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RAPID ESTIMATION OF CATECHOLAMINES, OCTOPAMINE AND 5-HYDROXYTRYPTAMINE IN BIOLOGICAL TISSUES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COULOMETRIC DETECTION

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SUMMARY

A rapid, convenient procedure is described for the simultaneous determination of catecholamines, monohydroxyphenolamines and 5-hydroxytryptamine in biological tissues. The procedure involves homogenization of tissue in perchloric acid, addition of heparin and centrifugation followed by direct injection of the supernatant onto a C_{18} reversed-phase high-performance liquid chromatographic column. The mobile phase employed sodium dodecyl sulfate as ion pair reagent with 20% acetonitrile and 10—12% methanol as organic modifier. Eluted fractions were detected electrochemically using dual coulometric electrodes operated in screen mode. The procedure has been applied to the analysis of norepinephrine, epinephrine, dopamine, octopamine, tyramine, 5-hydroxytryptamine and tryptophan in a variety of tissues including mammalian heart and brain and insect nerve cord.

INTRODUCTION

Abnormal levels of catecholamines, their monohydroxy equivalents and 5-hydroxytryptamine (5HT) in the brain have been implicated in a number of psychiatric disorders including schizophrenia, depression, migraine, aggression, irritability and Parkinsonism [1—5]. The importance of these compounds in brain function, together with their presence in mammalian myocardium [6], has resulted in intensive efforts to provide rapid, sensitive analytical procedures with which to determine tissue levels. High-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been particularly useful in this regard especially for the determination of catecholamines and 5HT [7]; however, few reports have described simultaneous determination of catecholamines, 5HT and monohydroxyphenolamines [8]. One constraint associated with simultaneous determinations of these compounds by HPLC—ED is that monohydroxyphenolamines require a higher electrode poten-

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tial than catecholamines in order to effect electro-oxidation [8]. This problem can be overcome to some extent by the use of multiple working electrodes which effectively extend the potential range of ED [9]. However, the relatively small surface of the tubular electrodes or thin-layer cells that are employed in amperometric detectors tends to result in fouling of the electrode surface at higher potentials. Coulometric detector electrodes provide a larger surface which is less susceptible to perturbations resulting from adsorption of reaction products and also confers greater redox efficiency [10]. Thus, a dual coulometric detector system offers advantages for detection of catecholamines, monohydroxyphenolamines and 5HT in a single biological sample.

The present report describes a convenient procedure for simultaneous analysis of catecholamines, 5HT and octopamine (OA) in biological tissues. The procedure involves direct inject of the sample onto a C_{18} reversed-phase HPLC column with the eluted fractions detected electrochemically using a dual-electrode coulometric detector. The utility of the described procedure is demonstrated by analysis of biogenic amine levels in rat heart and insect nerve cord and the brain of rat and mouse.

MATERIALS AND METHODS

Reagents

Norepinephrine bitartrate, epinephrine bitartrate, dopamine hydrochloride and tyramine hydrochloride were obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.), and octopamine hydrochloride, 5-hydroxytryptamine creatine sulfate complex, tryptophan and sodium heparin were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium dodecyl sulfate (SDS) (electrophoresis purity) was supplied by Bio-Rad Labs. (Richmond, CA, U.S.A.) and 3,4-dihydroxybenzylamine (DHBA), used as the internal standard, was donated by Bioanalytical Systems (West Lafayette, IN, U.S.A.). Solvents (HPLC grade) were obtained from Caledon Labs. (Georgetown, Canada) and all other chemicals (Baker Analyzed Grade) from CanLab (Toronto, Canada).

Catecholamine standards were prepared at 100 μg/ml in 0.1 *M* perchloric acid and working concentrations achieved by serial dilution in 0.1 *M* perchloric acid. Water used for the preparation of all solutions was deionized, distilled and re-distilled over potassium permanganate prior to use.

