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Note

Estimation of adrenal catecholamines by elevated-temperature liquid chromatography with amperometric detection

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High-performance liquid chromatography (HPLC) using amperometric detection is now widely used to measure catecholamines (CAs) and their congeners in biological tissues [1, 2]. The simultaneous determination of CAs in adrenal extracts presents a challenge to the chromatographer because of the very wide difference in the amounts of dopamine (DA) and epinephrine (EP) [3]. Published HPLC procedures [4, 5] for adrenal CAs have the disadvantages of lengthy chromatographic run time and/or suboptimal chromatographic conditions with respect to the amperometric detector response [6]. We report that adrenal DA content can be reliably measured along with EP and norepinephrine (NE) under optimized conditions by elevating the column temperature to shorten the DA retention time. Furthermore, the recommended conditions permit resolution of these CAs from some twenty other catechol compounds that might be present in adrenal tissue.

EXPERIMENTAL

Sample preparation

The adrenal glands of male Sprague—Dawley rats were removed under

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sodium methohexital sedation (Brietal, Eli Lilly, Toronto, Canada; 65 mg/kg body weight, subcutaneously). The glands were freed from fat and connective tissue, blotted on paper, weighed and homogenized together in 0.9 ml (or separately in 0.5 ml) of cold 0.3 *M* sucrose with a PTFE homogenizer. This medium allows one to measure concurrently adrenal CA biosynthetic enzymes, steroids and proteins [7]. Aliquots of 0.05 ml of homogenate were mixed with 0.10 ml of 3.9 m*M* sodium metabisulfite in acid-washed Eppendorf tubes and stored temporarily at −70° C.

Sample preparation was modified from that of Anton and Sayre [8] and Felice et al. [9] and involved initial deproteinization with cold 0.1 *M* perchloric acid for 5 min. The final sample volume of 0.25 ml contained also 300 ng of the internal standard, 3,4-dihydroxybenzylamine (DHBA; Sigma, St. Louis, MO, U.S.A.), dissolved in 0.1 *M* perchloric acid and 2.5 m*M* sodium metabisulfite. After centrifugation at 15 600 *g* for 25 min, CAs were adsorbed by shaking the supernatant fraction for 10 min with 20 mg of acid-washed alumina (Woelm neutral activity, grade I; ICN, Montreal, Canada) [8] previously buffered with 1.0 ml of a solution containing 0.5 *M* Tris—HCl, 27 m*M* disodium ethylenediaminetetraacetate (EDTA) and 2.6 m*M* sodium metabisulfite, pH 8.6 at 4° C. After the alumina had been washed twice with 0.5 ml of deionized water, the adsorbed amines were eluted by shaking for 15 min with 0.15 ml of 0.05 *M* phosphoric acid and 0.1 m*M* sodium metabisulfite; the eluates were centrifuged for 5 min to pellet alumina fines, and were stored at −70° C for at the most a few days.

Chromatographic system

A few microlitres of eluate were injected directly into the chromatographic system, which consisted of a Model 45 solvent delivery system a Model U6K manual injector and a μBondapak C_{18} column, 30 × 0.39 cm, obtained from Waters Scientific (Mississauga, Canada). A Co:Pell ODS guard column (Whatman, Clifton, NJ, U.S.A.) was included. The analytical column was enclosed in a water jacket (Alltech, Deerfield, IL, U.S.A.) thermostated at 30° C with a Lauda Model RM-3S circulator (Brinkmann Instruments, Rexdale, Canada).

The mobile phase was modified from that of Moyer and co-workers [6, 10]. It consisted of 8 parts of methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and 92 parts of a solution containing 0.1 *M* sodium phosphate (monobasic), 0.1 m*M* EDTA and 1.0 m*M* sodium octyl sulfate (Eastman Kodak, Rochester, NY, U.S.A.), pH 5.5, prepared in deionized water (Continental Waters Systems, El Paso, TX, U.S.A.) free of organic residues, filtered through 0.45-μm filters (Millipore, Bedford, MA, U.S.A.) and deaerated under vacuum. This solvent was sparged continuously with helium and pumped at a flow-rate of 3.0 ml/min. The inconvenience of column equilibration with the ion-pairing reagent was avoided by pumping overnight at 0.1 ml/min.

