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PLASMA CATECHOLAMINES IN HYPERTENSION AND PHEOCHROMOCYTOMA DETERMINED USING ION-PAIR REVERSED-PHASE CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

INVESTIGATION OF THE SEPARATION MECHANISM AND CLINICAL METHODOLOGY

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SUMMARY

The retention behavior of catecholamines (CAs) in ion-pair reversed-phase chromatography is examined. From the effects of pH, ionic strength and a secondary ion-pairing reagent (citric acid), under our chromatographic conditions, the retention behavior can be explained by assuming a mixed ion-exchange mechanism with octyl sulfate and citrate, on the column and in the mobile phase, respectively.

The developed separation method was applied to the analysis of CAs in plasma samples purified by alumina adsorption and detected amperometrically. This method provides the basis for the determination of the short-term magnitude of CA response to physical and physiological interventions, as well as the baseline CA levels in essential hypertension and pheochromocytoma. The results seen for norepinephrine and epinephrine are consistent with the functional roles of these CAs as hormones or peripheral transmitters.

INTRODUCTION

Under standardized conditions, circulating catecholamines (CAs) are believed to offer a reliable and accurate indication of the sympathetic tone and reactivity in man[1,2]. Thus, the assessment of CA levels may be of considerable importance in clinical investigations of disorders, such as hypertension, which involve the dysfunction of the sympathetic nervous system.

In recent years, the development of highly sensitive analytical methods has revolutionized the area of CA research. The determination of picogram quantities of

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neurotransmitters in physiological fluids, which is by no means a trivial matter, has necessitated the use of radioenzymatic[3] and double-isotope derivative[4] analyses, gas chromatography with mass spectrometry[5] and fluorometry[6,7].

The advent of reversed-phase liquid chromatography with amperometric detection has afforded yet another approach to the analysis of CAs[8,9]. Since CAs are present in their protonated form in the pH range most commonly employed with the chemically bonded reversed phases (2–7.5), they exhibit insufficient retention in the absence of ionic detergents. The addition of anionic ion-pairing reagents, such as octyl sulfate to the eluent has found widespread acceptance for modulation of retention and this technique is frequently referred to as ion-pair chromatography. However, the underlying thermodynamic equilibria have been a subject of considerable controversy. Thus, retention is believed to occur by dynamic ion exchange[10], ion-pair formation in the mobile phase[11], or dynamic complex exchange[12].

The main equilibria, generally recognized to be important in ion-pair chromatography of catecholamines, are the following:

(1) ion pairing in the mobile phase with the pairing ion, L^-

$$CA_m^+ + L_m^- \rightleftharpoons (CA^+ L^-)_m \quad (1)$$

followed by reversible binding to the non-polar surface:

$$(CA^+ L^-)_m \rightleftharpoons (CA^+ L^-)_s \quad (2)$$

where the subscripts m and s refer to the mobile and stationary phases.

(2) Dynamic ion exchange where the solute forms a complex with the ligand already adsorbed onto the non-polar surface:

$$CA_m^+ + L_s^- \rightleftharpoons (CA^+ L^-)_s \quad (3)$$

(3) Dynamic complex exchange where a metathetical exchange of the solute between the ion-pairing agent bound to the column and the ion pair formed in the mobile phase:

$$(CA^+ L^-)_m + L_s^- \rightleftharpoons (CA^+ L^-)_s + L_m^- \quad (4)$$

However, none of the above equilibria have been fully elucidated, due to experimental difficulties in obtaining unambiguous physicochemical evidence. Melander and Horváth[12], conclude that the proposed "mechanisms represent limiting cases and the retention process is not expected to follow any of them over a wide range of chromatographic conditions".

EXPERIMENTAL

Apparatus

The chromatographic equipment consisted of a Waters Model 6000A dual-piston pump and a U6K universal injector (Waters Assoc., Milford, MA, U.S.A.).

Chromatographic peaks were monitored by means of a Model 641 voltametric/amperometric detector (Brinkmann Instruments, Westbury, NY, U.S.A.) with a Model 656 detector cell made of polychlorotrifluoroethylene (Kel-F) and operating on a thin-layer wall-jet principle. The three-electrode potentiostatic system contains glassy carbon working and auxiliary electrodes (type 6.0805.010) and a Ag/AgCl reference electrode. The effective cell volume is approximately 1 μl. The chromatographic column was a prepacked, stainless-steel Ultrasphere ODS (15 cm × 4.6 mm I.D.) with average particle size of 5.0 μm (Altex Scientific, Berkeley, CA, U.S.A.). The guard column (5 cm × 4.6 mm I.D.) was prepacked with 5-μm packing material, also from Altex. Chromatographic peaks were integrated electronically using a Hewlett-Packard 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

The determinations of the pK_a'' values for citric acid were carried out using a Metrohm E 636 Titroprocessor, a microprocessor-controlled equilibrium titrator, equipped with the Metrohm EA 120 combination glass electrode (Metrohm, Herisau, Switzerland).

