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SENSITIVE METHOD FOR DETERMINATION OF PICOGRAM AMOUNTS OF EPINEPHRINE AND OTHER CATECHOLAMINES IN MICRODISSECTED SAMPLES OF RAT BRAIN USING LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Liquid chromatography with high-sensitivity electrochemical detection has been employed to measure picogram amounts of epinephrine and other catecholamines in microdissected samples of the rat hypothalamus. Tissue catecholamines are purified by solvent extraction; this provides better selectivity and recovery than methods involving alumina. The solvent extraction technique has been modified in order to eliminate its major disadvantage, the presence of electroactive substances separating with catecholamines. Detection limits of below 1 pg allow for analysis of catecholamines including epinephrine in very small brain samples such as micropunches

INTRODUCTION

Despite extensive research on the neurobiology of catecholamines, the role of epinephrine (E) in the mammalian central nervous system (CNS) remains obscure. The adrenergic innervation shown by immunohistochemical studies within the hypothalamus suggests an involvement of E in the regulation of food and water intake, sleep, body temperature and reproductive processes [1–3]. Since E is present in the brain at a relatively low concentration [with a ratio of E to norepinephrine (NE) of approximately 1:50 in the rat], there are considerable problems concerning the specificity and sensitivity of the analytical methods used for its measurement. These problems are particularly important when attention is focused on "discrete" subregions of the brain, but they have received little attention apart from recent descriptions of E measurement using microbore high-performance liquid chromatography (HPLC) [4,5].

We have employed HPLC with coulometric detection because it has been shown

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to offer the necessary high sensitivity and stability in the measurement of NE and dopamine [6]. Our development of this technique allows for quantification of less than picogram amounts of E and other catecholamines in microdissected samples of the rat hypothalamus. Prior to the chromatographic analysis the tissue catecholamines are purified by solvent extraction; this provides higher recovery and selectivity than methods involving alumina [4,7]. Purification of catecholamines was preferred to the increasingly popular direct analysis of the tissue extracts for several reasons. Firstly, the low ratio of E to NE necessitates clear separation between E and abundant NE; this requires resolution using a relatively slow mobile phase. Eluent of this kind, if applied to a crude tissue extract, containing amine metabolites and indoles, results in an unacceptably long elution time, mainly because of the high retention of serotonin. Secondly, the presence of a broad electrochemically active solvent front, typical for non-purified tissue samples, often affects the quality of quantification of NE (see ref. 6) and when working at very high sensitivities this can complicate the measurement of E. Thirdly, purification of tissue extracts contributes to an extended lifetime of both the analytical cell and column and reduces problems of maintenance. Our modifications of the solvent extraction method have now eliminated its major disadvantage, the presence of endogenous electroactive substances separating with catecholamines, and have made it compatible with electrochemical detection at the highest sensitivities. A preliminary report of this technique has already been presented [8].

EXPERIMENTAL

Reagents

Epinephrine bitartrate (E), norepinephrine free base (NE), 3,4-dihydroxyphenylethylamine hydrochloride (dopamine, DA), 3,4-dihydroxybenzylamine hydrobromide (DHBA), acid alumina (type WA4), diphenylboric acid–2-amino ethyl ester (diphenylborate–ethanolamine, DPBE) and sodium metabisulphate were purchased from Sigma (Poole, U.K.). Acetic acid, ammonium chloride, ammonia solution, disodium ethylenediaminetetraacetic acid (EDTA), *n*-heptane, hydrochloric acid, methanol (HPLC grade), monosodium dihydrogen orthophosphate (Aristar grade), orthophosphoric acid, tetraoctylammonium bromide (TOABr), trichloroacetic acid (TCA) and water (HPLC grade) were purchased from BDH (Poole, U.K.). Sodium-1-octane sulphonic acid (OSA) was purchased from Eastman-Kodak (Liverpool, U.K.). Octanol (HPLC quality) was purchased from Aldrich (U.K.). LY 134046 was obtained from Eli Lilly (Indianapolis, IN, U.S.A.). All reagents were analytical quality unless otherwise stated.

