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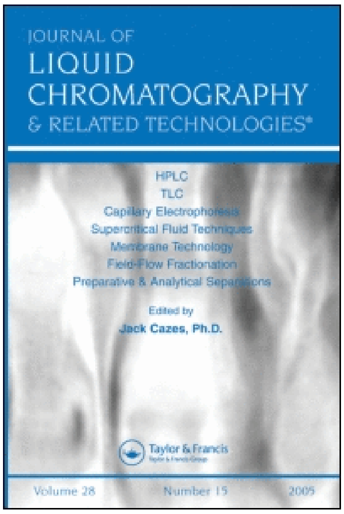

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CATION EXCHANGE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION AS A METHOD FOR THE ANALYSIS OF ENDOGENOUS CATECHOLAMINE CONCENTRATIONS IN PLASMA OR SERUM

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ABSTRACT

A chromatographic system which permits determination of catecholamines, isolated from a few ml of plasma or serum, is described in detail. Some factors of importance for the improvement of the signal-to-noise ratio at subpicomole detection levels have been carefully investigated.

INTRODUCTION

Determination of catecholamines in normal human plasma or serum still represents an advanced problem from the analytical point of view. A prerequisite for all non-enzymatic methods in use is the performance of the isolation procedure by adsorption on alumina as described by Anton and Sayre (1). Of the different assay methods that may be used, the fluorimetric (1-3) and radio-enzymatic (4) ones are the commonest. None of these makes use of separation by high performance liquid chromatography (HPLC), except for a recently published modification of the radio-enzymatic procedure (5).

In the last few years, however, attention has been drawn to the use of electrochemical detection (EC) in HPLC (LCEC) for the trace analysis of electro-oxidizable compounds at a graphite paste anode (6). In particular, its use for catecholamine determination in brain tissue (7) and urine (8) has been investigated, and other studies on the use of LCEC for the determination of catecholamines (9) and cysteinyl-DOPA compounds (10,11) are now in progress.

Because of the high sensitivity of the EC-detector (12), a combination of low background current and noise with highly efficient HPLC columns would yield signal-to-noise ratios high enough to generate acceptable chromatograms of even sub-picomole amounts. In this paper some results of efforts to perfect the technique to allow the simultaneous determination of norepinephrine (NE), epinephrine (E) and dopamine (DA) in normal human plasma or serum are presented.

EXPERIMENTAL

Instrumentation

The liquid chromatograph was constructed from an Altex mod. 100 constant flow solvent pump, a Rheodyne mod. 7120 sample injection valve provided with a 20 or 50 µl loop, a 4.6 x 170 mm Altex stainless steel column (all available from Altex Scientific Co., Berkeley, Ca.), slurry packed with Nucleosil SA 10 µ (Machery and Nagel Co., Düren, G.F.R.), and an electrochemical detector (Bioanalytical Systems Inc., West Lafayette, In.). The detector consisted of a plexiglass cell, housing the graphite paste anode and mounted with a 50 µ teflon spacer, a RE-1 reference electrode compartment equipped with an Ag/AgCl electrode, and an LC-2A electrochemical control unit (potentiostat and operational amplifier). A silicone oil based carbon paste (CPS) was used as anode material. The detector output was connected to a Linear mod. 264 potentiometric recorder. The mains voltage supply to the LC-2A unit was stabilized via a Philips mod. PE 1401/12 voltage transient supressor. At all operations at high sensitivity a 5 m long 1/16" I.D. teflon coil, inserted between the pump and injector, was used as a pulse dampening and electrical noise screening device. The separation column was protected by means of a Rheodyne 2 µ exchangeable filter element between the injector valve and the column. The injector, column, and detector parts were all monted within a Faraday cage.

Column Packing Procedure

The column was packed with the balanced density slurry packing technique, according to Majors (13). The use of the upward-slurry packing method described by Bristow (14) did not improve the column effeciency significantly.

Chemicals and Solvents

Distilled water was redistilled from alkaline permanganate before its use for buffer preparation. The mobile phase, pH 5.2, was prepared from citric acid (23.0 g), anhydrous sodium acetate (16.6 g), acetic acid 4.20 ml) (all from E. Merck, Darmstadt, GFR), and sodium hydroxide (9.6 g) diluted with the ultrapure water to 4 l. Standard solutions (1-0.01 µM) of NE, E, and DA (Sigma Chem. Co., St Louis, Mo.) were prepared in 0.1 M perchloric acid (PCA) and used for the determination of retention and detector response data of the chromatographic system. Acid washed alumina prepared by the method of Anton and Sayre (1) as well as a commercially available grade (Bioanalytical Systems Inc., West Lafayette, In.) was used.

