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BRIEF COMMUNICATION

Rapid and Simultaneous Assay of Monoamine Neurotransmitters and Their Metabolites in Discrete Brain Areas of Mice by HPLC with Coulometric Detection

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SAITO, H., S. MURAI, E. ABE, Y. MASUDA AND T. ITOH. *Rapid and simultaneous assay of monoamine neurotransmitters and their metabolites in discrete brain areas of mice by HPLC with coulometric detection.* PHARMACOL BIOCHEM BEHAV **42**(2) 351-356, 1992.—For simultaneous assay of the three monoamine neurotransmitters, norepinephrine, dopamine, and serotonin, and four respective metabolites in brain tissue, a rapid and simple method using high-performance liquid chromatography with coulometric detection is described. Because the present method permits the determination of these target substrates within 10 min or less in one chromatographic run, 150 samples can be analyzed using an autosampler and an integrator in a 24-h period. Within-run coefficients of variation for the target substrates in the standard solution and the whole brain sample were less than 3% and 2% (n = 40), respectively. The quantitative detection limits were 0.01-0.1 pmol. The present procedure was applied to measure the target substrates in several discrete brain areas in mice.

High-performance liquid chromatography   Coulometric detector   Monoamine neurotransmitters
Related metabolites   Mouse brain areas

RECENTLY, high-performance liquid chromatography with electrochemical detection (HPLC-ECD) has been widely used for the determination of various neurochemical substrates. Two kinds of detection systems are currently utilized for the electrochemical detectors: a single-electrode amperometric detector, and dual- or multiple-electrode coulometric detectors. For simultaneous determination of monoamine neurotransmitters and their metabolites in brain tissue samples and brain microdialysis samples, the amperometric detection system is the most popular. This system is simple and inexpensive, but has difficulty in clearly resolving the early eluting and interesting metabolite of norepinephrine, such as 3-methoxy-4-hydroxyphenylglycol (MHPG) because of interference with the large solvent front peak and/or tyrosine peak, as previously reported by us (11) and others (9,15). On the other hand, coulometric detection systems are relatively expensive, but a series of two or three coulometric electrodes provide the capability to screen out these interfering peaks (8,10,14). This potential ability of the coulometric detection system is based on a reversible oxidation/reduction of the electroactive substrates in the electrodes and may allow improvement of separation of the MHPG peak without prolongation of a single chromatographic analysis time.

Several papers have already described the HPLC method using the coulometric detector for simultaneous determination of monoamine neurotransmitters and their metabolites in brain samples (1,2,7,8,10,13,14,16). However, these are not satisfactory with respect to the analysis time and/or selectivity for the simultaneous determination of these monoamine-related substrates including MHPG (8,10,13,14,16).

Here, we report a rapid HPLC method using a coulometric detector for simultaneous determination of NE, dopamine (DA), serotonin (5-HT), MHPG, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) in discrete brain areas of mice. Because the method is also simple and practical without loss of sensitivity and accuracy, it is suitable for routine laboratory analysis when many samples are prepared.

