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SIMPLE METHOD FOR THE SIMULTANEOUS DETERMINATION OF ACETYLCHOLINE, CHOLINE, NORADRENALINE, DOPAMINE AND SEROTONIN IN BRAIN TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A simple method for the simultaneous determination of acetylcholine, choline, noradrenaline, dopamine and serotonin in brain tissue was developed by using high-performance liquid chromatography with electrochemical detection. These compounds are analysed in a single chromatographic run within 30 min with a simple sample clean-up procedure. The detection system consists of two electrochemical detector cells aligned in series: a glassy-carbon electrode for catecholamines and serotonin, and a platinum electrode for acetylcholine and choline. For the detection of the latter compounds, they were converted enzymatically into hydrogen peroxide through a column reactor with immobilized acetylcholinesterase and choline oxidase. A column of boronic acid gel was placed just ahead of the immobilized enzyme column to remove catecholamines, which caused interfering responses on the platinum electrode. Two equivalent analytical columns and a column switching were employed to speed up the serotonin assay. Simultaneous determination of these major neurotransmitters in rat brain regions was successfully carried out with the system described.

INTRODUCTION

Cholinergic, noradrenergic, dopaminergic and serotonergic neurons are the principal neurons in the brain, and function through their close synaptic interactions. It is important to measure the level of neurotransmitters in the same brain region in order to investigate these neuronal activities and functions. A large number of publications have appeared on the determination of catecholamines and indoleamines in the brain and body fluids using high-performance liquid chromatography (HPLC)[1],

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involving the simultaneous determination of catecholamines, indoleamines and their metabolites[2–6]. However, none of the published methods allows the simultaneous determination of these biogenic amines and acetylcholine (ACh), because of the absence of a suitable detection system for ACh. Potter *et al.*[7] have recently developed a highly sensitive and simple assay of ACh and choline (Ch) by HPLC with electrochemical detection (ED). They converted ACh and Ch enzymatically into hydrogen peroxide through the post-column reaction with acetylcholinesterase and Ch oxidase. By applying this HPLC–ED method, we previously developed highly sensitive assays for choline acetyltransferase[8] and more recently, by using an immobilized-enzyme column as a post-column reactor, acetylcholinesterase activities[9]. This report describes a method for the simultaneous single chromatographic determination of ACh, catecholamines and serotonin in brain tissue.

EXPERIMENTAL

Materials

L-Noradrenaline (NA), L-adrenaline (AD) bitartrate, dopamine (DA) hydrochloride, ACh chloride, sodium 1-octanesulphonate (SOS) and tetramethylammonium (TMA) chloride were obtained from Nakarai Chemicals (Kyoto, Japan). Ch chloride was from Sigma (St. Louis, MO, U.S.A.). 5-Hydroxytryptamine (5-HT) creatinine sulphate was from Merck (Darmstadt, F.R.G.). 3,4-Dihydroxybenzylamine (DHBA) and 3-aminopropylethoxysilane were from Aldrich (Milwaukee, WI, U.S.A.). Immobilized enzyme of acetylcholinesterase and Ch oxidase was prepared as described in detail elsewhere[10]. Briefly, porous glass beads (particle size, 30–40 μm; pore size, 400–500 Å; Electronucleonics, Fairfield, NJ, U.S.A.) were washed with 5% nitric acid, followed by water, dried and then refluxed overnight with 3-aminopropylethoxysilane in toluene. Acetylcholinesterase and Ch oxidase were covalently immobilized onto the alkylamino glass beads with glutaraldehyde through Schiff base formation. The life-time of this enzyme column was *ca.* 2 months at 4°C, and 300–500 assays were possible. Boronic acid gel (particle size, 10 μm; polyvinyl copolymer immobilized with dihydroxyboryl group), and ethylhomocholine (EHC) were kindly supplied from BAS (Tokyo, Japan). All other chemicals used were of analytical-reagent grade. Glass-distilled water was further purified by using a Milli-QII system (Millipore, Bedford, MA, U.S.A.). Male Fischer rats (body weight, *ca.* 400 g) were sacrificed by microwave irradiation (Toshiba Model TMW-6402 A) for 1.5 s at a power of 5 kW. The brains were removed and immediately frozen, and then stored at −80°C until use. The whole brain was dissected on a glass plate over ice into six parts: cerebral cortex, striatum, hippocampus, midbrain, pons-medulla oblongata and cerebellum.