Reagents

Analytical reagent-grade chemicals were used without further purification. Norepinephrine (NE), epinephrine (E), dopamine (DA), 3,4-dihydroxybenzylamine (DHBA) and citric acid were purchased from Sigma (St. Louis, MO, U.S.A.). Reference solutions containing 20 ng/ml of the three CAs and the internal standard were prepared in 0.05 *M* $HClO_4$, which contained 10 mg/l of $NaHSO_3$. These solutions were kept frozen at −20°C until the analysis.

Potassium dihydrogen phosphate was purchased from Mallinckrodt (St. Louis, MO, U.S.A.), methanol, distilled-in-glass, from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), sodium octyl sulfate from Eastman-Kodak (Rochester, NY, U.S.A.), and alumina, activity grade I, (for chromatography) from Alpharm Chemicals (New Orleans, LA, U.S.A.).

Chromatographic conditions

Separation of plasma extracts was achieved isocratically. The eluent consisted of 0.0347 *M* KH_2PO_4, 0.030 *M* citric acid, 3.0 m*M* sodium octyl sulfate and 14% (v/v) anhydrous methanol. The pH was adjusted with a KOH solution to 4.85, prior to the addition of methanol. The volume of KOH needed to adjust the pH was kept to a minimum. Furthermore, this volume was kept constant for every eluent in order to avoid variations in ionic strength. The aqueous mixture was always filtered through Millipore membrane filters, type HA, with pore size of 0.45 μm (Millipore, Bedford, MA, U.S.A.). The methanolic solution was always purged with helium in order to expell dissolved air. The flow-rate was 1.2 ml/min, and the temperature was ambient in all cases. Amperometric detection was carried out at +0.500 V *vs.* Ag/AgCl, at the sensitivity of 7.5 nA full scale. The attenuation setting on the Hewlett-Packard integrator was 7 (128 mV ± 10% full scale).

Activation of alumina prior to adsorption

Approximately 100 g of chromatographic grade alumina, activity grade I, was suspended in 500 ml of 2 *M* HCl. The suspension was heated with constant stirring

for 45 min at 90–100°C. The mixture was then allowed to cool and settle, and the supernatant was withdrawn and discarded. The alumina was washed twice with 2 *M* HCl and resuspended in 500 ml of 2 *M* HCl for 15 min at 70°C. The supernatant was again discarded and the alumina was washed repeatedly with water until the pH of the washings reached 3.4. Excess water was evaporated by heating the alumina for 3–4 h at 215–300°C. The activated alumina was kept in tightly capped vials until use.

Preparation of plasma samples

Subjects were assessed in the sitting, recumbent and upright positions. Blood samples were drawn by venipuncture and transferred quickly into tubes containing glutathione and ethylene-bis(oxyethylene-nitrilo)tetraacetic acid. Samples were then centrifuged at 600 *g* at 4°C, the plasma was removed and stored frozen at −20°C until the analysis. Samples (2–4 ml) were added to tubes containing 2.0 ng of DHBA (internal standard) and 50.0 mg of acid-washed alumina. The pH was adjusted to 8.6 with Tris–HCl buffer, and the tubes were capped and shaken mechanically for 15 min. The samples were centrifuged at 600 *g* for 5 min, the supernatant was discarded, and the alumina was washed three times with distilled–deionized water. After centrifugation, the washings were discarded and CAs were eluted with 500 μl of 0.05 *M* $HClO_4$, containing 10 mg/l of $NaHSO_3$ (antioxidant). The samples were kept frozen at −20°C until the analysis. No significant decrease in catecholamine levels was observed over a period of one to two months under our storage conditions.

Clinical studies

Control subjects. Control subjects consisted of the Mount Sinai laboratory employees whose health was confirmed by routine physical examination. Absence of the following diseases was insured in this group: cardiac, pulmonary, hepatic, endocrine, gastrointestinal, neurologic, hypertension (diastolic blood pressure greater than 90 mm Hg), obesity and medication (except aspirin).

Hypertensive subjects. This group had diastolic pressure greater than 90 mmHg, with no history of neural crest tumors. In addition, they were not taking any medication.

Pheochromocytoma. The existence of this tumor was confirmed by histologic examination of tissue samples removed at surgery.

All subjects were in the age group between 35 and 50 years.

Identification of chromatographic peaks

The adsorption of CAs onto alumina at basic pH is highly selective and this approach has been widely used for sample preconcentration and cleanup. The resulting chromatograms contain only compounds with a catechol moiety, which facilitates considerably both the separation and characterization of solutes. In addition, the catechol compounds can be readily oxidized to corresponding orthoquinones at relatively low potentials where interfering compounds may exhibit a low or negligible electrochemical response. Thus, both the retention and electrochemical behavior are more informative than in highly populated chromatograms. Moreover, chromatographic solutes can be characterized online by means of their hydrodynamic voltammograms. If the responses (current) are monitored at different oxidation potentials and each response divided by the maximal response, the current ratio (ø) can be

plotted *versus* potential applied. The result is a characteristic sigmoid curve, which is highly characteristic of a particular compound.

Quantification

Quantitative analysis was performed using the standard addition method. Increasing amounts of the three CAs were added to aliquots of a plasma extract containing 2.0 ng of DHBA. After chromatography, ratios of responses of the CAs to DHBA were calculated for each sample and plotted *versus* the amount of CA reference added. The straight lines were described by mathematical equations which were later used to calculate CA levels in plasma samples under study.