Animals and tissue collection

Male rats of the Sprague-Dawley strain, weighing between 220 and 330 g, were obtained from a colony maintained in the Department of Biology, University of Waterloo, on a 12 h:12 h light:dark cycle and provided with food (Purina Labs.) and water ad libitum. Rats were isolated from the colony 12 h prior to sacrifice by decapitation. Brains and hearts were excised and frozen in liquid nitrogen before being stored at −75°C.

Male mice of the C-57 strain, weighing between 20 and 27 g, were obtained from a colony maintained in the Department of Psychology, University of Waterloo, under similar conditions to those described for rats. Brain tissues were obtained and stored in a similar manner to that described for rats.

Adult male cockroaches were taken at 1—3 months after the adult moult

from a colony of *Periplaneta americana* maintained under standard conditions in this laboratory [11]. Insects were isolated 12 h prior to removal of nerve cord tissues (including thoracic and abdominal ganglia). Nerve cords were rinsed in cockroach saline [12] and transferred to a preweighed microcentrifuge tube containing 250 μl of ice cold 0.1 *M* perchloric acid and the internal standard, dihydroxybenzylamine (DHBA).

Sample preparation

Brain and heart tissues were weighed and broken in the frozen state before being placed in 1.5 ml (rat tissues) or 1.0 ml (mouse brain) of ice-cold 0.2 *M* perchloric acid containing DHBA. All tissues were homogenized by ultrasonic disintegration over ice using a Branson Sonifier Model 200 (75 W, 50% pulsed power for 2 min). Sodium heparin (rat tissues, 2000 units; mouse brain, 750 units; insect nerve cord, 200 units) was added to facilitate lipoprotein precipitation [13]. Samples were centrifuged at 40,000 *g* for 30 min and the supernatant was then diluted appropriately with water to reduce the amount of contaminating material injected onto the HPLC column.

Apparatus

Chromatography was achieved using a Spectra-Physics Model 740B solvent delivery system equipped with an extra pulse dampener. Samples were introduced through a Valco CV-6-UHPa-N60 injection valve fitted with a 50-μl sample holding loop. Separations were performed on an Ultrasphere IP C_{18} column (150 × 4.6 mm I.D., 5-μm particle size) (Beckman, Toronto, Canada) protected by a Brownlee guard column (30 × 2.1 mm I.D., 5-μm particle size) (Technical Marketing Assoc., Mississauga, Canada).

The electrochemical detection system (ESA 5100A, Bedford, MA, U.S.A.) comprises a coulometric guard cell and a solid-state analytical cell containing dual coulometric working electrodes made from porous graphite (Fig. 1). The guard cell is installed before the injector and, by operating it at a high poten-

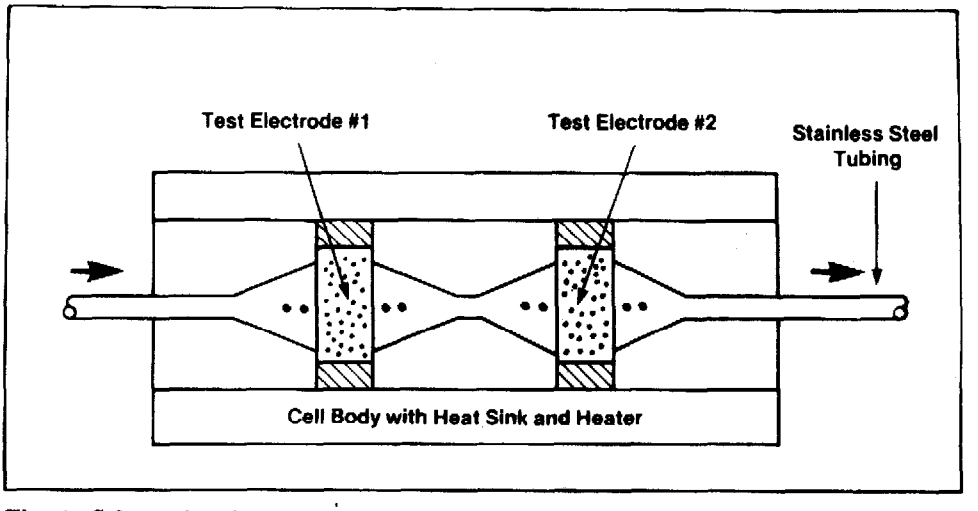

Fig. 1. Schematic of cell design of dual-electrode electrochemical detection system (reproduced with permission from ESA, Bedford, MA, Brochure No. 182-5100).