Sample preparation

The procedure for sample preparation from brain tissue was essentially the same as that used for the assay of ACh and Ch as described previously[11–14]. Brain tissue (20–100 mg) was sonicated for *ca.* 30 s at 0°C (Cell Disruptor 200, Branson, Danburg, CT, U.S.A.) in a polypropylene centrifugation tube containing 1 *M* formic acid–acetone (15:85; 1 ml/100 mg tissue) and 500 pmol of DHBA and 5 nmol of EHC as internal standards. After 30 min in an ice-bath, the mixture was centrifuged

at 20 000 *g* for 15 min at 0°C. The supernatant was transferred to another glass conical tube, and an equal volume of diethyl ether was added as described by Ikarashi *et al.*[14]. The mixture was vortexed and centrifuged at 1600 *g* for 5 min at 4°C to clarify the interface. The organic phase was carefully aspirated off and the remaining aqueous phase was dried at 40°C under nitrogen. The dried sample was stored at −20°C or dissolved immediately in 200 μl of water with vigorous mixing, and passed through a membrane filter (Millipore HV4, 0.45 μm). A 10–20-μl aliquot was injected into the chromatograph.

HPLC system and chromatographic conditions

The entire HPLC system used in the present study is illustrated schematically in Fig. 1. It consisted of a PM 30A dual-piston pump (P1), LC-3A and LC-4B amperometric detectors (ED1 and ED2) equipped with a TL-5A glassy-carbon (GC) and a TL-10A platinum (Pt) electrodes, respectively (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Rheodyne 7125 injector (I) with a 20-μl sample loop, a Rheodyne 7000 high-pressure six-way valve (V) (Berkely, CA, U.S.A.), two equivalent analytical columns packed with Chemcosorb 7 ODS-L-300 (C1 and C2) (particle size, 7 μm; pore size, 300 Å; 75 × 4.6 mm I.D. each; Chemco Scientific, Osaka,

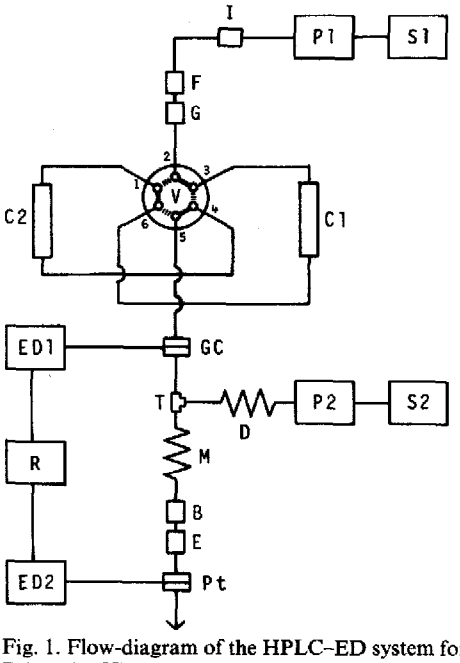


Fig. 1. Flow-diagram of the HPLC–ED system for the simultaneous determination of Ch, ACh, NA, AD, DA and 5-HT. S1 = Mobile phase; P1 = pump 1 (Bioanalytical systems, PM 30A); I = injector (Rheodyne 7125); F = filter (1 μm); G = guard column (Develosil ODS 15/30, 10 × 4 mm I.D.); V = high-pressure six-way valve (Rheodyne 7000); *C1* and *C2* = analytical columns (Chemcosorb 7 ODS-L-300, 75 × 4.6 mm I.D.); GC = glassy-carbon electrode (Bioanalytical Systems, TL-5A); T = tee, S2 = second solution; P2 = pump 2 (Japan Servo, DHCR 80 HP); D = damper (PTFE tubing, 15 m × 0.25 mm I.D.); M = mixing coil (PTFE tubing, 2 m × 0.5 mm I.D.); B = immobilized boronic acid column (10 × 4 mm I.D.); E = immobilized enzyme column (10 × 4 mm I.D.); Pt = platinum electrode (Bioanalytical Systems, TL-10A); ED1 and ED2 = electrochemical detectors (Bioanalytical Systems, LC 3A and LC 4B, respectively); R = two-pen recorder. The chromatographic conditions are detailed under Experimental.