RESULTS AND DISCUSSION

Effect of ionic strength on the pK_a^0 values

Regardless of the exact equilibrium responsible for retention, it is of critical importance that the solute molecules be charged in order for the interaction with the ion-pairing ions to take place.

The effect of ionic strength (μ) on the pK_a (OH) values for CAs and citric acid can be evaluated from the modified Debye–Hückel equation, the Davis equation (ref. 13):

$$pK_a' = pK_a^0 - \frac{(2n + 1)\, A\mu^{\frac{1}{2}}}{1 + \mu^{\frac{1}{2}}} + 0.1\,(2n + 1)\,\mu \qquad (5)$$

where pK_a' and pK_a^0 are the pK values at a given ionic strength and at $\mu = 0$, respectively; n is the stage of the ionization; A is a constant (0.512 at 25°C).

The pK_a (OH) values for CA and citric acid in the mobile phase without methanol are shown in Tables I and II, respectively. Table II also illustrates the effect of ionic strength and methanol on the pK_a values for citric acid, determined using eqn. 5 and potentiometrically.

TABLE I

THE pK_a^0 VALUES FOR CATECHOLAMINES

NE	E	DA
pK_a^0 (OH) 8.58	pK_a^0 (OH) 8.55	pK_a^0 (OH) 8.93

Combined effects of methanol and ionic strength on the pK_a^0 values

Eqn. 5 applies only to purely aqueous solutions and a more complicated expression must be used for methanol–water mixtures[14]. Alternatively, the pK_a'' values

TABLE II

THE pK_a VALUES FOR CITRIC ACID IN PURE WATER (pK_a^0) IN AQUEOUS SOLUTION AT INDICATED IONIC STRENGTH (pK_a'), AND IN 14% METHANOL AT INDICATED IONIC STRENGTH (pK_a'')

	pK_a^0 $\mu = 0$	pK_a' *No* CH_3OH $\mu = 0.0377$	pK_a'' *14%* CH_3OH $\mu = 0.120$
$HOOCCH_2C(OH)(COOH)CH_2COOH \rightleftharpoons HOOCCH_2C(OH)(COO^-)CH_2COOH +$	3.13	2.89	—
$HOOCCH_2C(OH)(COO^-)CH_2COOH \rightleftharpoons HOOCCH_2C(OH)(COO^-)CH_2COO^- + H^+$	4.76	4.373	4.82
$HOOCCH_2C(OH)(COO^-)CH_2COO^- \rightleftharpoons {}^-OOCCH_2C(OH)(COO^-)CH_2COO^- + H^+$	6.40	5.844	6.37

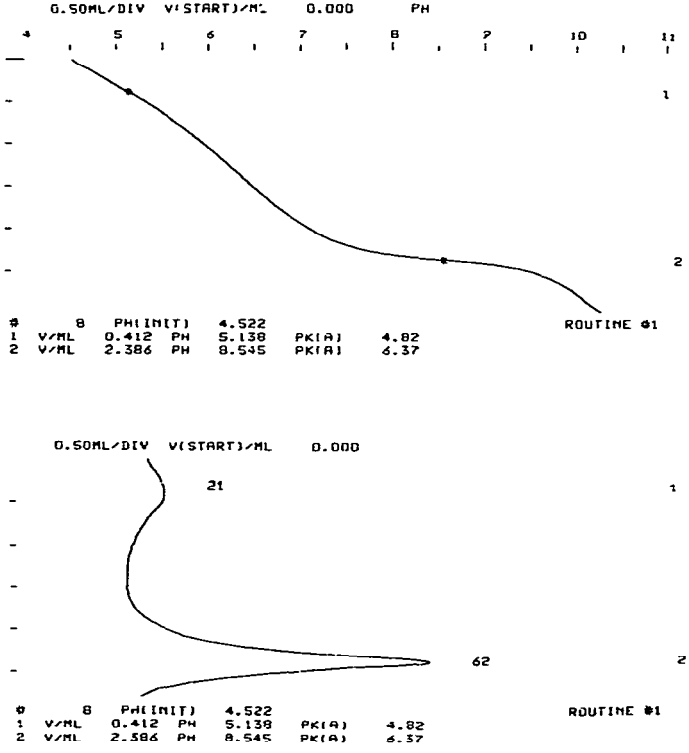


Fig. 1. Titration of citric acid with 0.1070 *M* KOH using a microprocessor-controlled equilibrium titrator. Upper curve: pH *vs.* ml KOH; lower curve: $\Delta pH/\Delta V_{KOH}$ *vs.* ml KOH.

can be determined potentiometrically, provided that the differences between the liquid-junction and glass-electrode potentials in the solvent containing 14% methanol and a purely aqueous solvent are not significant[15]. The titration curve for citric acid in a medium composed of 14% (v/v) methanol, 0.030 *M* citric acid, 3.0 m*M* octyl sulfate and 0.0347 *M* KCl is shown in Fig. 1. The upper part of the curve is a plot of pH *versus* ml KOH, and the lower part is the first derivative curve. Potassium phosphate was replaced with KCl in order to maintain the same ionic strength, while avoiding the titration of the phosphate salt. It is obvious from the pK_a'' values that at a pH of 4.85 an approximately equimolar mixture of -1 and -2 charged species is present in the eluent (see Table II).