tial (0.9 V), can preoxidize mobile-phase contaminants thereby reducing the noise generated by the buffer at the analytical detector.

For detection of catecholamines in brain and heart tissues the first coulometric detector was set at 0.1 V to reduce interference by contaminating electroactive compounds at the second detector which was set at 0.3 V, the potential required for electro-oxidation of norepinephrine (NE), epinephrine (E), dopamine (DA), 5HT and the internal standard, DHBA (Fig. 2A). Detection of OA requires a higher oxidation potential [8]; therefore, for the analysis of OA the first detector was set at 0.5 V for detection of NE, E, DA, 5HT, some tryptophan (TP), and DHBA, whereas the second detector was set at 0.75 V and detected OA, tyramine (TA), 5HT and TP (Fig. 3). Signals from the coulometric detectors were recorded and integrated using an SP4270 computing integrator for detector 1 and an SP4100 computing integrator for detector 2 (Spectra Physics, San Jose, CA, U.S.A.).

Chromatography

The mobile phase employed for catecholamine and 5HT determinations in brain and heart tissues contained 0.02 *M* trichloroacetic acid (TCA), 0.075 *M* sodium phosphate, 1.5 μM EDTA with 1.5 m*M* SDS as ion-pair reagent and 20% acetonitrile and 10% methanol as organic modifier. The buffer, final pH 3.1, was continually degassed with a stream of helium and pumped at a flow-rate of 1.0 ml/min. When operating at higher potentials for the determination of OA, the mobile phase contained 2.0 m*M* SDS and 12% methanol with the remaining buffer components as described above. The flow-rate was reduced to 0.8 ml/min when OA determinations were required.

RESULTS AND DISCUSSION

Chromatographic system

A previous report [8] demonstrated that baseline separation of DA, NE, TA and OA was achieved using a 150 mm × 4.6 mm I.D. Ultrasphere RP-18 IP column packed with 5-μm particles, an acetic acid—ammonium hydroxide buffer containing octane sulfonic acid (OSA) for ion pairing and amperometric detection. Subsequently it was found that replacement of OSA with SDS provided better separation of amines, a quieter baseline at high oxidation potentials due to the lower amounts of ion-pair reagent required and, therefore, lower limits of detection for OA and TA ($\leqslant$100 pg with signal:noise ratio $\geqslant$4).

Use of the ESA coulometric detector in the present study requires a buffer system that generates low currents at both working electrodes and resulted in adoption of the TCA—phosphate buffer containing 1.5 μM EDTA; this provides currents of less than 1 μA at 0.75 V. The separation and peak symmetry obtained for various amines using TCA—phosphate buffer was further improved by the use of high concentrations of SDS (1.5—2.0 m*M*) together with high organic solvent content ($\geqslant$30% mobile phase). This improvement was achieved without any increase in the capacity factor for TA, a relatively non-polar amine. The present buffer system affords elution of 5HT within