Japan), and a Develosil ODS 15/30 guard coumn (G) (10 × 4.6 mm I.D., Nomura Chemical, Seto, Japan), a Model DHCR 80 HP dual-piston pump (P2) (Japan Servo, Tokyo, Japan), a column of immobilized boronic acid gel (B) (10 × 4 mm I.D.), and an immobilized enzyme column (E) (10 × 4 mm I.D.).

The chromatographic conditions were essentially the same as those reported by Potter *et al.*[7], as described previously[8,9]. The mobile phase (S1) was 0.01 *M* sodium acetate buffered to pH 5.0 with citric acid, containing 1.2 m*M* TMA and 30 mg/l SOS, which was passed through a 0.20-μm membrane filter (Schleicher & Schull, Dassel, F.R.G.) and degassed with a water-aspirator for a few minutes prior to use. The flow-rate was 1.0 ml/min. Catecholamines and 5-HT were first detected by a glassy-carbon electrode with the potential set to +0.65 V against an Ag/AgCl reference electrode. The second solution (S2), 0.2 *M* potassium phosphate buffer (pH 8.5), was filtered and degassed as described above and pumped at a flow-rate of 0.6 ml/min. This second buffer was mixed with the column effluent through the tee (T) in a mixing coil (M) (Teflon tubing, 2 m × 0.5 mm I.D.). Catecholamines were then trapped on the boronic acid gel. ACh, Ch and EHC in the effluent from the coil were enzymatically converted into hydrogen peroxide at room temperature (20–25°C) when they were passed through the column reactor on which acetylcholinesterase and Ch oxidase were immobilized covalently. The hydrogen peroxide thus produced was detected by the electrochemical detector cell equipped with a platinum electrode set to the potential +0.5 V against an Ag/AgCl reference electrode. These two immobilized columns were washed with the second buffer (S2) and stored at 4°C after every use.

At the first position of the six-way valve, all components except 5-HT are separated by passing through the two columns C1 and C2. When DA has eluted from the second column, 5-HT is still retained on the first column. Therefore, 5-HT can be directly eluted from the first column without passing through the second column by switching the valve. The output currents from the two electrochemical detectors were recorded with a two-pen recorder (R).

RESULTS

Standard compounds of Ch, EHC, ACh, NA, AD, DHBA, DA and 5-HT could be all assayed with this system in less than 30 min in a single chromatographic run (Fig. 2). Fig. 2 also shows the effectiveness of the boronic acid gel on the analysis of ACh and Ch. It is clear that the boronic acid gel removed the second response of catecholamines on the platinum electrode, and that no significant effect was observed on the resolution of ACh, Ch and EHC (Fig. 3). The column-switching was made at *ca.* 15 min after the injection. Under these conditions, the retention times were Ch (4.5 min), EHC (6.7 min), ACh (7.8 min), NA (4.5 min), AD (6.9 min), DHBA (7.9 min), DA (12.6 min) and 5-HT (23.0 min). The peak-height responses of the electrochemical detectors were linear over the following ranges: 5–2500 pmol for ACh, Ch and EHC; 1–1000 pmol for NA, AD, DHBA and DA; 1–500 pmol for 5-HT. The detection limits were *ca.* 1 pmol for catecholamines and 5-HT, and 5 pmol for ACh and Ch at the signal-to-noise ratio of 5.

