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Note

Determination of catecholamines in biological tissues by liquid chromatography with coulometric detection

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Changes in the activity of monoaminergic systems have been implicated in many nutritional, physical, toxicological and pathological states [1,2]. The three most important substances are dopamine (DA), norepinephrine (NE) and epinephrine (E), which have widely different functions depending on their location in the organism. For this reason it is valuable to have a method for analysis of endogenous concentrations of these biogenic amines in discrete regions of brain and in adrenals, heart, plasma, whole blood and other tissues or fluids. Usually the samples are small and contain only picogram amounts of these amines. High specificity and sensitivity are therefore required. For instance, the basal catecholamine levels in human plasma are approx. 10–100 pg/ml [3], and other samples from animals or humans (biopsies) have even lower levels. More and more laboratories other than those involved in fundamental biochemical research are interested in measuring catecholamines routinely. Thus the method also needs to be simple, accurate and rapid.

The method described here requires relatively simple equipment and is both rapid and accurate. The monoamines investigated were DA, NE and E. After liquid chromatographic separation, the detection system was based on a coulometric principle [4,5]. The advantages of this detection are stability of the system, low noise, and good sensitivity and reproducibility. The three amines were measured in several tissues and fluids, and the method could be extended to a wide variety of other samples.

EXPERIMENTAL

Chemicals and reagents

The sources were as follows: 3-hydroxytyramine hydrochloride or DA and 3,4-dihydroxybenzylamine hydrobromide (DHBA) as internal standard (both Sigma,

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St. Louis, MO, U.S.A.), *d,l*-norepinephrine hydrochloride, 1-epinephrine, methanol, potassium dihydrogen phosphate, orthophosphoric acid, 1-octanesulphonic acid sodium salt monohydrate, hydrochloric acid, and perchloric acid (PCA) (all Fluka, Buchs, Switzerland), trishydroxymethylaminomethane (Tris) and aluminium oxide 90 active neutral (Merck, Darmstadt, F.R.G.). Water was deionized and glass-distilled.

Equipment

The high-performance liquid chromatographic (HPLC) determinations were carried out using a Constametric III (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) isocratic pump, a Coulochem Esa 5100 A (Bedford, MA, U.S.A.) with a 5010 analytical cell connected to a LDC/Milton Roy CI-10 recording integrator. Potential differences were +0.60 V and −0.22 V. The principles of the system were described by Martin et al. [6]. Samples were introduced via a 25-μl injection loop (Model N 60 Valco). Separations were carried out using a 250×4 mm I.D. Hibar column packed with LiChrosorb RP 18 (7 μm average particle size).

Chromatographic conditions

The mobile phase (pH 2 adjusted with orthophosphoric acid) contained 20 m*M* potassium dihydrogen phosphate, 0.4 m*M* octanesulphonic acid and 14% methanol (v/v). The effects of pH, electrolyte and methanol concentration were investigated (Table I). The mobile phase was prepared each day, filtered through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.) and then degassed under vacuum before use. A flow-rate of 1 ml/min (150 bar) at ambient temperature (20±2°C) was used in all experiments.

Standards

Standards solutions of DA, NE, E and DHBA were prepared at a concentration of 5 mg/ml in 0.01 *M* hydrochloric acid and stored at −80°C. From these stock solutions, dilutions were made each day in the mobile phase in order to inject between 10 pg and 10 ng, and the amount of internal standard was adjusted. Standard curves were constructed with the DA/DHBA, NE/DHBA, and E/DHBA area responses. From the integration parameters, the corresponding regression equations were calculated. Reproducibility for identical samples was evaluated. Chromatographic parameters were calculated [7] between contiguous compounds, i.e. NE versus E, E versus DHBA, DHBA versus DA.