Control of separation

From the pK_a^0 (OH) values listed in Table I, it is evident that CAs are in their protonated form in the useful pH range for chemically bonded reversed-phases. For this reason, NE and E are eluted near the void volume and DA is only slightly retained (Fig. 2A). Change in the eluent pH from 2 to 7.5 does not have any significant effect on the degree of retention. Increase in the ionic strength of the buffer upon addition of citrate ions facilitates the protonic equilibria in the mobile phase which improves the peak shapes with no effect on retention (Fig. 2B).

However, addition of an anionic ion-pairing reagent, such as octyl sulfate, to the mobile phase affects dramatically the retention behavior of CAs. It has been shown by several investigators[10,16] that the chemical nature and concentration of the pairing reagent are important in determining the extent of retention. However, high concentrations and excessively long chain lengths of the pairing reagent should be avoided due to increase in equilibration times. Octyl sulfate seems to give optimal selectivity with minimal equilibration time (20 min). By using a mobile phase containing 0.0347 *M* phosphate and 3.0 m*M* octyl sulfate, pH 4.85, the retention time of NE changes from 3 min (phosphate only) to 11 min. However, E, DHBA and particularly DA exhibit excessively long retention times under these conditions, which gives rise to considerable peak broadening. Thus, it is necessary to add an organic modifier, such as methanol, to the eluent in order to obtain optimal k' values. Addition of methanol was reported to cause a decrease in the dielectric constant of the medium, resulting in increased ion-pair formation in the mobile phase[17]. Melander *et al.*[18] have confirmed the existence of ion pairs in the eluent and determined the formation constants. They concluded that, in view of the low coverage (a few percent) of the non-polar surface with the pairing ions, the existence of ion pairs might play a significant role in the thermodynamics of chromatographic retention. However, Knox and Hartwick[10] believe that the predominant factor in the overall retention is the interaction of the charged solutes with the pairing ions, dynamically coated to the non-polar surface. It is to be expected, however, that neither mechanism will predominate if the chromatographic conditions (type and concentration of pairing ion, percent organic modifier, etc.) are varied over a wide range. Thus, neither model can explain the data in a consistent way and several broader concepts have been proposed in order to accommodate both types of behavior without combining the two models[12].

If it is assumed that the pairing ions are adsorbed onto the non-polar surface of the column packing material, they should be well spaced from one another due to electrostatic repulsions. The nature of the column surface would thus change and the protonated CAs could be transferred through the electrical double layer and retained

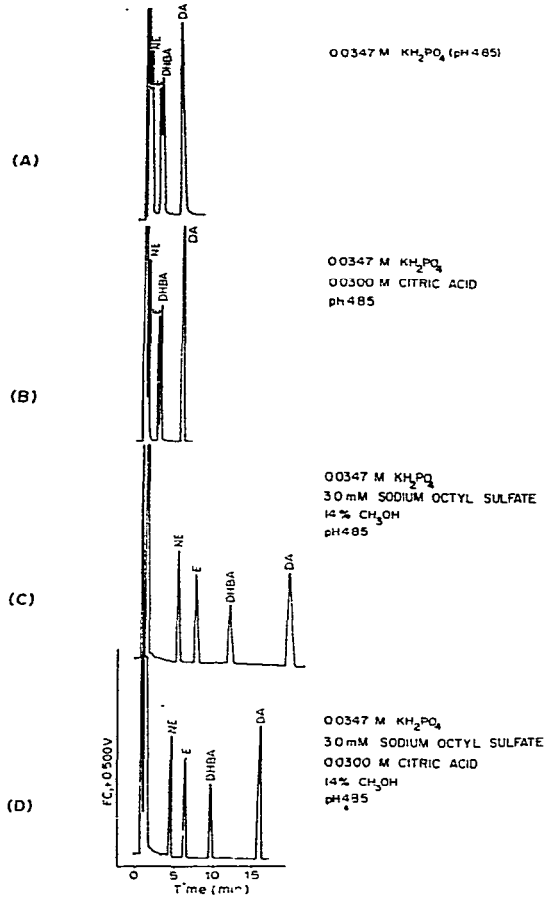


Fig. 2. Separation of a synthetic mixture of norepinephrine (NE), epinephrine (E), 3,4-dihydroxybenzylamine (DHBA) and dopamine (DA). Chromatographic conditions: column: Ultrasphere ODS (15 cm × 4.6 mm I.D.), 5-μm average particle size; flow-rate: 1.2 ml/min; temperature: ambient; detection: amperometric at +0.500 V *vs.* Ag/AgCl; detector sensitivity: 7.5 nA f.s.; eluents: (A) 0.0347 *M* KH_2PO_4, pH 4.85; (B) 0.0347 *M* KH_2PO_4, 0.030 *M* citric acid, pH 4.85; (C) 0.0347 *M* KH_2PO_4, 3.0 m*M* sodium octyl sulfate, 14% (v/v) methanol, pH 4.85; (D) 0.0347 *M* KH_2PO_4, 3.0 m*M* sodium octyl sulfate, 0.030 *M* citric acid, 14% (v/v) methanol, pH 4.85.