Fig. 4A shows one example of simultaneous analysis of the extract from rat midbrain region. ACh, Ch, NA, DA and 5-HT were clearly separated from each

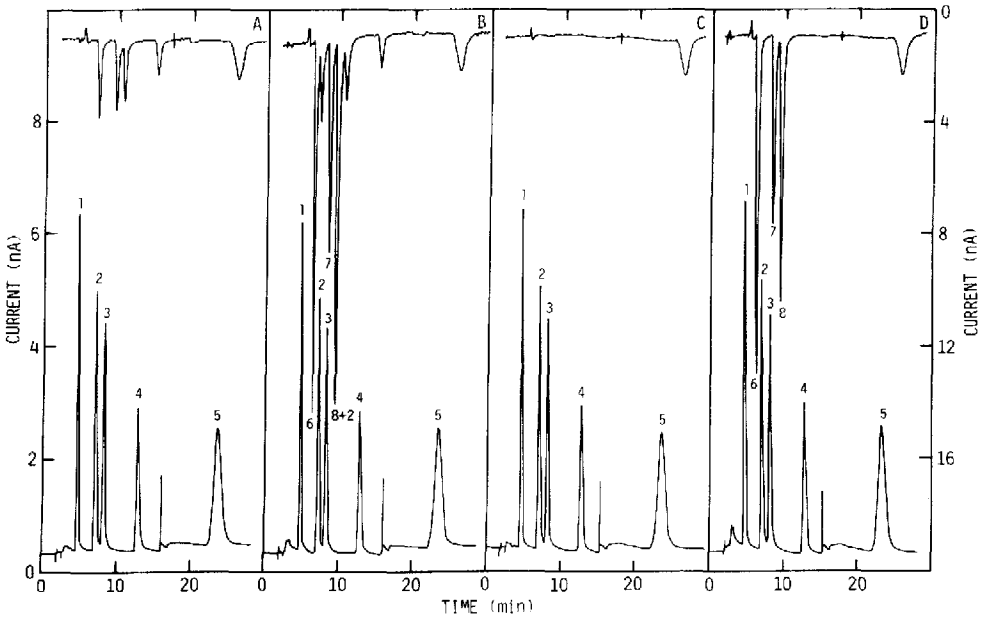


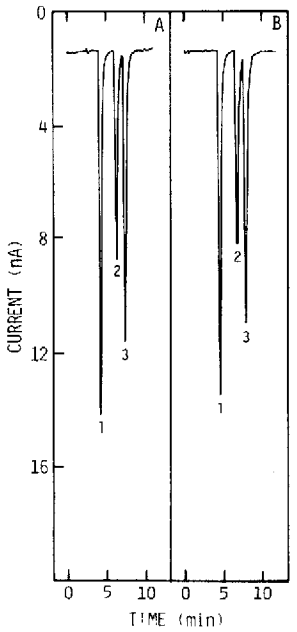
Fig. 2. HPLC elution profiles of the standard compounds. Analyses were carried out using the HPLC–ED system illustrated in Fig. 1 in the absence (A and B) or presence (C and D) of the immobilized boronic acid column. Upper chromatograms show the peak current from the platinum electrode (right ordinate), and the lower chromatograms from the glassy-carbon electrode (left ordinate). (A and C) A mixture of catecholamines and 5-HT (25 pmol each) was injected. (B and D) A mixture of catecholamines, 5-HT (25 pmol each) plus Ch, ACh and EHC (200 pmol each) was injected. Note that the second response due to catecholamines on the platinum electrode (A and B) was successfully removed by the boronic acid gel (C and D). Column switching was made at *ca.* 15 min after injection. Peaks: 1 = NA; 2 = AD; 3 = DHBA; 4 = DA; 5 = 5-HT; 6 = Ch; 7 = EHC; 8 = ACh.

other. AD was hardly detectable under the conditions used; this is the case for tissue from other brain regions as well.

To confirm the identification of ACh and Ch, the immobilized enzyme column was omitted from the system. As shown in Fig. 4B, no peaks are observed at the retention time of these compounds. This indicates that the system is highly specific for ACh and Ch.