Sample preparation

Samples were obtained from dogs or rats. In dogs, blood was collected by venipuncture in a centrifuge tube containing heparin. The plasma, separated by centrifugation at 1000 *g* at 4°C for 30 min, was either analysed immediately or stored frozen at −80°C. In rats, after decapitation, blood was collected in a centrifuge tube containing heparin and centrifugated. Plasma or erythrocytes were stored at −80°C until analysis. Adrenals, heart, and brain (cerebellum and hemispheres separated) were rapidly removed and immediately frozen at −80°C until analysis. Samples were prepared in different ways. For blood, erythrocytes and

TABLE I

EFFECTS OF pH AND ELECTROLYTE AND METHANOL CONCENTRATIONS IN THE MOBILE PHASE ON SELECTIVITY AND RESOLUTION. COMPARISON WITH MONOAMINES IN RAT ADRENALS AND DOG PLASMA (STANDARDIZED CONDITIONS)

Selectivity: $\alpha = \frac{t_{R_2} - t_0}{t_{R_1} - t_0}$; resolution: $R_S = 2\frac{t_{R_2} - t_{R_1}}{w_2 + w_1}$

where t_0 = dead volume, t_R = retention time at peak maximum and w = width at base of peak.

	Selectivity, α			Resolution, R_S		
	NE/E	E/DHBA	DHBA/DA	NE/E	E/DHBA	DHBA/DA
Buffer 0.1 *M*	1.53	3.41	5.40	5.89	6.05	11.16
Buffer 0.05 *M*	2.07	1.45	8.64	15.74	10.77	13.29
Buffer 0.020 *M*	2.23	1.68	1.96	4.74	5.55	10.48
pH 2.5	1.77	2.96	7.06	6.84	5.98	12.95
pH 2	1.30	1.67	3.75	4.94	5.57	9.37
Methanol 6%	2.07	1.45	8.64	15.74	10.77	13.29
Methanol 12%	1.77	2.10	4.25	5.50	5.70	10.93
Methanol 14%	0.90	1.25	2.57	4.50	6.25	10.28
Adrenals	1.24	1.39	1.98	12.92	22.84	35.51
Dog plasma	1.31	1.27	1.47	27.75	19.19	35.84

plasma (1 ml), 0.25 vol. of 1 *M* PCA containing DHBA as internal standard was added. The mixtures were then homogenized in a Vortex (Vortex Genie, Springfield, MA, U.S.A.) and centrifuged (10 min, 7000 *g*). Supernatants were decanted, added to a tube containing 100 mg of alumina and 1 ml of 1 *M* Tris–HCl buffer (pH 8.6), and gently agitated for 15 min. Catecholamines were eluted from alumina with the HPLC mobile phase (2×100 μl). After centrifugation, supernatants were injected into the HPLC system.

For adrenals, homogenization was carried out in 1 ml of 0.3 *M* PCA containing DHBA in a Thomas potter, followed by centrifugation. The supernatant was diluted (1:10) with the mobile phase and then injected into the HPLC system. For the heart and brain samples, the method was similar except that dilution in the mobile phase was adjusted according to the detector response. Dilution in the mobile phase reduced interference from perchloric acid.

In all cases, the extraction were carried out an ice-water bath and the samples were filtered through 0.22-μm membrane filters (Millipore) before the HPLC analysis.

RESULTS AND DISCUSSION

As can be seen from the results shown in Fig. 1, all compounds were conveniently separated. Table I shows the optimal conditions for assay of catechol-