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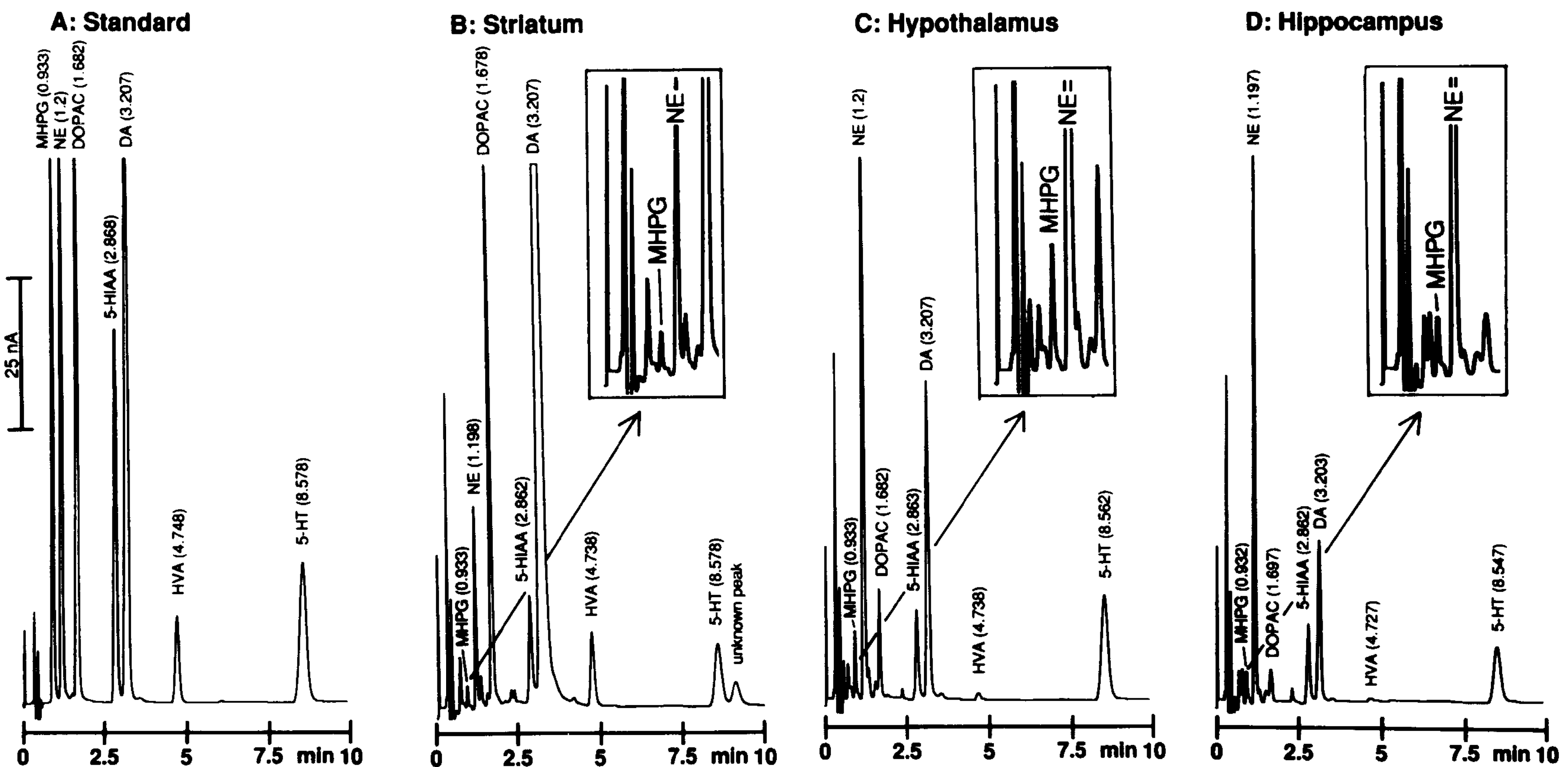

FIG. 1. Typical chromatograms of a standard mixture (10 pmol each), a striatum (equivalent to 0.38 mg tissue weight), a hypothalamus (0.3 mg), and a hippocampus (0.4 mg) extract of mouse brain. The detector settings were: conditioning cell +0.4V; #5051 cell 1, +0.02V; #5051 cell 2, −0.3V. The values in parentheses represent the retention time (min). See text for abbreviations.