Table I shows the recoveries for the compounds after 250 pmol each of catecholamines and 5-HT, and 2 nmol each of ACh and Ch were added to the whole brain homogenate. The absolute recovery for catecholamines and 5-HT was *ca.* 80% and that for ACh and Ch was *ca.* 75%. In both cases for catecholamines/5-HT and ACh/Ch, the recovery relative to the respective internal standards was *ca.* 100%. These results indicate that extraction with 1 *M* formic acid–acetone followed by ether washing is a useful procedure for the simultaneous determination of ACh, Ch, catecholamines and 5-HT.

The results of the simultaneous analysis for these neurotransmitters in several rat brain regions are summarized in Table II. It was found that the values obtained here agreed reasonably well with the previous data[14–18], which were obtained by individual measurements.



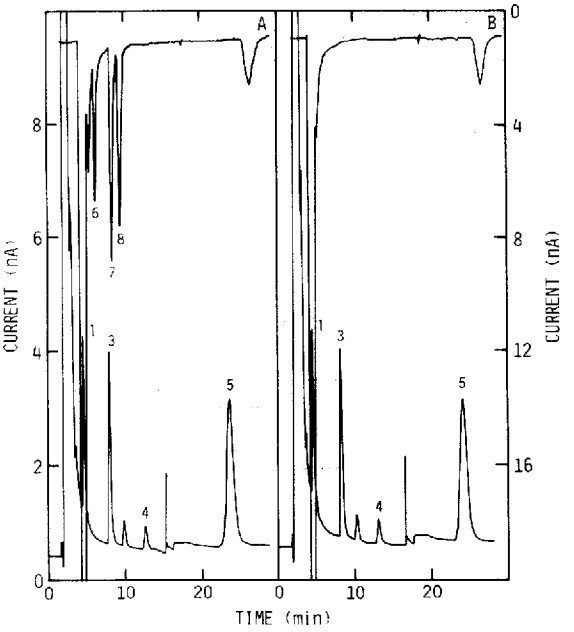


Fig. 3. The effect of the immobilized boronic acid column on the resolution of Ch, EHC and ACh. Standard compounds (200 pmol each) were analysed in the absence (A) or presence (B) of the immobilized boronic acid column. Peaks: 1 = Ch; 2 = EHC; 3 = ACh.

Fig. 4. HPLC elution profiles of the extract from rat midbrain region. Rat midbrain (106 mg) was homogenized and processed as described under Experimental. Analyses were carried out in the presence (A) or absence (B) of the immobilized enzyme column. Note that there are no peaks at the retention times of Ch, EHC and ACh in B. Peak numbers as in Fig. 2; injection volumes, 10 μl.

TABLE I

RECOVERIES OF Ch, ACh, CATECHOLAMINES AND 5-HT FROM RAT BRAIN HOMOGENATE AFTER THE ADDITION OF KNOWN AMOUNTS OF THE COMPOUNDS

Microwave-irradiated rat whole brain was homogenized as described under Experimental. To the experimental sample, 2 nmol each of Ch, EHC and ACh, and 250 pmol each of NA, AD, DHBA, DA and 5-HT were added. Relative recovery was calculated from absolute recovery, taking the value of the internal standard as 100%. Results represent mean ± S.E. for 4–5 experiments.

Compound	*Absolute recovery (%)*	*Relative recovery (%)*
Ch	74.2 ± 2.0	101.4 ± 1.5
EHC	73.2 ± 1.6	100
ACh	76.6 ± 2.1	104.7 ± 2.0
NA	80.6 ± 0.9	103.0 ± 0.4
AD	80.5 ± 0.8	102.8 ± 0.2
DHBA	78.3 ± 0.7	100
DA	86.4 ± 1.1	110.3 ± 1.1
5-HT	81.9 ± 3.2	104.4 ± 1.3

TABLE II

AMOUNTS OF Ch, ACh, NA, DA AND 5-HT IN RAT BRAIN REGIONS

Microwave-irradiated rat brain was dissected and processed as described under Experimental. Results represent mean ± S.E. for a group of 4–5 animals. The amounts are expressed in nmol/g wet tissue.