HPLC equipment

The liquid chromatographic system included a Spectroflow 400 Kratos pump (Kratos Analytical, U.K.) equipped with a pulse dampener, a Model 7025 injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 100-μl loop, and a Coulochem ESA 5100A controller with a 5020 guard cell and a 5011 analytical

cell (Environmental Sciences Assoc., Bedford, MA, U.S.A. and Severn Analytical, U.K.).

The dual-electrode system was used in screen mode. In the case of the mobile phase routinely used and described below, the first detector was operated at +0.02 V and the second quantifying detector at +0.32 V. A potential of +0.45 V was usually applied to the guard cell. Details of the system have been described by Kilpatrick et al. [6].

Several reversed-phase analytical columns were examined: a μBondapak C_{18} cartridge (100 mm×8 mm, Waters Millipore, U.K.) and a Resolve 10-μm C_{18} cartridge (100 mm×5 mm, Waters Millipore), both compatible with a Z-module compression chamber (Waters Millipore), and an Excel Spherisorb 5 ODS 2 stainless-steel column (100 mm×4.6 mm I.D., Hichrom, U.K.). The analytical column was preceded by a Resolve 10-μm Guard Pak C_{18} (Waters Millipore). A high-pressure in-line filter containing a 0.2-μm graphite element (ESA, Severn Analytical) was used before the guard cell and also before the detection cell.

The HPLC system was kept in an earthed Faraday cage and the detector was additionally protected by a 3-A mains filter (Vero-Speed, U.K.).

Signals from the detector were recorded by a double-pen chart recorder (SE 120 Goerz Metrawatt, Austria). The identification and quantification of the catecholamines were performed by comparing their retention time and peak height with the standards.

Standards

Standard stock solutions of NE, E, DA and DHBA were prepared at a concentration of 1 mg/ml in 0.1 *M* hydrochloric acid which had been pre-filtered (0.2 μm) and degassed; they were stored in the dark at +4°C for up to one month. Standard working solutions were made each day by dilution of the stock solutions with 0.05 *M* TCA or the mobile phase.

Mobile phase

The best performance was achieved using a mobile phase containing 0.05 *M* monosodium dihydrogenphosphate, 0.1 m*M* EDTA, 0.4 m*M* OSA and 5% methanol, pH 3.25, together with an Excel Spherisorb 5 ODS 2 column. It was found that a slight increase in the concentration of OSA may be needed to compensate for changes in column characteristics with time. The mobile phase was prepared using HPLC-grade water and methanol, and the pH was adjusted with concentrated orthophosphoric acid after adding methanol. The buffer was then filtered through a 0.2-μm filter (Millipore) and degassed in a sonic bath. A volume of 0.5 l was prepared as required and the eluent was recycled in a closed system for up to one month unless the background current increased earlier. In the system used, background currents of 10±5 nA at a potential of +0.32 V on detector 2 were normally observed. A flow-rate of 1.0 ml/min ($6 \cdot 10^6$ Pa, 60 bar) at ambient temperature (19–25°C) was used.

Animals and tissue collection

Female Wistar rats (250–300 g) were given two intraperitoneal injections of the vehicle (2.0 ml water per kg) or LY 134046 (20 mg/kg) with a 3-h interval

between the treatments. The animals were decapitated and the brains were rapidly removed 2 h after the second injection. Hypothalami were dissected according to Coen and Coombs [9]. For the micropunched samples the brains were prepared according to Palkovits [10]. Brains and hypothalami were frozen in dry ice and stored at −80°C until further analysis.

Transverse sections of the brains (300 μm) were cut in a cryostat (Bright Instrument, U.K.) at −10°C and microsamples from the preoptic area were punched from the frozen sections using a stainless-steel needle of 0.5 mm I.D. [11]. Micropunched samples were maintained frozen in dry ice until sonication on the same day.