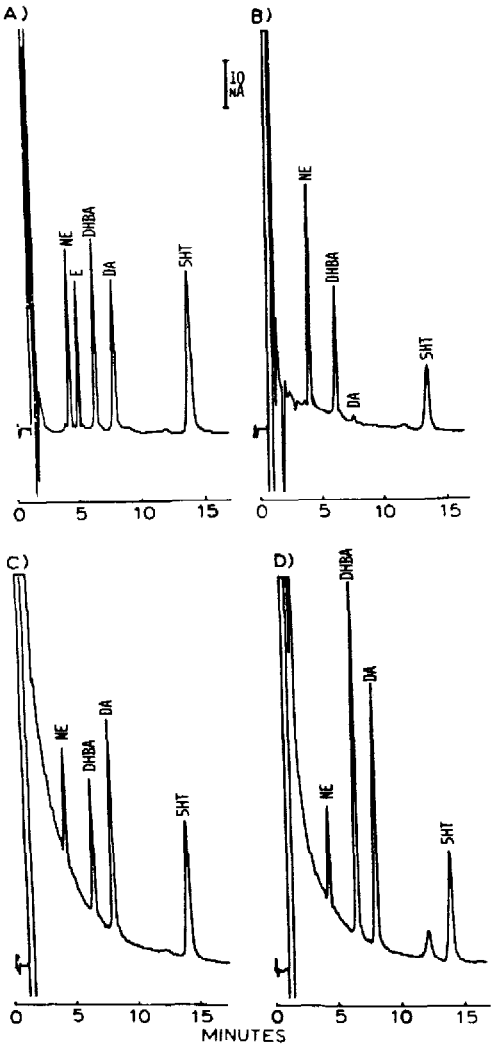

Fig. 2. Chromatograms of 1 ng of standards (A), rat heart (B), rat brain (C) and mouse brain (D) with second detector set at 0.3 V. Flow-rate 1.0 ml/min; other chromatographic conditions are defined in the text.

15 min while still retaining NE for at least 4.5 min (Fig. 2). The increased resolution obtained with high SDS concentration may be explained by its strong adsorption to the bonded-phase packing so that at 2.0 m*M* the surface coverage of the bonded-phase column particles approaches the coverage of the chemically bonded C_{18} groups. The critical micelle concentration (CMC) for SDS in a mobile phase containing 20% methanol is 2.5 m*M* [14], thus the concentration of SDS used in the present study (2.0 m*M*) for mobile phase containing 32% organic solvent is below the CMC. The high concentration of organic solvent maintained reproducible column retention of amines even when the mobile phase (2 l) was recycled for over 100 injections and also provided a fast run-time per sample even at high potentials. The direct injection of biological samples revealed few slowly eluting fractions; indeed TP,

which was detected at the 0.75-V electrode at about 20 min, demonstrated the highest capacity factor among the compounds of interest.

Sample preparation

Tissue samples were normally processed within 24 h following dissection; however, samples could be stored at −75°C for several months without any significant change in amine levels.

Several workers have described the use of dilute perchloric acid to precipitate proteins as an early step in the preparation of samples for biogenic amine determinations using HPLC—ED [15—18]. However, samples in which proteins were precipitated with perchloric acid and then centrifuged reveal a large number of early peaks when the supernatant is injected directly onto a reversed-phase column with ED. These peaks interfere with the detection and quantification of NE, thus only a few studies have exploited the convenience of the technique [9, 16—20]. The present study demonstrates that the addition of heparin to 0.2 *M* perchloric acid increases the amount of precipitation and results in a cleaner chromatogram (Figs. 2 and 3). Heparin is known to form insoluble complexes with lipoprotein molecules [13, 21] and may also co-precipitate electroactive molecules which would otherwise elute from a reversed-phase column. It is appropriate to indicate that the buffer employed in this study also contributes to a cleaner signal at the front of the chromatogram through the rapid elution of many molecules that are not retained by an ion-pair mechanism because of the high content of organic solvent.

Recovery

An internal standard, DHBA, was included in the sample to accommodate dilution of supernatant by tissue fluid. Determinations of amine recoveries with and without the addition of heparin demonstrate that heparin has no effect on the recovery of tissue amines; therefore, the amine levels reported in Tables I and II indicate 100% recovery.