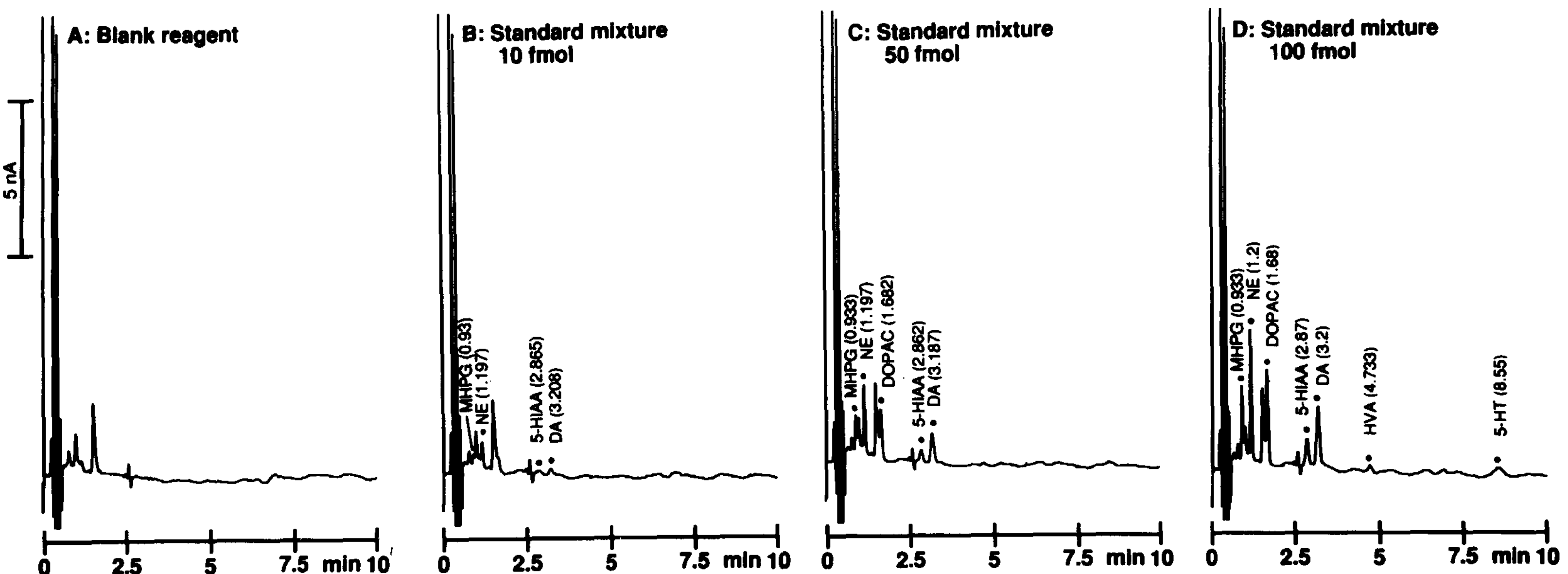

FIG. 2. Chromatograms of diluted standard mixtures. The values in parentheses represent the retention time (min). See the legend to Fig. 1 for other details.

METHODS

Chromatography

The HPLC system consisted of a solvent delivery pump (Model EP-10S, Eicom, Kyoto, Japan), an autosampler (Model AS-4000, Hitachi, Tokyo, Japan), and an analytical column (C18 Ultrasphere, 75 mm × 4.6 mm ID, 3-μm particle size, Beckman, San Ramon, CA) protected by a guard column (Eicom prepak, 5 mm × 4.6 mm ID, 7-μm particle size). The ESA model 5100A Coulochem electrochemical detection system (ESA Inc., Bedford, MA) consisted of a model 5021 conditioning cell (detector setting, +0.4 V) followed in sequence by a model 5011 dual electrode analytical cell (cell 1, +0.02 V; cell 2, −0.3 V). These oxidative and reductive potentials are basically set from current-voltage curves reported by Kurata et al. (8) and Takeda et al. (14). The output signal from the final electrode is amplified by the 5100A controller and relayed to the integrator (Model C-R6A, Shimadzu, Kyoto, Japan). The temperature of the analytical and guard columns was controlled by a column jacket connected to a thermostatic water-bath (Model UC-65, Tokyo Rika Kikai, Tokyo, Japan) and maintained at 35°C.

The mobile phase was 0.02 M sodium acetate/0.0125 M citric acid buffer, pH 3.7, containing 8% (v/v) methanol, 0.042% heptanesulfonic acid (C_7), and 0.1 mM Na_2EDTA. The buffer solution was filtered through a 0.45-μm membrane filter and degassed. The flow-rate was set to 2.4 ml/min, which yielded a pressure of 190 kg/cm^2. The mobile phase was recycled and replaced when baseline noise of the detector output attained unacceptable levels.