Sample preparation

Hypothalami. Dissected rat hypothalami (55 ± 5 mg wet mass) were sonicated in 10 volumes of 0.05 *M* TCA, 0.15% sodium metabisulphate and 0.5 m*M* EDTA containing DHBA as an internal standard at a concentration of 5 pg/μl. Homogenates were prepared using a Vibracell soniprobe (Sonics & Materials, U.S.A.) for 3×15 s on ice. Samples were centrifuged (+4°C, 6000 *g*, 15 min) and the supernatant was collected for further analysis; if necessary the latter may be frozen on dry ice and stored at −80°C for up to three weeks without any observable decomposition of catecholamines. Pellets were left for protein assay [12].

Micropunched samples. Three or six punched microsamples removed from the appropriate area of the brain sections (containing approximately 7 μg protein each) were pooled and sonicated in 200 μl of 0.05 *M* TCA, 0.15% sodium metabisulphate and 0.5 m*M* EDTA containing 50 pg DHBA (3×10 s on ice). These supernatants were usually analysed immediately afterwards but it was possible to store them at −80°C for several days without any significant loss of catecholamines. Protein content was measured as above.

Solvent extraction of catecholamines from TCA supernatants

The methods of Smedes et al. [7] and Durkin et al. [4] were modified.

Preparation of reagents. A simple and quick procedure was developed to remove electroactive trace impurities from the diphenylborate complexation solution and from the ion-pairing TOABr. TOABr (0.35%, m/v) and 1% octanol (v/v) in *n*-heptane were combined with 0.65 volumes of 0.08 *M* acetic acid and shaken for 2 min. The mixture was left to separate the phases, the acetic acid (bottom) phase was discarded and 0.5 volumes of water were added to remove the remaining acid. The mixture was shaken and separated again as before. The purified organic phase (heptane phase, TOABr) was used for the extraction of catecholamines.

DPBE complex (0.2%) and 0.5% EDTA (both m/v) in 3 *M* ammonium chloride–ammonia, pH 8.8, were treated with alumina (100 mg per 10 ml) and shaken for 10 min. The alumina was sedimented and the supernatant filtered through a 0.2-μm Gelman filter disc (Gelman, U.K.). The filtrate was combined with 0.4 volumes of purified heptane phase and shaken for 2 min. The phases were separated and the bottom one was collected and further used as the purified DPBE solution.

Acetic acid (0.08 *M*) and 0.5 *M* monosodium dihydrogen phosphate were fil-

tered through 0.2-μm filter discs (Gelman). All the solutions used for extraction of catecholamines were kept at +4°C avoiding direct light and no changes in their quality were noticed up to four weeks after purification.

Procedure. The protein-free supernatants (20 or 200 μl) derived from the whole hypothalami or from micropunched samples, respectively, and corresponding to 2 or 0.2–0.4 mg tissue, respectively, were placed in 1.5-ml Eppendorf-type tubes (Sterilin-Greiner, U.K.). When appropriate, the volume was adjusted with 0.05 *M* TCA to 200 μl. Use of perchloric acid should be strictly avoided since it precipitates the ion-pairing reagent and reduces the recovery of catecholamines. A 250-μl sample of the purified DPBE solution and 200 μl of the purified heptane phase were added and the mixture was shaken vigorously for 2 min and then centrifuged for 2 min to separate the phases. The heptane (top) phase, containing the diphenylborate–catecholamine complex ion-paired with tetraoctylammonium cation, was carefully collected avoiding any contamination by the water phase and transferred into a new tube (as before). A 70-μl aliquot of 0.08 *M* acetic acid and 120 μl of octanol were added and the mixture was shaken for 2 min. In order to improve the compatibility of the catecholamine extract with the HPLC mobile phase, 35 μl of 0.5 *M* monosodium dihydrogen phosphate were added and the tubes were shaken briefly and centrifuged as before. The organic (top) layer was collected carefully and discarded and 120 μl of octanol were added to the remaining water phase that contained catecholamines. The mixture was again shaken for 2 min and centrifuged. The octanol extraction was then repeated. The double treatment with octanol of the catecholamine extract was found to remove some electroactive contaminants formed during the earlier procedures. The tubes were usually left for several minutes on ice and the catecholamine-containing water phase was carefully aspirated with a Hamilton microsyringe, strictly avoiding any contamination from the meniscus. Should octanol contaminate the catecholamine extract, a short spin can be repeated and the water phase withdrawn afterwards. Approximately 80 μl of the catecholamine extract can be routinely collected and used for the HPLC analysis. All the reagents and catecholamine extracts were kept on ice, protected from direct light while the centrifugation and shaking were done at room temperature.

