limit

The minimum detectable limits, at a signal-to-noise ratio of 3:1, under the conditions described were: 3.5 ng/g wet weight for VMA, NE, E, DOPA, DA, 5-HIAA and HVA, 9 ng/g for 5-HT, 140 ng/g for Hyp, 290 ng/g for Ino and 80 ng/g for Ado.

3.3. Linearity

The relationship between peak area and concentration was linear in the following ranges: VMA, NE, E, DOPAC, DA, 5-HIAA and HVA, 0.75–7.5 ng; 5-HT, 2.5–25 ng; Hyp, Ino and Ado, 25–250 ng. The regression equation and correlation coefficient for each analyte were as follows: VMA, $y = 6833x - 55$, $r^2 = 0.9964$; NE, $y = 10820x - 91$, $r^2 = 0.9982$; E, $y = 8933x - 50$, $r^2 = 0.9985$; DOPAC, $y = 4556x + 22$, $r^2 = 0.9968$; DA, $y = 11911x - 25$, $r^2 = 0.9996$; 5-HIAA, $y = 8570x + 96$, $r^2 = 0.9995$; HVA, $y = 8940x - 18$, $r^2 = 0.9978$; 5-HT, $y = 2640x - 71$, $r^2 = 0.9992$; Hyp, $y = 82x - 27$, $r^2 = 0.9981$; Ino, $y = 77x + 4$, $r^2 = 0.9996$; and Ado, $y = 119x + 21$, $r^2 = 0.9996$.

4. Conclusions and applications

The HPLC separation system coupled with combined UV–ED described here represents an improvement of the systems currently found in the literature since it provides improved and complete resolution for the eleven compounds tested. The improved selectivity of the detection allowed direct injection of crude biological samples. Consequently, it is possible to routinely analyse a large number of samples per day.

Because of the close interactions between the Ado and the DA systems at the striatal level (see Introduction), the method reported here may help to elucidate some aspects of the basal ganglia physiopathology.

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