Detection

The applied potential required to oxidize the biogenic amines with the coulometric detector used in the present study is considerably lower than that required for amperometric detection [8]. Many commercial amperometric detectors use a silver/silver chloride reference electrode, the half-cell potential of which is subject to drift due to diffusion of chloride ions from the reference electrode into the mobile phase [18]. By contrast, the proprietary solid-state reference electrode used in the ESA coulometric detector provides potential control through a H_2/H^+ couple and this, together with the close proximity (only 1 mm apart) of the reference and working electrodes, results in lower applied potentials, greater electrode stability and minimal uncompensated potential drop (IR) over the solution between the working and reference electrodes. Consequently, the optimized applied potential for detection of octopamine in the present study was 0.75 V whereas an amperometric detector with a silver/silver chloride reference electrode required an optimal applied potential of 0.9 V [8].

Operation of the detector in a screen mode offers increased selectivity particularly for compounds which oxidize at high applied potentials. Fig. 3

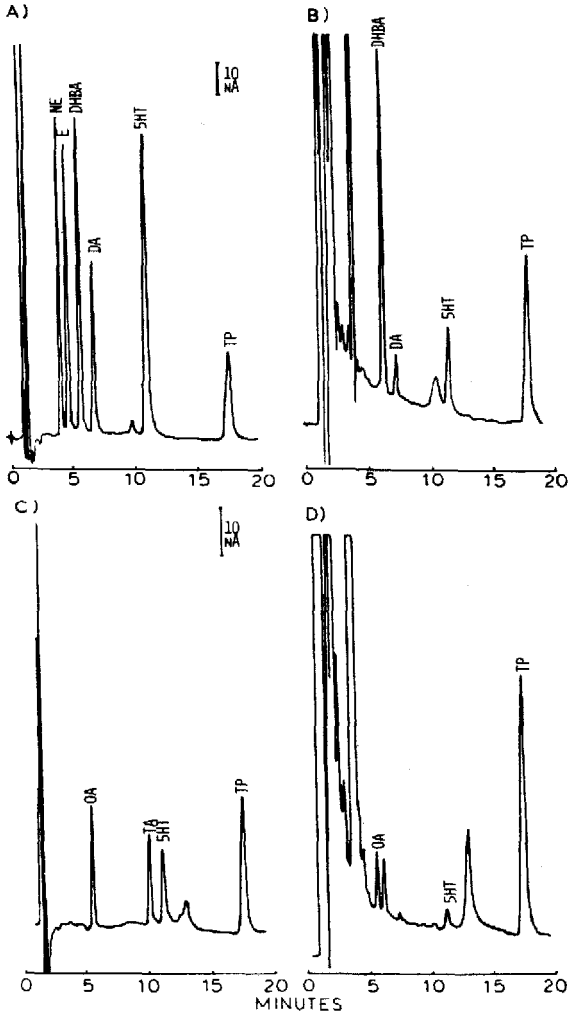

Fig. 3. Chromatograms of 1 ng of standards (A, C) and cockroach nerve cord extracts (B, D) with detector 1 set at 0.5 V (A, B), detector 2 at 0.75 V (C, D). Flow-rate 0.8 ml/min; other chromatographic conditions are defined in the text. (The peak appearing at 13.10 min on detector 2 is an unidentified contaminant originating from the injector seal material.)

illustrates the detection of NE, E, DHBA, DA, 5HT and TP at the first coulometric detector (set at 0.5 V) whereas TA, OA, 5HT and TP are detected at the second detector (set at 0.75 V). Thus, many compounds that oxidize at the lower potential are screened from the second detector. The advantages of this system are evident by reference to OA which appears at detector 2 at 5.80 min without any interference from DHBA which is retained for 6.16 min but which is totally oxidized at detector 1. Dual-electrode amperometric detector systems oxidize only a fraction of the electroactive compound and, therefore, could not provide this degree of selectivity. Fig. 3 demonstrates also that 5HT and TP undergo oxidation reactions at both detectors. The first oxidation of 5HT occurs at the 5-hydroxy group of the indole while the second reaction involves oxidation of ring nitrogen to N^+ [22, 23]. The nature of the dual detection of TP has not been elucidated.