Chromatographic Conditions

Degassing of the buffer was continuously effected by maintaining it at a temperature of 50°C. At a constant solvent flow rate (0.40 or 0.60 ml/min) through the system the background current output from the detector was allowed to stabilize for at least 3 h. Background compensation was generally of the order 1.0-1.5 nA. At very high sensitivity settings (1 nA full scale or less) baseline instability caused by many different factors constitutes a very important and difficult problem. Electronic noise from the pump motor, however, could be reduced effectively by connecting the outlet steel capillary tube to ground. Only slight further improvement was obtained by the teflon coil, which suggests that flow rate variations from the pump are less important than electronic factors. Further, secure fixation of the cord containing the cables to which the detector cell is connected is a vital factor.

Preparation of Samples

Two modifications of Anton and Sayre's original method were used. In the first, 2.0 ml of freshly prepared plasma was adjusted to pH 8.6 and shaken with 30 mg of alumina for 15 min. After two washings with water the alumina was dried by suction and the catecholamines liberated by the addition of 50 µl of 0.1 M PCA. After centrifugation 20 µl of the supernatant was injected into the chromatograph. In the second modification 6.0 ml of serum was first completely deproteinized by the addition of 600 µl of 4 M PCA, after which 400 mg of alumina was added to the supernatant obtained on centrifugation after its dilution with 20 ml of 0.4 M PCA and adjustment to pH 8.6. The subsequent procedure was analogous to that in the first modification. 1.5 ml of 1 M PCA was used for desorption, however, and the whole isolation procedure was readily carried out in a teflon-stoppered 100 x 10 mm I.D. glassfilter funnel. 50 µl of the PCA filtrate was injected. In both procedures sodium bisulphite as antioxidant and EDTA are present in the solutions during the adsorption process (1).

RESULTS AND DISCUSSION

The detector response curves shown in Fig. 1 were obtained from studies of injected standard solutions. The linear range extends to ca. 500 pmoles injected material for all three catecholamines. An increase in the applied potential above 0.55 V caused a certain increase in peak height but also in background current, especially above 0.70 V. The results pertaining to the detector response and retention characteristics are shown in Table 1.

The retention properties of the ion-exchange material are important because NE has to be completely separated from the solvent front containing less-retained compounds, and because buffer dilution (to increase V_R) will soon cause noise problems. Unfortunately, large variations may occur between different batches of the same material. Of the Nucleosil SA ion exchangers with mean particle diameters of 5 µm and 10 µm, respectively, only one batch of the latter material was found to give a capacity factor for NE high enough to be satisfactory.

TABLE 1

Retention and Detector Response Data

Compound	V_R ml	$k' = \frac{V_R - V_0}{V_0}$	Peak height mm per pmol at 0.55 V and 0.5 nA/V full scale	Peak area mm^2 per pmol at 0.55 V and 0.5 nA/V full scale
NE	4.6	1.55	71	107
E	7.7	3.3	32	97
DA	10.4	4.8	28	143

Of the many difficulties associated with the determination of catecholamines in plasma or serum by HPLC, the following problems are of particular importance. The aluminum oxide extraction process yields, except for the three catecholamines of interest here, a number of other electroactive compounds (e.g. L-DOPA and derivatives) which appear close to the solvent front and which may partially overlap the catecholamine peaks, especially NE. Because the extent to which this occurs varies with factors such as the aluminium oxide used, the sample to be extracted and the final washing procedure, we used slight modifications of Anton and Sayre's original method to study the effect of deproteinization of the sample in this respect and on catecholamine recovery as well. The recoveries obtained by the two procedures were studied by accurate radiolabelling experiments and the results are shown in Table 2.