as a result of electrostatic attraction. At low concentrations of the pairing ion in the mobile phase, a significant fraction of the surface would be unaffected by the presence of the pairing ions and, thus, solvophobic interactions may also play an important role in the overall retention. We believe that, under chromatographic conditions shown in Fig. 2C, both the dynamic ion-exchange and solvophobic interactions can account for the retention of CAs. The addition of citrate ions to the mobile phase containing octyl sulfate has a profound effect on retention (Fig. 2D), due to secondary equilibria in the mobile phase. From the differential curve shown in Fig. 1 it is evident that the ratio of the concentration of citrate ions with −2 charge to the concentration of the

— 1 ions is approximately 1. It should be pointed out that the difference between Fig. 2C and D is not due to the ionic strength effect, since all compounds exhibited identical behavior to the one illustrated with Fig. 2C when citric acid was replaced with an equivalent amount of KCl. This behavior leads us to believe that ion pairing takes place on the column surface covered with octyl sulfate and with citrate ions in the mobile phase.

If the pH of the mobile phase containing phosphate, octyl sulfate, citric acid and methanol is varied over the range of 4.85 to 3.01, several interesting effects can be seen (Fig. 3). By lowering the pH, the ionization of citric acid is suppressed and thus its effective charge is decreased. The concurrent increase in hydrophobic character of citric acid species results in increased interaction of citric acid with the free non-polar surface. This is facilitated by the diminished repulsions between the undissociated citric acid and the negatively charged octyl sulfate groups on the surface. The result is shown in Fig. 3, where the unlabeled peak represents citric acid. Decreased ionization of citric acid would also diminish its role as a secondary pairing agent in the mobile phase. Thus, the combined effect of increased protonation of CAs at lower pH values and diminished ion-pairing with citrate ions in the mobile phase results in enhanced electrostatic interaction between CAs and the negatively charged surface.

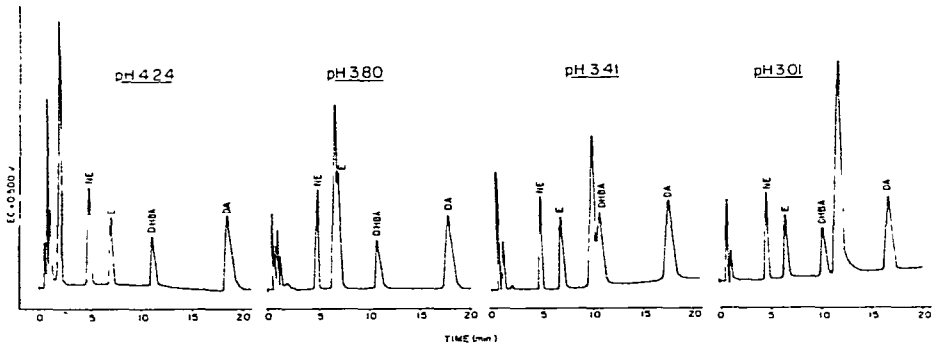
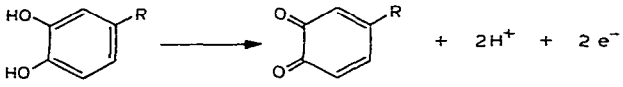


Fig. 3. Effect of eluent pH on the separation of a synthetic mixture of CAs. Chromatographic conditions same as in Fig. 2(D), except for eluent pH.

Determination of plasma catecholamines

Since the mobile phase containing 0.0347 *M* phosphate, 3.0 m*M* octyl sulfate, 0.030 *M* citric acid, 14% (v/v) methanol, pH 4.85 afforded the best combination of resolution, analysis time and peak shapes, it was adopted for the assessment of CA levels in samples of human plasma. Because plasma levels of NE are several hundred hundred picograms per ml, and those of E and DA are at least one order of magnitude lower, accurate determinations of neurotransmitter levels necessitate the use of a highly sensitive detection technique. Judging from the evergrowing number of publications on the use of amperometric detection for the analysis of electroactive species in physiological fluids, it is now evident that this detection method holds great promise in CA research. CAs can be easily oxidized to the corresponding orthoquinones at relatively low oxidation potentials, according to the following reaction:



(6)

This enables sensitive and accurate analyses needed for clinical investigations. Prior to the establishment of the protocol procedure, the detection potential was optimized in order to achieve the best compromise between the sensitivity and electrode lifetime. Therefore, voltammograms were obtained for the four compounds under study (Fig. 4). It has been shown previously[16] that the ionic strength of the solvent and a change in the pH of the medium can affect both the half-wave potential ($E_{\frac{1}{2}}$) and the rate of electron transfer; a change in the pH from 2 to 6 results in a marked increase in catecholamine response. However, above pH 6.0, the response is diminished, probably due to the oxidation of the catechol ring prior to its detection[16]. It is obvious from the sigmoid voltammetric waves shown in Fig. 4 that the optimal oxidation potential for CAs under these chromatographic conditions is +0.500 V *vs.* Ag/AgCl. By operating the detector on the mass-transport limited portion of the curve, the advantages are two-fold. Firstly, small shifts in potential resulting from reference electrode drift or the internal resistance of the solution will have a minor effect on the response (current). Secondly, low oxidation potentials extend the electrode lifetime. Our electrode system was used over a period of one year with periodic resurfacing with Alox powder (aluminum oxide. Brinkmann Instruments), with no loss in sensitivity due to surface degradation.