Tissue Preparation

Mice were killed by microwave irradiation (4 kW, 1.2 s, Model TMW-6402A, Toshiba, Tokyo, Japan). Each brain was removed and dissected into the four brain regions (cortex, hippocampus, hypothalamus, and striatum) as previously described by Glowinski and Iversen (4). The dissected tissues were quickly frozen on dry ice, weighed, and stored in a 1.5-ml microtube at −80°C until extraction. Tissues were homogenized for 10 s with an ultrasonic cell disruptor (Model 200, 60 W, 50% pulsed power, Branson, Danbury, CT) in 400 μl ice-cold 0.05 M perchloric acid (PCA) solution for striatum; 200 μl for hypothalamus; and 300 μl for hippocampus. Homogenates were centrifuged at 12,000 *g* for 15 min at 4°C, and the clear supernatants were decanted and filtered through a 0.45-μm filter (Type HV, Nihon Millipore, Yonezawa, Japan) and stored at −80°C until assayed.

TABLE 1

DETECTION LIMIT AND PRECISION FOR THE HPLC DETERMINATION OF THE SEVEN MONOAMINE-RELATED SUBSTRATES

Substrate	Detection limit (pmol)*	Within-run C.V. (%)†		
		Standard	Striatum	Hippocampus
NE	0.01	±0.7	±1.0	±0.7
MHPG	0.01	±0.4	±1.8	±0.9
DA	0.025	±1.0	±0.6	±2.1
DOPAC	0.05	±0.3	±0.6	±0.8
HVA	0.1	±2.1	±1.1	±3.7
5-HT	0.1	±3.0	±2.9	±2.7
5-HIAA	0.05	±1.3	±1.1	±2.2

*Injected amount of standards giving a signal-to-noise ratio higher than 3.

†Within-run coefficients of variation were calculated on 40 consecutive injections of the standard solution, striatal samples, and hippocampal samples.

TABLE 2

LEVELS OF MONOAMINE NEUROTRANSMITTERS AND MAJOR METABOLITES IN DISCRETE BRAIN AREAS OF MICE

Substrate	Striatum	Hippocampus	Frontal cortex	Hypothalamus
NE	199 ± 6	445 ± 20	372 ± 13	1673 ± 30
MHPG	37 ± 1	54 ± 1	37 ± 1	109 ± 3
DA	9465 ± 191	98 ± 9	708 ± 35	537 ± 29
DOPAC	523 ± 8	21 ± 1	88 ± 5	138 ± 5
HVA	904 ± 22	35 ± 2	187 ± 13	216 ± 12
5-HT	631 ± 13	693 ± 27	698 ± 16	1728 ± 37
5-HIAA	369 ± 12	357 ± 9	180 ± 6	572 ± 15

Data represent mean ± SE (n = 7) and substrates levels are expressed in ng/g tissue weight.

Standards and Calculations

The working standard solutions were prepared monthly in 0.1 M PCA containing 0.1 mM Na_2EDTA and stored at −80°C. Peaks were identified by comparing the retention time of each peak in the sample solution to that of individual peak in the standard solution, and by superimposing the chromatograms of the samples spiked with and without known amounts of the standards. The levels of substrates in each brain sample were calculated by an integrator from comparison of sample peak heights with external standard peak heights.

Precision and Detection Limits

Within-run coefficients of variation (C.V.) were calculated on 40 consecutive injections of the standard solution, striatal samples, and hippocampal samples. The detection limits of sensitivity for the substrates based on the signal-to-noise ratio higher than 3 were determined by injections of diluted working standard solutions.

MATERIALS

Chemicals were obtained from the following sources: NE bitartrate and 5-HT creatinine sulfate were obtained from Wako Chemicals (Osaka, Japan); DA hydrochloride from Kanto Chemical (Tokyo, Japan); HVA and MHPG hemipiperazine salt from Sigma Chemical (St. Louis, MO); DOPAC, 5-HIAA, and C_7 sodium salt from Aldrich (Milwaukee, WI). All other reagents and solvents were analytical grade and used without further purification.

Animals

Male ddY mice, weighing 25–35 g, were purchased from Nihon SLC (Hamamatsu, Japan). All animals were housed at a room temperature of 22 ± 1°C and humidity of 55 ± 5%. The light cycle was from 0700 to 1900. The animals were given free access to both water and standard laboratory chow.