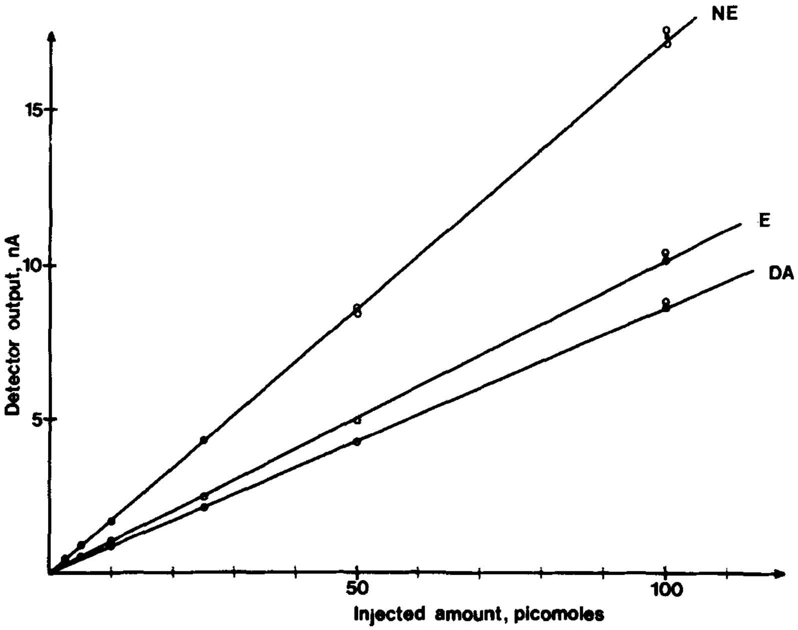

Fig. 1. Graph showing the detector response as a function of the amount injected. The column used was not the same as the one used for the data presented in Table 1.

TABLE 2

Recoveries Obtained in the Extraction Procedures Described in the Experimental Part. Method A denotes the procedure without deproteinization of the sample, B the other procedure.

Compound	Method	Adsorption yield, %	Total recovery, %
NE	A	76	74
	B	86	66
E	A	79	60
	B	89	69
DA	A	82	67
	B	91	68

Complete base-line separation of the catecholamines from the less-retained material requires a high column efficiency and a stationary phase which can yield sufficiently high capacity factors. This can only be achieved by the use of a metallic column and a packing material of small diameter. Of the materials suitable for electrochemical detection, reversed phase and ion-exchange microparticles, the latter has the advantage of separating in order of basicity, a prerequisite to avoid interference with the less basic amino acids (10, 11).

Figs. 2 and 3 illustrate some typical results. In Fig. 2 chromatograms of the three catecholamines isolated from 2 ml of non-deproteinized plasma and from a solution of standards are compared. According to the previously determined detector response factors, the E/NE and DA/NE ratios would be 0.19 and 0.20. The absolute concentrations in nmol/l of NE, E and DA which may be calculated from a 70 % recovery are 6.9, 1.4 and 1.3, respectively. Fig. 3 shows a similar result from the analysis of another sample in which 6 ml of PCA-deproteinized serum was used for adsorption on alumina. Here the E and DA concentrations are too low to be evaluated from the chromatogram. The NE peak, however, corresponds to a serum concentration of ~ 20 nmol/l.

We have also shown by separate experiments that internal standardization by the addition of 3,4-dihydroxybenzylamine (DHBA) to the sample for accurate re-