Since plasma is a complex matrix which contains many electroactive species, optimization of detection potential for the compounds under study does not afford sufficient selectivity and sample cleanup is mandatory. Alumina adsorption under alkaline conditions is highly specific for CAs. Thus, other potentially interfering substances can be eliminated and samples are also preconcentrated. This was found especially useful for the determination of the usually low catecholamine levels in normotensive subjects. Prior to the analysis of plasma samples, the overall recovery of the adsorption procedure was evaluated by liquid chromatographic and radioisotopic methods. The average recovery for all catecholamines, as well as DHBA, was approximately 60%. Individual recoveries were as follows: NE, 62 ± 10%; E, 60 ± 10%; DHBA, 58 ± 10% and DA, 56 ± 10%. It should be pointed out that, even under strictly controlled conditions, the recoveries were found to vary up to ±10%. Thus, the use of an internal standard is essential for quantitative analysis.

The levels of plasma CAs can be affected by many variables: patient condition, medication, diet and posture during blood collection. Fig. 5 shows the effects of posture during sample collection (recumbent *vs.* upright) and physical stress on the plasma CA levels. In addition, the method of blood collection (venipuncture *vs.* indwelling catheter), blood/plasma storage and analytical methodology must be specified in order to achieve meaningful interlaboratory correlation of quantitative data.

The neural crest tumor, pheochromocytoma, is a rare cause of hypertension. This catecholamine-secreting lesion, fatal if undetected, has all the clinical symptoms of essential hypertension which makes the diagnosis difficult or impossible without the exact knowledge of the secretory and inactivation pathways. CAs and their metabolites have been successfully used as diagnostic markers of adrenomedullary hyperfunction and in follow-up treatment of neural crest tumors. The tremendous variability in the clinical presentation of pheochromocytoma results from the wide spectrum of catecholamine secretion and changes in the balance between their release and inactivation. Fig. 6 illustrates the CA levels in plasma samples from a patient before

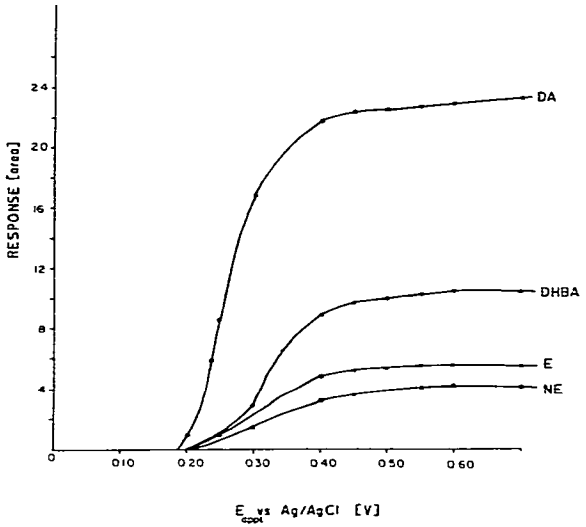


Fig. 4. Voltammograms of the three CAs and internal standard (DHBA). E_{appl} vs. current ratio (∅)