RESULTS AND DISCUSSION

Solvent extraction of catecholamines

We have introduced various modifications to the solvent extraction method described first by Smedes et al. [7] and developed in a micro-version by Durkin et al. [4]. The extraction system involves complexation between diphenylborate and the diol groups of the catecholamines in alkaline medium, ion-pair formation with tetraoctylammonium cations and extraction of the ion-paired complexes into organic solvents, from which the catecholamines can be freed into acetic acid [7]. A major disadvantage of this method concerns the presence of unknown electroactive substances separating with catecholamines [4]. Previously suggested modifications of the chromatographic conditions in order to avoid interference with the peaks of interest [4] do not obviate the problem when

electrochemical measurements are performed at very high sensitivities and a detection limit of below 10^{-12} g is needed.

Our studies suggest that the electroactive contaminations afflicting the HPLC of catecholamines originate partly from the chemical reagents used for the solvent extraction, and partly from the procedure itself, presumably at the stage of the catecholamine recovery from the organic phase into acetic acid, i.e. during the dissociation of the complex of DPBE–catecholamines, ion-paired with tetraoctylammonium. We found that the trace impurities of the reagents can be removed by a quick and simple pre-purification of the solutions used for the complexation and ion-pairing. A short extraction of tetraoctylammonium organic solution with acetic acid at the concentration used during the subsequent procedure (0.08 *M*) and a short extraction of the DPBE–EDTA alkaline solution (alumina-pretreated) with the purified organic phase can eliminate a part of the electroactive contaminations from the HPLC profile (Fig. 1A and B). This pre-cleaning of the reagents is not time-consuming and does not affect the recovery of catecholamines; furthermore, we have used the purified solutions for up to four weeks (stored at +4 °C) without any observable changes in their quality. It is also the case that some polypropylene reaction vials used for solvent extraction can contribute to an erratic background when using electrochemical detection at the highest sensitivity. We found the 1.5-ml tubes purchased from Sterilin-Greiner to be satisfactory. Electroactive substance(s) originating during the solvent extraction procedure can be removed by a short double extraction of the acetic acid

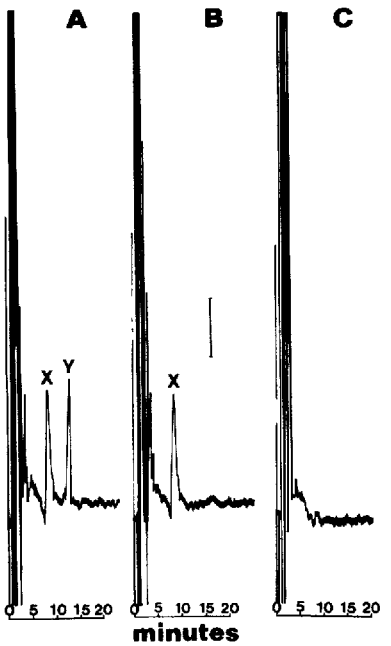

Fig. 1. Chromatograms of a blank sample from the solvent extraction, 0.1 nA/bar. (A) Unmodified procedure: two electroactive peaks are present. (B) Purified reagents: one of the peaks is absent. (C) Purified reagents and "octanol treatment": both contaminating peaks are absent.

phase with octanol (Fig. 1C). The treatment is quick (2×2 min) and the whole procedure remains fast.