Preoxidation of electroactive material in the mobile phase was achieved by means of a guard cell set at 0.9 V. This resulted in lowered background currents at the detector electrodes and improved the signal:noise ratio.

The coulometric detector employed in the present study should not be used at high potentials when any part of the chromatographic system contains graphite-impregnated PTFE material because this releases electroactive substances which contribute unidentified peaks to the chromatogram. An unidentified peak originating from the manual injector is evident in Fig. 3C and D at about 13 min; however, this contaminant can be separated from peaks of interest.

Biogenic amines in tissues

The levels of NE, DA and 5HT in rat heart, rat brain and mouse brain are presented in Table I. The catecholamine levels found in the heart fall within the ranges reported in previous studies [6, 16, 20] while the 5HT levels are, to the best of our knowledge, the first to be described for this organ using HPLC—ED. The biogenic amine levels reported for other tissues in Table I are also consistent with earlier studies on rat brain [17, 24, 25] and mouse brain [26, 27]. The close correlation of the data reported in Table I with those of previous investigations provides vindication of the current procedure for estimating tissue levels of catecholamines and 5HT.

By contrast to the widespread use of HPLC—ED for estimation of catecholamines, most determinations of monohydroxyphenolamines have employed radioenzymatic procedures [28]. Indeed, HPLC—ED has been applied only to the estimation of OA, TA and synephrine in plant products [29, 30] and HPLC with fluorometric detection has been used to monitor OA in ganglia of *Aplysia californa* [31]. Table II shows the levels of OA, DA, 5HT and TP obtained for nerve cords of the american cockroach *Periplaneta americana* using the HPLC—ED procedure described in this study. The levels are slightly lower than those reported by other workers using radioenzymatic techniques [32], possibly due to the greater specificity that is afforded by the present technique. In addition to providing increased specificity, the present proce-

TABLE I

CONCENTRATIONS OF NOREPINEPHRINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE IN RAT HEART, RAT BRAIN AND MOUSE BRAIN ESTIMATED BY HPLC—ED

Values indicate mean ± S.D. for the number of determinations shown in parentheses.

Tissue	Amine concentration (ng/g)		
	Norepinephrine	Dopamine	5-Hydroxytryptamine
Rat heart	725.2 ± 123.2 (10)	23.1 ± 2.1 (2)	253.1 ± 73.5 (10)
Rat brain	376.4 ± 27.5 (12)	905.9 ± 69.6 (12)	340.8 ± 46.8 (12)
Mouse brain	473.1 ± 24.4 (16)	1423.7 ± 114.9 (16)	586.8 ± 54.7 (16)

TABLE II

CONCENTRATIONS OF OCTOPAMINE, DOPAMINE, 5-HYDROXYTRYPTAMINE AND TRYPTOPHAN IN COCKROACH NERVE CORD ESTIMATED BY DUAL COULOMETRIC ELECTROCHEMICAL DETECTION OF HPLC-SEPARATED FRACTIONS

Values indicate mean ± S.D. for the number of determinations shown in parentheses. Average weight of nerve cords = 8.4 ± 1.7 mg.

Amine	Amine concentrations (ng/g)	
	Detector 1 (0.5 V)	Detector 2 (0.75 V)
Octopamine	—	888.3 ± 127.8 (8)
Dopamine	311.5 ± 64.9 (8)	—
Serotonin	335.9 ± 77.4 (8)	368.8 ± 105.2 (8)
Tryptophan	4634.0 ± 1399.0 (8)	4499.2 ± 1069.8 (8)

dure enables simultaneous estimation of other biogenic amines contained within the biological sample. The sensitivity of the HPLC—ED technique described herein also compares favourably with the radioenzymatic procedure and permits OA estimates in single ganglia.

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