Brain region	*Ch*	*ACh*	*NA*	*DA*	*5-HT*
Cerebral cortex	13.8 ± 1.0	16.4 ± 0.5	2.32 ± 0.10	2.50 ± 0.27	3.48 ± 0.09
Striatum	23.1 ± 2.8	39.0 ± 2.1	1.37 ± 0.11	58.6 ± 1.8	3.82 ± 0.23
Hippocampus	21.3 ± 2.7	19.5 ± 1.2	2.59 ± 0.16	0.84 ± 0.15	3.17 ± 0.23
Midbrain	20.9 ± 2.2	23.1 ± 0.9	3.20 ± 0.03	1.65 ± 0.19	4.99 ± 0.19
Cerebellum	18.2 ± 1.0	4.6 ± 0.2	1.95 ± 0.04	0.15 ± 0.02	0.98 ± 0.08
Pons-medulla oblongata	15.9 ± 0.8	18.2 ± 0.4	2.92 ± 0.07	0.39 ± 0.04	3.72 ± 0.13

DISCUSSION

This paper describes a simple method for the simultaneous determination of ACh, Ch, NA, DA and 5-HT in the brain by HPLC–ED. Smith *et al.*[19] have previously reported the concurrent measurement of ACh, Ch, DA, NA, 5-HT and other several amino acids in rat brain. They separated these compounds by using three different ion-exchange columns, and measured ACh and Ch by a radioenzymatic assay and catecholamines and 5-HT by fluorometric assays —a laborious and time-consuming method. As an extraction solvent, they used 1 *M* formic acid–acetone (15:85), which had been preferentially employed for the assay of ACh and Ch[11–14]. In the present work we also used this solvent system. It was found that the absolute recoveries were sufficient (*ca.* 80% for catecholamines and 5-HT, and *ca.* 75% for ACh and Ch) and the relative recoveries were excellent (almost 100% against DHBA or EHC). The sample clean-up procedure by washing with ether was very simple; it effectively removed lipids and other interfering components. It was found that NA and Ch were separated sufficiently from a large front-peak. In the present method it was inevitable that major acidic metabolites, 3,4-dihydroxyphenylacetic acid, homovanilic acid and 5-hydroxyindoleacetic acid, were almost washed out during this ether washing under the acidic conditions.

We encountered two particular problems. The first was the interfering response due to catecholamines on the platinum electrode; the assay of ACh, Ch and EHC was much interfered with in the absence of the boronic acid gel (Fig. 2A and B). However, these ghost responses on the second detector cell were removed successfully by the boronic acid column, which was placed just ahead of the immobilized enzyme column (Fig. 2C and D). These two immobilized columns had a back-pressure of *ca.* 10 Kg/cm^2. It is known that boronic acid can be complexed with catechols most effectively under weakly alkaline conditions (*ca.* pH 7–8)[20]. Therefore, the chromatographic conditions optimized for the assay of ACh and Ch (pH 8) were found to be suitable for the complex formation of boronic acid with catechols.

The second problem was that 5-HT was strongly retained on the octadecylsilane (C_{18}) column owing to the use of an ion-pairing reagent, which was necessary for the analyses of ACh and catecholamines. Although we examined several different columns and different compositions of the mobile phase, it was difficult to analyse

all the components in a relatively short time without adversely affecting the resolution. Fortunately, since there was no substance eluted between DA and 5-HT, it was possible to speed up the elution of 5-HT by employing two equivalent analytical columns and a column-switching device (Fig. 1). This also increased the sensitivity to 5-HT because of the improved elution profile.

The present system has many advantages compared with the previous method[19]. (1) The procedure for sample preparation is simple: there is no pretreatment such as column chromatography. (2) The analysis is rapid (within 30 min). (3) The sensitivity is high (less than 1 pmol of catecholamines and 5-HT, and 5 pmol of ACh and Ch). The system would be useful for neurochemical studies dealing with small amounts of tissue samples from the same brain region.

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