The incompatibility between an acidic sample and the HPLC mobile phase has been recognised as a problem at the higher sensitivities [6,13], and we have overcome this problem by adding 0.5 *M* monosodium dihydrogen phosphate to the acetic acid phase (1:2, v/v) following the final stage of the extraction procedure. This modification adjusted the pH of the catecholamine-containing material used for the HPLC, without affecting the recovery of the catecholamines. The stability of catecholamines in this medium was considerable, i.e. at least 6 h at +4°C.

Table I shows the percentage recovery of the catecholamines through the solvent extraction step and transference into the acetic acid phase. The efficient collection of the acetic acid phase depends solely on manual skills and this step can decrease the overall recovery by 15–25%.

The high recoveries found are in agreement with previous publications [7,14–16] and they are decidedly better than those obtained by adsorption on alumina, which are approximately 60% [17,18]. The relative recoveries of all four catecholamines from the same sample were essentially the same and the absolute recovery did not depend on the amount of amine extracted (Table I); in each of these respects this method is preferable to those using adsorption onto alumina [14].

Another advantage of the solvent extraction method is its good capacity; this allows for a reduction in the amount of reagents used and thus cleaner HPLC profiles are achieved. In comparison with the micro-version of Durkin et al. [4] we have reduced the volume of DPBE by half and were still able to extract quantitatively over 17 ng of catecholamines per tube. The solvent extraction method has been described as highly selective for catecholamines, in contrast to that of adsorption onto alumina [4,14]; nevertheless, we found that the complexation

TABLE I

RECOVERY OF CATECHOLAMINES BY SOLVENT EXTRACTION

Data are expressed as means ± S.D. Absolute recoveries of catecholamines are expressed as percentage of the original amounts of the amines. Standard solutions of catecholamines in 0.05 *M* TCA were used for extraction.

Amount of each amine (pg)	Absolute recovery (%)				Relative recovery		
	NE	E	DA	DHBA	NE/DHBA	E/DHBA	DA/DHBA
E and DHBA: 20	89.5	86.3	87.8	86.7	1.03	0.99	1.01
NE and DA: 200 ($n=4$)	±6.0	±8.3	±6 7	±7.6	±0.12	±0.03	±0.02
E and DHBA 200	90.4	95.7	93.2	94 6	0.96	1.01	1.00
NE and DA· 2000 ($n=5$)	±7.4	±6.0	±4.1	+6.0	±0.05	±0.04	±0.03
E and DHBA: 800	98.3	97.2	91.4	92.0	1.05	1.04	0.98
NE and DA: 8000 ($n=3$)	±3.8	±4.0	±4.5	±4.1	±0.01	±0.01	±0.02

with DPBE occurs also with 3,4-dihydroxyphenylacetic acid (DOPAC) which is extracted together with the catecholamines, but at considerably lower recovery (approximately 50%). This phenomenon is not caused by the modifications introduced into the method and could not be eliminated when TOABr was replaced by tetraheptylammonium bromide as used by Durkin et al. [4]. In our opinion the partial extraction of DOPAC is the only drawback of the solvent extraction method and as a consequence the HPLC system must allow for a clear separation of DOPAC from the catecholamines.