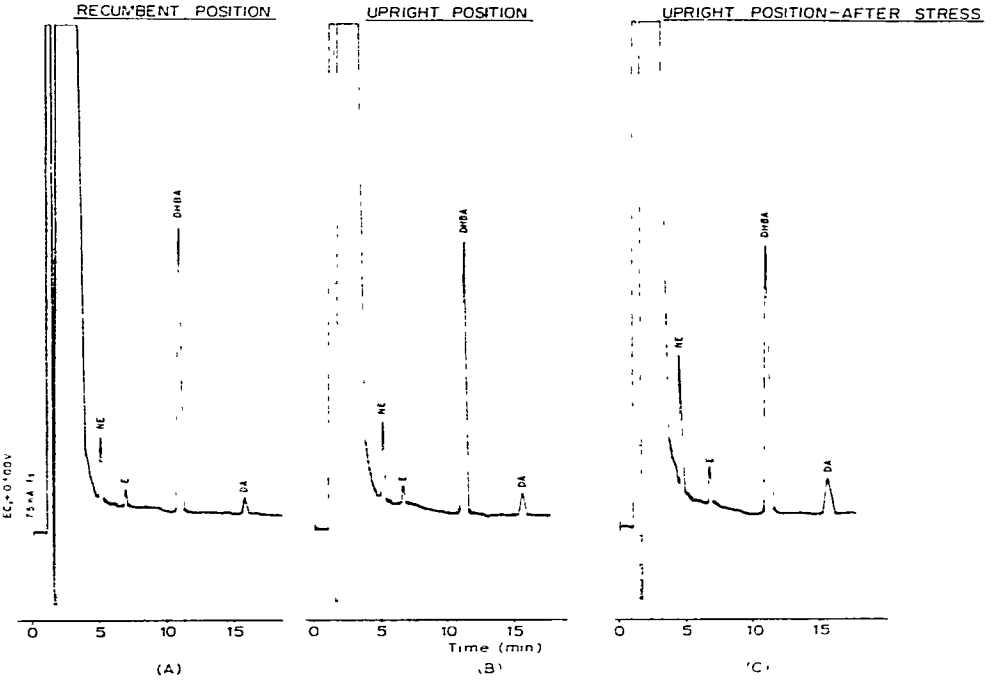


Fig. 5. Effects of posture (recumbent vs. upright) and physical stress on plasma CA levels in a normotensive subject. Chromatographic conditions same as in Fig. 2(D). Volumes injected: 100 μl (corresponding to 400 μl plasma); CA levels: (A) NE, 210 pg/ml; E, 19.5 pg/ml; DA, 40 pg/ml; (B) NE, 251.0 pg/ml; E, 21.0 pg/ml; DA, pg/ml; (C) NE, 430 pg/ml; E, 50 pg/ml; DA, 110.0 pg/ml.

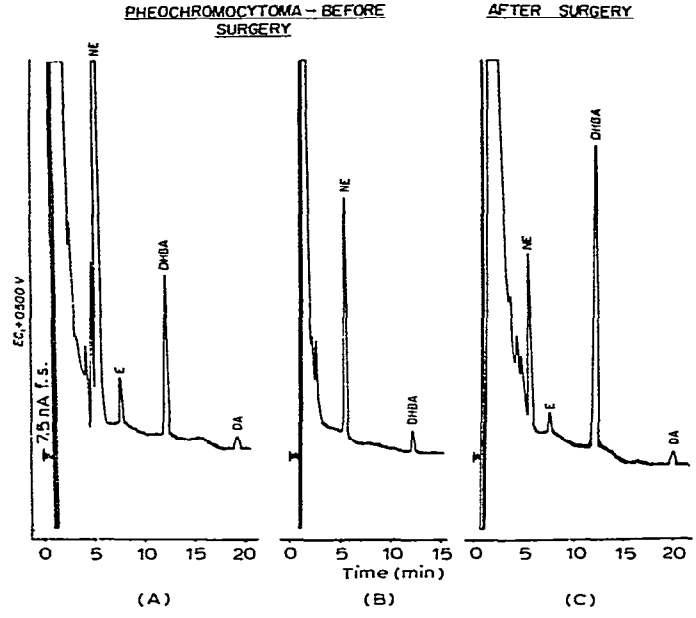


Fig. 6. Chromatograms of plasma samples from a patient with pheochromocytoma, before (A, B) and after (C) surgical removal of the tumor. Chromatographic conditions same as in Fig. 2 (D). Volumes of extract injected: 100 μl; volumes of plasma extracted by alumina adsorption: (A), 0.300 ml; (B), 0.030 ml; (C), 0.800 ml. CA levels: (A), (B) NE, 14.5 ng/ml; E, 180 pg/ml; DA, 85 pg/ml; (C) NE, 350 pg/ml; E, 25 pg/ml; DA, 40 pg/ml.

(A, B) and after (C) surgical removal of the NE-producing tumor. It is interesting to note that the considerably elevated levels of NE in the tumor patient returned to normal after surgery. The plasma samples from this patient illustrate an insignificant change in the free E and DA. This is in agreement with the literature reports[18] which demonstrate that conjugation during passage in the venous blood appears to be one of the major inactivation pathways of CAs secreted from a pheochromocytoma.

Prior to the quantitative analysis, the purity and identity of chromatographic peaks must be determined. Since alumina adsorption is highly specific under alkaline conditions, the characterization of peaks is considerably facilitated.

In addition, amperometric detection offers an attractive possibility for online characterization of electroactive solutes. By replicate injections of the sample detected at several potentials, hydrodynamic voltammograms can be obtained for the sample peaks and compared with those for the reference compounds. Fig. 7 illustrates the use of this method for characterization of the NE peak in the plasma sample shown in Fig. 6A, B.

The standard addition curve used for the quantification of catecholamines is shown in Fig. 8. The endogenous levels for this sample were found by extrapolating the straight lines. For all other samples, the lines for the three catecholamines were