The CAs were detected by means of an oil-impregnated carbon paste electrode (in a Model TL-4 5-mil thin-layer cell) set, with a Model LC-4 electronic controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.), at a sensitivity of 5 nA/V and at +0.70 V against a silver/silver chloride reference electrode. Quantitation [9] was done with a Model SP4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.) by determination of peak

area ratio to the internal standard and by inverse linear regression analysis of a calibration curve constructed with standards taken through sample preparation. Levels of unconjugated CAs were expressed in terms of the free base as μg of EP and NE, or ng of DA per single adrenal or pair of adrenals.

Chemicals

Many catechol compounds were purchased from Sigma, but 3,4-dihydroxyphenylserine was from Calbiochem (Los Angeles, CA, U.S.A.), 3,4-dihydroxyphenylethanol from Regis (Morton Grove, IL, U.S.A.), dobutamine from Eli Lilly and apomorphine from MacFarland-Smith (Edinburgh, U.K.). Dihydroxyphenylalanine, carbidopa and α-methyldopamine were donated by Merck Sharp & Dohme (Kirkland, Canada), the α-methyl and α-ethyl derivatives of norepinephrine by Sterling-Winthrop Institute (Rensselaer, NY, U.S.A.), N-methylepinephrine by Dr. R.W. Fuller of Eli Lilly (Indianapolis, IN, U.S.A.) and fusaric acid (5-butylpicolinic acid) by Schering (Bloomfield, NJ, U.S.A.).

RESULTS AND DISCUSSION

Moyer and co-workers [6, 10] have optimized the chromatographic conditions for analysis of CAs with respect to both their resolution and detector response. We have raised the proportion of methanol to 8% to resolve the DA peak from that of a contaminant present in low amount in the internal standard (Fig. 1). Because the run-time had to be shorter in order to detect the DA peak reliably we had to choose between two further measures. (A) Additional supplementation with methanol to shorten the retention time. This

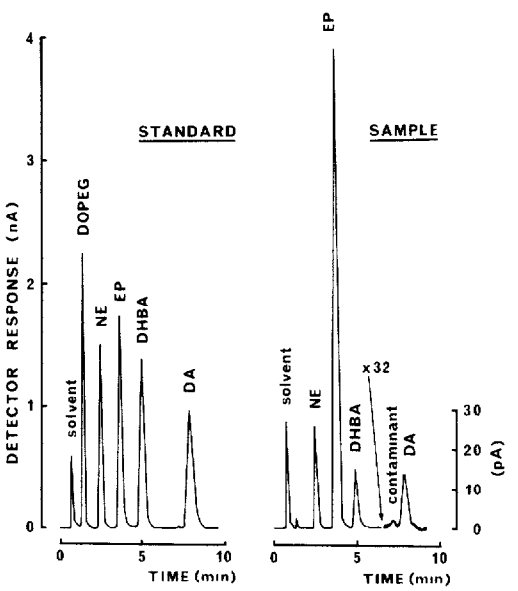

Fig. 1. Chromatogram of CAs from standards taken through sample preparation and from an adrenal extract with 300 ng of DHBA added to it. The arrow indicates when the recorder scale was magnified 32 times. This sample contained 4.9 μg of NE, 19.4 μg of EP and 120 ng of DA per adrenal

would have required the use of glassy carbon electrodes or carbon pastes other than the oil-impregnated one, with which the highest sensitivities are achieved. (B) In view of that limitation, we found it advantageous to operate above ambient temperature because this not only decreased the retention times but also produced sharper and higher peaks, thereby lowering the detection limits of the method. Thus, with the present method, run-times can be reduced by 20% by simply raising the column temperature from ambient to 30°C (Fig. 2). At the higher temperature DA is eluted within 9 min (Fig. 1). This run-time is 45% shorter than that of another procedure for adrenal CAs [4]. For a large number of samples to be analyzed at one time, such a reduction in run-time is especially useful. Even higher temperatures, up to 60°C, can be used in assaying 3,4-dihydroxyphenylacetic acid and homovanillic acid [11]. As the column temperature is raised, the mobile phase must be pre-heated to this temperature to preserve resolution and peak symmetry [12]. On the other hand, lowering the temperature can compensate for the decrease of capacity factors after months of column use.