Chromatographic analysis

A satisfactory chromatographic system was achieved with an Excel Spherisorb 5 ODS 2 (5 μm) analytical column. This provided a good separation of NE from the solvent front, a safe position for the E peak without a risk of overlapping with the preceding abundant NE and a non-conflicting position for the partly re-

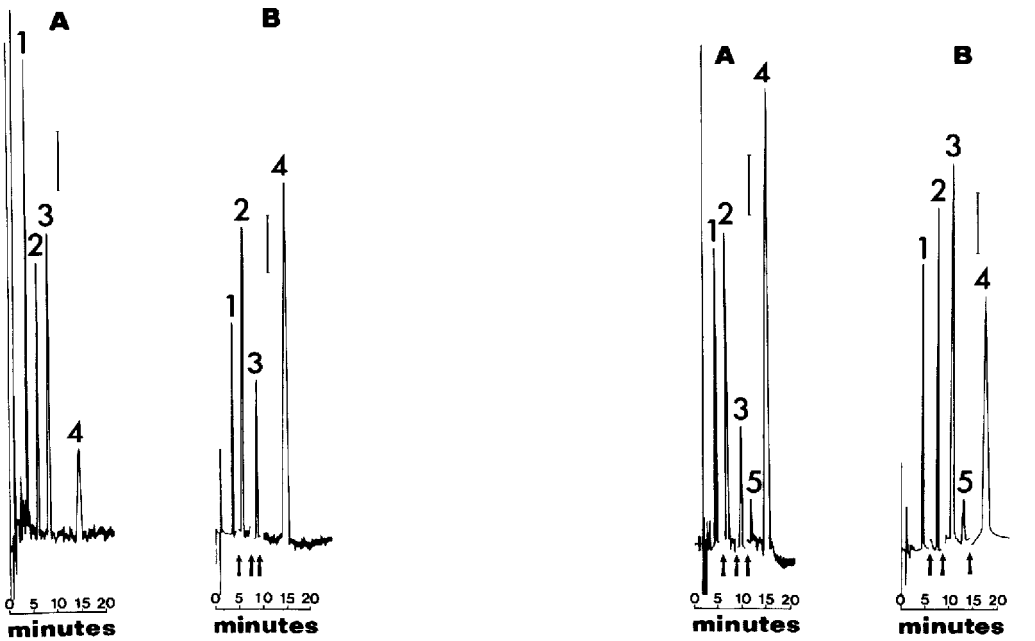

Fig. 2. Chromatograms of standard mixtures of catecholamines. (A) Peaks: 1 = NE; 2 = E; 3 = DHBA; 4 = DA; 10 pg each, 0 1 nA/bar. (B) Peaks: 1 = NE, 500 pg, 10.1 nA/bar; 2 = E, 10 pg, 0.1 nA/bar; 3 = DHBA, 50 pg, 1.01 nA/bar; 4 = DA, 50 pg, 0.1 nA/bar (i.e. proportions of catecholamines comparable with those in tissue extracts). Arrows represent changes in amplifications.

Fig. 3. (A) Chromatogram of catecholamines extracted from micropunches from the preoptic area of the rat hypothalamus. The material injected was obtained from a tissue sample containing 45 μg protein. Peaks 1 = NE, 694 pg, 10 1 nA/bar; 2 = E, 11 pg, 0.1 nA/bar; 3 = DHBA, 35 pg, 1.01 nA/bar; 4 = DA, 61 pg, 0 1 nA/bar; 5 = DOPAC. Arrows represent changes in amplification. (B) Chromatogram of catecholamines extracted from the rat whole hypothalamus The material injected was obtained from a tissue sample containing 200 μg protein. Peaks: 1 = NE, 3440 pg, 40 nA/bar; 2 = E, 69 pg, 0.4 nA/bar; 3 = DHBA, 72 pg, 0.4 nA/bar; 4 = DA, 1414 pg, 4 nA/bar; 5 = DOPAC. Arrows represent changes in amplification.

TABLE II

PRECISION OF THE HPLC DETERMINATION OF CATECHOLAMINES FOLLOWING THE SOLVENT EXTRACTION

Samples of standard catecholamines containing 50 pg of E and DHBA and 500 pg of NE and DA were analyzed following the solvent extraction. The rat whole hypothalami were purified by solvent extraction procedure and samples corresponding to 1 mg of the tissue were taken for analysis.