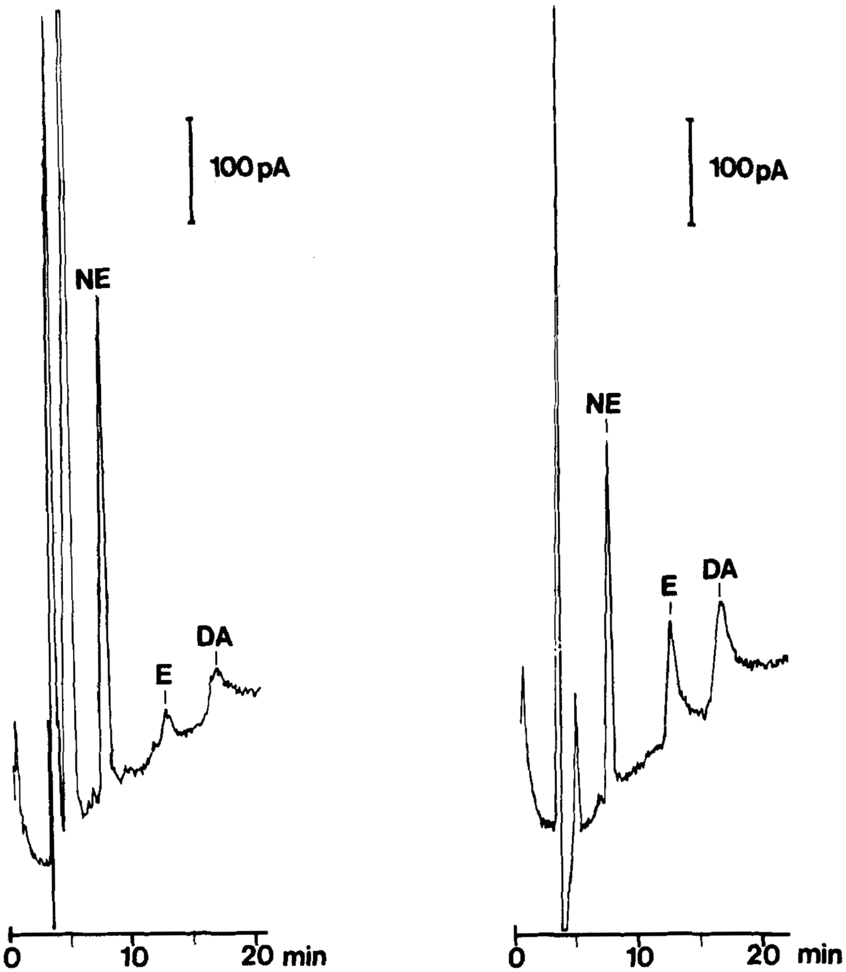

FIGURE 2

Representative chromatograms of the desorbed material from alumina extraction of a) a 2 ml non-deproteinized plasma sample (left) and b) catecholamine standards (right). The chromatographic recordings were performed under identical conditions - flow rate 0.60 ml/min corresponding to a pressure of 680 psi, chart speed 20 cm/hr, sensitivity 1 nA/V at 1 V recorder input, background offset 1.2 nA.

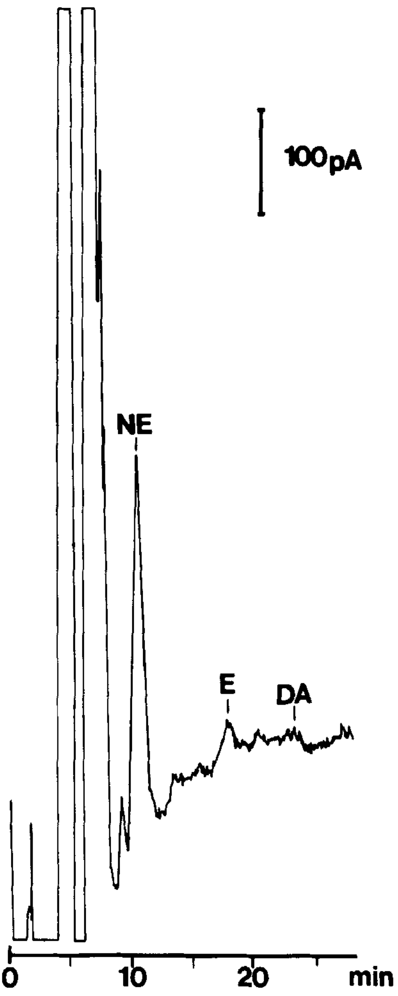

FIGURE 3

Chromatogram of the desorbed material from alumina extraction of 6 ml of deproteinized serum recorded at a flow rate of 0.40 ml/min. Other conditions as stated under Fig. 1.

covery determinations is possible because the DHBA peak is obtained well enough resolved from its neighbours NE and E.

Under the conditions used for the chromatographic recordings presented in Fig. 2, a signal-to-noise (S/N) ratio of 10 corresponds to injected amounts of 0.4 pmol of NE, 0.9 pmol of E and 1.0 pmol of DA. The limits of detection (S/N=2) were 80, 180 and 200 fmol, respectively.

In our opinion, although the method has to be evaluated further with respect to accuracy and precision, the results show that the three catecholamines studied can be completely resolved from each other and from the other extracted compounds in the leading peaks and quantitated by standardization techniques. We are convinced that with the use of even more efficient columns and with further improvement in baseline stability by means of meticulous detector isolation, this method will be sensitive enough to permit plasma catecholamine studies if sample volumes are of the order 2-10 ml.

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