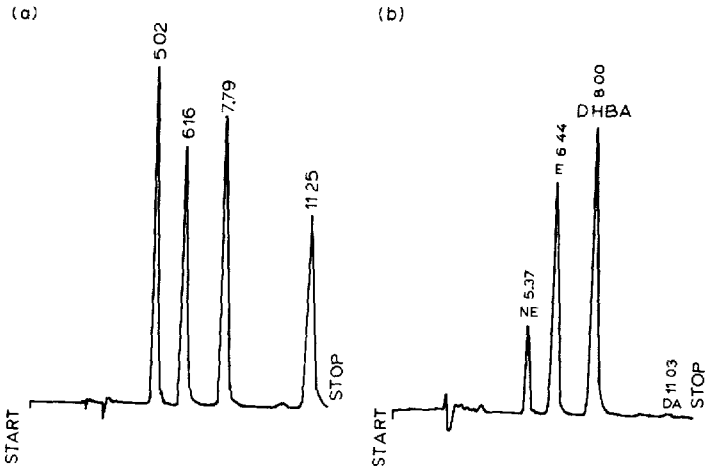

Fig. 1. Chromatographic separation of (a) the reference compounds (norepinephrine, epinephrine, dihydroxybenzylamine and dopamine, 2 ng each) and (b) adrenal extract, from rat. (a) gain, 1000; response time, 2 s; (b) gain, 300; response time, 4 s.

amines. The pH and the concentrations of the electrolyte and methanol play a role in the chromatographic separation, as shown by Svendsen and Greibrokk [8]. Acidic conditions are usable [8]. Satisfactory conditions [7] are obtained with a selectivity factor of over 0.8. The resolution should be above 1.0, and it can be seen from the table that this value was considerably exceeded in our assay. Table II shows the regression lines of the standard curves for the three catecholamines. Linearity was checked statistically.

For high sample concentrations, adsorption on alumina can be avoided without introducing baseline instability. This avoids loss of sample and is quicker. Conversely, if the concentrations are low (e.g. in plasma), the alumina extraction stage concentrates the compounds of interest and even lower levels can be esti-

TABLE II

LINEARITY OF RESPONSE OF CATECHOLAMINES AS A FUNCTION OF CONCENTRATION (FROM 10 pg TO 10 ng)

$$y = \frac{\text{DA, NE or E area}}{\text{DHBA area}}$$

x = DA, NE or E concentration (pM)

Catecholamine	Regression equation
Dopamine	$y = 0.220x - 0.013$ ($r = 0.99$)
Norepinephrine	$y = 0.192x - 0.004$ ($r = 0.99$)
Epinephrine	$y = 0.205x - 0.010$ ($r = 0.99$)

Probability of non-linearity = not significant. $P < 0.05$.

TABLE III

CATECHOLAMINES LEVELS IN SOME SAMPLES IN RAT AND IN DOG PLASMA

Sample	*n*	DA	NE	E
Whole blood (ng/ml)	12	–	3.08 ±0.92	5.34 ± 0.90
Erythrocytes*	8	–	0.39 ±0.07	2.32 ± 0.48
Plasma*	10	0.49 ±0.045	1.99 ±0.22	3.31 ± 0.24
Dog plasma (ng/ml)	27	5.96 ±1.326	18.65 ±3.634	101.4 ±27.73
Adrenals (ng/ml)	16	0.244±0.0466	3.84 ±0.335	13.32 ± 2.350
Heart (μg)	9	0.007±0 0008	0.540±0.035	0.025± 0.0019
Whole brain (μg)	7	1.408±0.138	0.418±0.111	–
Cerebellum (μg)	8	0.032±0.020	0.110±0.036	–
Hemispheres (μg)	6	0.85 ±0.176	0.340±0.054	–

*Expressed in ng with respect to whole blood.

mated. Addition of an internal standard before extraction provides a way of estimating recovery. Using the standards, the recovery was estimated to be 70–75%. Using the biological samples, the recovery was 70%. Detection was considered meaningful when the peak heights were at least twice the noise amplitude. The detection limits were about 2–5 pg with day-to-day coefficients of variation of less than 10%. The sensitivity is adequate, although lower detection limits are attainable [9].

Table III shows a few experimental values obtained using this method. Adrenals are rich in NE and especially in E, and brain is rich in DA. For heart and brain we found levels similar to those reported in the literature [6]. Circulating levels are low in the rat, but it is known that they can be even lower and variable depending on the emotivity and the level of stress at the time of sampling [10].

This HPLC method with coulometric detection can be used for all types of biological sample containing different amounts of catecholamines, ranging from a few picograms to tens of nanograms. It represents an improvement over the existing method [11] because the selectivity and resolution are high. It is sensitive, reproducible and easy to carry out. Thirty samples can easily be measured per day.

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