Compound	Coefficient of variation (%)			
	Within-assay ($n=7$)		Between-assay ($n=5$)	
	Standard mixture	Tissue	Standard mixture	Tissue
NE	2.25	2.50	3.99	4.25
E	3.69	2.64	2.32	4.21
DA	2.91	1.88	2.75	2.67
DHBA	2.70	3.62	2.63	4.89

TABLE III

CATECHOLAMINE CONTENT IN MICROPUNCHED SAMPLES FROM THE HYPOTHALAMIC PREOPTIC AREA IN RATS TREATED WITH VEHICLE OR LY 134046

LY 134046 was given intraperitoneally, 2×20 mg, 5 and 2 h before sacrifice.

Group	n	Concentration (mean ± S.D.) (ng/mg of protein)		
		NE	E	DA
Control	6	16.95 ± 5.09	0.332 ± 0.191	1.76 ± 0.49
LY 134046	6	15.15 ± 2.60	0.152 ± 0.039*	1.74 ± 0.28

*Significantly different from the control ($P<0.05$, Student's t-test).

covered DOPAC. As a result of the simple purification of reagents and final catecholamine extract, the chromatograms were free of unwanted peaks and the quality of the separation of the biological samples was as high as that of the catecholamine standards (Figs. 2 and 3). The total elution time varied between 14 and 19 min depending on the ambient temperature and the age of the analytical column.

The attempts to use μBondapak and Resolve C_{18} (both 10 μm) cartridges were disappointing. μBondapak exhibited a limited selectivity for catecholamines and a discouraging peak shape. Furthermore, the flow-rate of 2 ml/min needed for the 8 mm I.D. cartridge caused higher back-pressure and thus increased the background noise at the highest sensitivities. Resolve C_{18} showed a satisfactory selectivity for catecholamines, nevertheless as a non "end-capped" resin, with a high retention of amines, it needed the addition of dibutylamine to the mobile phase to improve peak shape. Dibutylamine caused some extra noise at the baseline and is believed to have an adverse effect on the analytical cell, resulting in a more frequent need for cleaning. Furthermore, at the concentration of OSA used for

appropriate separation of catecholamines, Resolve C_{18} did not provide a convenient position for DOPAC which was adjacent to E.

Detection limits at a maximum working system gain of ×99 000 (1.01 nA full scale) with a signal-to-noise ratio of 2 were: 0.4 pg for NE, 0.7 pg for E, 0.6 pg for DHBA and 2.3 pg for DA. They are comparable with detection limits obtained with microbore liquid chromatography–electrochemical detection [4] and lower than those achieved in other coulometric detections [6,13]. The electrochemical response of the detector was linear over the range of 50 000 pg for all catecholamines. Precision of the determination of catecholamines following the solvent extraction is shown in Table II.

Applications

The highly sensitive HPLC system in combination with the improved solvent extraction offers a reliable method for determining E and other catecholamines in very small brain samples, such as micropunches. The highest sensitivities allowing for detection of less than picogram amounts of catecholamines are routinely used for analysis of biological samples containing as little as 25–45 μg of protein (Fig. 3A). When larger amounts of the brain tissue are available (e.g. whole hypothalamus) the microanalysis of small portions of 1–2 mg wet mass (Fig. 3B) is advantageous since it extends the life of the analytical cell and chromatographic column and also reduces the amount of reagents used.

Table III shows the concentrations of the catecholamines in the micropunched samples from the hypothalamic preoptic area in rats injected with LY 134046, an inhibitor of phenylethanolamine N-methyltransferase (PNMT), the enzyme synthesizing E from its precursor NE. The treatment resulted in a specific and significant decrease in the E content.

It should be noted that the quantification of E in extremely small tissue samples was feasible even when the level of the amine was decreased by around 50%. Since this range of depletion is usually sufficient to estimate the turnover of catecholamines, the described method offers the possibility of studying E turnover in subregions of the brain, and thus can contribute to the understanding of the role of this amine in the mammalian CNS.

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