RESULTS

Figure 1 shows typical chromatograms of (A) the standard, (B) striatum, (C) hypothalamus, and (D) hippocampus of mice brain. The three monoamine neurotransmitters (NE, DA, and 5-HT) and the four major metabolites (MHPG, DOPAC, HVA, and 5-HIAA) were well separated from each other and tissue-derived interferences, and detected within 10 min in one chromatographic run. No solvent front and tyrosine peaks interfering with separation of the early eluting MHPG peak are apparent.

Figure 2 shows chromatograms of the diluted standard mixture. The peaks of target substrates represent 0.01, 0.05, and 0.1 pmol, respectively.

Under the present chromatographic conditions, the detector responses were linear from 0.01 to 100 pmol for NE, DA, MHPG, DOPAC, and 5-HIAA, and from 0.1 to 100 pmol for HVA and 5-HT. The correlation coefficients of standard curves were between 0.99994 and 0.99999 for each substrate. The quantitative detection limits based on a signal-to-noise ratio higher than 3 was 0.01 pmol for NE, DA, MHPG, DOPAC, and 5-HIAA, and 0.1 pmol for HVA and 5-HT. The within-run coefficients of variation for the substrates in the standard and the brain samples were less than 3% and 2% (n = 40), respectively. These data are presented in Table 1.

The present procedure was applied to measure the target substrates in several discrete brain areas in mice. The results are shown in Table 2.

DISCUSSION

For simultaneous assay of monoamine neurotransmitters and their metabolites, the direct injection of crude PCA-extracts of brain tissues onto the HPLC system is a very simple and time-saving technique, and can eliminate a possible source of technical error and loss of recovery. However, a serious problem of interference with the solvent front and the peaks of nontarget substrates must be resolved because of the absence of a purification step for target substrates. Especially, it has been difficult to resolve the early eluting MHPG peak without prolongation of one chromatographic run time. MHPG is a major metabolite of NE and frequently used as a valid index for assessment of NE turnover (6). Thus, it is important to assay monoamine-related substrates concomitant with MHPG.

The dual-electrode coulometric detection system used in this study has some distinct advantages over the widely used amperometric detection system. The most obvious advantage is elimination of the solvent front and early eluting of some peaks, such as tyrosine. This elimination is easily attained using reductive detection of substrates following oxidation and may allow a clearer separation of the MHPG peak. Thus, in this study we investigated the optimal chromatographic conditions for simultaneous assay of monoamine-related substrates including MHPG in mice brain.

Under the present chromatographic instrumental and mobile phase conditions, all of the target substrates were well separated within less than one 10-min chromatographic run. Thus, with the use of an autosampler and an integrator, 150 samples set in an autosampler tray can be automatically analyzed in a 24-h period. The present analysis time is much shorter than those reported for similar coulometric HPLC methods designed to simultaneously measure the monoamine-related substrates including MHPG (1,7,8,10,13,14, 16). Although the present analysis time is almost the same as the time for the amperometric HPLC method that we previously reported (11), the separation of the MHPG peak in the present method was almost completed in comparison to that of the MHPG peak that was eluted as a shoulder peak of tyrosine in the previous method (11).

The limits of quantitative detection of the target substrates were 0.01–0.1 pmol and are almost comparable to the values previously reported by other coulometric HPLC methods (2,13,14). The major drawback of the coulometric detection is that it has relatively less sensitivity for HVA in comparison with other target substrates (8,14). Although this resulted in an apparently increased C.V. value for HVA (Table 1), the present method has good within-run variation and high and quantitative recovery for all of the target substrates without use of the internal standard (11).

Provided the guard column is changed after 200 analyses, the separation efficiency of the analytical column remains satisfactory after almost 2,000 sample injections. However, because the elution time gradually shortens concomitant with aging of an analytical column, the concentrations of C_7, the ion-pairing agent, must increase slightly, depending on aging of the column.

The present method was applied to measuring the steady-state levels of the target substrates in discrete brain areas of the mouse. The present data are in agreement with the values previously reported for mice brain (3,5,11,12). Thus, the present rapid analytical method provides simplicity, high sensitivity, and high reliability, and may be suitable for the routine laboratory determination of monoamine neurotransmitters and their main metabolites, of which many samples are prepared.

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