A linear detector response for peak area ratios of DA, NE and EP was observed between 0.3 and 150 ng (correlation coefficients greater than 0.9998), the curves for injected standards being co-linear with those obtained from spiked homogenates (not shown). The detector response for these substances was two- to five-fold greater [6] when the pH of the mobile phase was 5.5, as used here, than at pH 2.8 as in the other procedures for adrenal CAs [4, 5], therefore increasing the sensitivity of the method. Lower noise level and better

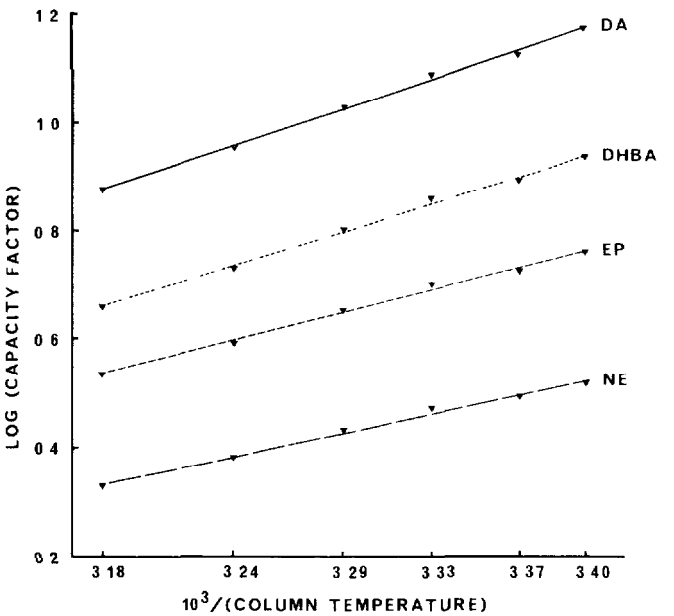

Fig. 2. Van't Hoff plots depicting the effect of temperature on the capacity factor for CAs. The abscissa represents temperatures between 20 and 40°C, graphed as 10^3 °K^{-1}. The capacity factors on the ordinate are a measure of the time spent by a solute on stationary phase divided by that in mobile phase. Standard errors were smaller than the data points; the slopes for NE, EP, DHBA and DA were 0.87, 1.03, 1.25 and 1 35, respectively.

sensitivities were also achieved by helium sparging of the mobile phase and by electrical grounding of the chromatographic column.

No interference may be expected for major sample components and DHBA, as evidenced from the capacity factors of the many catecholic and related compounds listed in Table I. This table also shows that the relative detector response, related to the oxidation current produced by various catechols, differs by two-fold at the most.

Data in Table II confirm the identity of DA, NE and EP peaks after powerful inhibition of DA hydroxylation by fusaric acid [13]. The presence of 3,4-dihydroxyphenylglycol (about 300 ng per adrenal in 425-g rats, Fig. 1) and of traces of N-methyldopamine (epinine) [14] was suggested by co-elution experiments but their amounts were not affected by administration of fusaric acid.

TABLE I

RETENTION AND DETECTION DATA OF CATECHOLS AND RELATED COMPOUNDS

These data were obtained by duplicate injections of 30 pmol (about 5 ng) of compounds with 15 pmol of DHBA. The mobile phase was thermostated at 22° C and pumped at 2.5 ml/min

Compound	Retention time (min)	Capacity factor	Relative detector response*
DL-3,4-Dihydroxymandelic acid (DOMA)	1.2	0.22	(0.52)
DL-*threo*-3,4-Dihydroxyphenylserine (DOPS)	1.3	0.36	(0.66)
3,4-Dihydroxyphenylacetic acid	1.6	0.66	0 79
L-3,4-Dihydroxyphenylalanine	1.8	0.81	0.75
DL-3,4-Dihydroxyphenylglycol	2.0	1.0	0 87
L-α-Methyldopa	2.4	1.4	0.67
L-Carbidopa	2.8	1.9	1 4
L-Norepinephrine (NE)	3.8	2.9	0.96
L-α-Methylnorepinephrine	5.8	5 0	1.2
3,4-Dihydroxyphenylethanol	5.9	5.1	1.0
L-Epinephrine (EP)	6 0	5 2	1.1
N-Methylepinephrine (LYO 18839)	7.1	6.4	1.5
6-Hydroxydopamine	7 9	7.2	0.78
3,4-Dihydroxybenzylamine (DHBA)	8.6	7.8	1
N-Acetyldopamine	9.9	9.2	0 84
α-Ethylnorepinephrine (butanephrine)	13.0	12.3	0.77
Dopamine (DA)	14.2	13.7	1.1
N-Methyldopamine (epinine)	18.5	18.0	0.92
L-Isoproterenol	27.3	27.2	0.83
5,6-Dihydroxytryptamine	27.6	27.7	1.3
L-α-Methyldopamine	35.3	35.6	0.96
DL-α-Propyldopacetamide	>60		
Dobutamine	>60		
Apomorphine	>60		

*The relative detector response is the peak area ratio of a given substance compared to an equimolar amount of internal standard. The DOMA and DOPS peaks were, respectively, seven and twenty times larger than co-eluting solvent front peaks; their response factors are obtained after substracting the area of these solvent peaks.

TABLE II

ADRENAL CATECHOLAMINE LEVELS FOLLOWING INHIBITION OF DOPAMINE-β-HYDROXYLASE

Rats weighing 325–340 g were given a single intraperitoneal injection of fusaric acid, 100 mg/kg body weight, dissolved in saline pH 6.0, and their adrenals were removed 3 h later. Mean values ± standard errors are presented; data were subjected to logarithmic transformation in order to minimize the heterogeneity of their variances [18], before Student's *t*-tests were carried out: $p < 0.001$ for DA and EP.

Group	Number of rats	DA (ng/pair of adrenals)	NE (μg/pair of adrenals)	EP (μg/pair of adrenals)
Saline	4	287 ± 57	5.44 ± 0.58	26.4 ± 2.2
Fusaric acid	6	1210 ± 188 (420%)	4.90 ± 0.45 (90%)	14.9 ± 1.7 (56%)

Many precautions have been taken during sample preparation. The adrenals were removed under anesthesia with sodium methohexital because barbiturates do not raise the concentrations of plasma CAs [15] and, therefore, probably do not deplete the adrenals of their CA contents. The final perchloric acid concentration, 0.1 *M*, was insufficient to hydrolyse conjugated DA, which amounts to 5% of DA in the rat adrenal [4]. The purification of CAs on alumina was mandatory because adrenal steroids would otherwise cause deterioration of the reversed-phase HPLC column. This adsorption was done in the presence of EDTA, a condition that improves markedly the extraction of DA from tissues [8]. The use of phosphoric acid rather than perchloric acid provides 92–98% elution of all CAs tested [9, 16]. However, we obtained only 68–71% recovery of CAs, whether tissue matrix was present or not; differences in the preparation or type of alumina used could account for this discrepancy. Phosphoric acid also produced smaller void volume detector response and better resolution of CAs with short retention times [16].

The present method compares favorably with the hydroxyindole fluorometric technique of Laverty and Taylor [17]. In experiments [7] with intact rats weighing 340–450 g, the values for DA by liquid chromatography range from 184 to 352 ng/pair of adrenals, those for NE and EP from 8.0 to 11.0 and from 33.5 to 43.5 μg/pair of adrenals, respectively. Using the hydroxyindole method in separate experiments, we found contents of 171–245 ng/pair of adrenals for DA, 10.3–13.3 μg/pair of adrenals for NE, and 27.8–34.2 μg/pair of adrenals for EP in rats weighing 370–410 g killed by diethyl ether overdose. The minimal detectable quantity of DA (at a signal-to-noise ratio of 5) was about 75 ng per adrenal for the present method, 625 ng [17] for the other. The precision of duplicate sample measurement was greater than 2, 2 and 7% for NE, EP and DA, respectively; corresponding figures with the fluorometric assay were 5, 8 and over 10%. The improved precision and sensitivity are important for the measurement of very low amounts of DA in adrenal extracts.

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