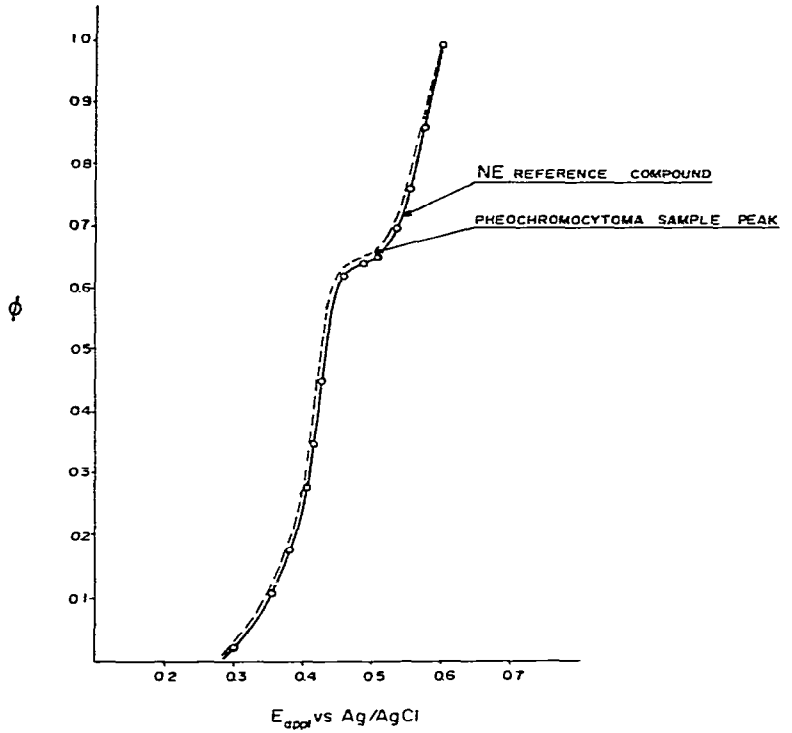


Fig. 7. Hydrodynamic voltammograms for the NE reference compound and the peak in the sample from a patient with pheochromocytoma shown in Fig. 6(A). *x*-Axis: oxidation potential *vs.* Ag/AgCl; *y*-axis: ratio of response (current) at a particular potential to the maximal response.

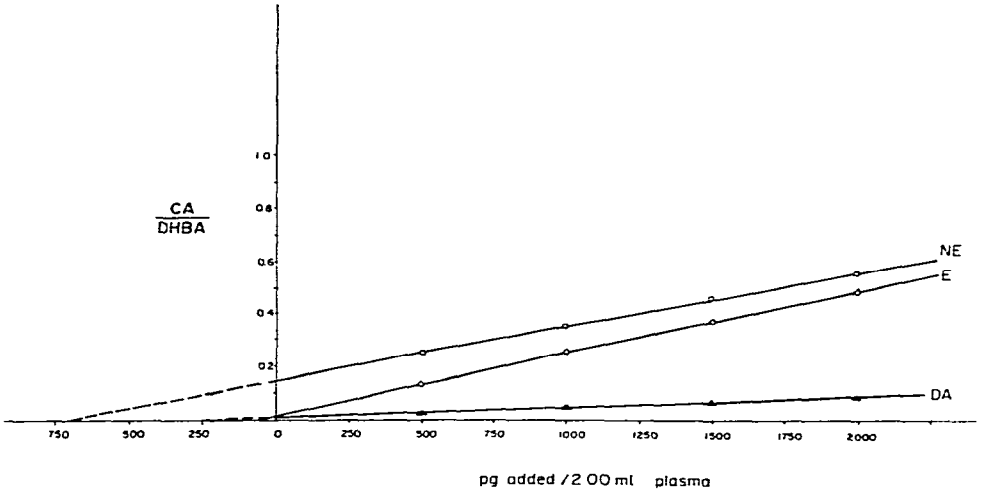


Fig. 8. The standard addition curve obtained by addition of increasing amounts of reference compounds to aliquots of a plasma sample from a control subject. *x*-Axis: pg of CAs added to 2.0 ml plasma samples; *y*-axis, ratio of responses of each CA to that of DHBA. NE, 350.0 pg/ml; E, 22.0 pg/ml; DA, 87.0 pg/ml.

described by mathematical equations which were used for determination of CA levels:

$$\text{ratio } \frac{\text{CA}}{\text{DHBA}} = mx + b \tag{7}$$

where m is the slope and b the intercept with the y-axis. The ratio of the peak height for a CA to that of the internal standard was calculated for each sample and the concentrations of CAs were determined from the following equations:

$$\frac{\text{NE}}{\text{DHBA}} = 0.000207x + 0.146 \tag{8}$$

$$\frac{\text{E}}{\text{DHBA}} = 0.000238x + 0.013 \tag{9}$$

$$\frac{\text{DA}}{\text{DHBA}} = 0.000040x + 0.010 \tag{10}$$

Within-run precision of quantitative analyses was 10% under carefully controlled conditions of sample preparation and chromatographic parameters. The reproducibility of peak heights was 1.2%, and the day-to-day precision of quantitative results was 0.7%. The quantitative data are shown in Table III. The ranges for NE, E and DA in normotensive subjects were 100–600, 10–80 and 10–50 pg/ml, respectively. Physical exercise (stress) resulted in elevation of NE, while E and DA levels remained within the normal range. Measurements of individual CAs in the venous outflow from hypertensive patients is characterized by pronounced increase in NE (1.6–1.8 ng/ml). The three subjects with clinically-diagnosed pheochromocytoma prior to surgery produced an extreme elevation in NE with a moderate increase in E and DA. Significant differences in the free NE levels between the normotensive and hypertensive subjects indicate the clinical significance of CAs as an index of increased activity of the sympathetic nervous system. In addition, changes in plasma CAs in subjects with pheochromocytoma reflect the hyperfunction of the adrenal medulla, the most common site of neural crest tumors.

TABLE III

QUANTITATIVE DATA FOR CATECHOLAMINES IN CONTROL SUBJECTS (NORMOTENSIVES), CONTROL SUBJECTS UNDER PHYSICAL STRESS, HYPERTENSIVES AND PATIENTS WITH PHEOCHROMOCYTOMA

	NE	*E*	*DA*
Control subjects (normotensive) (n = 20)	100–600 pg/ml	10–80 pg/ml	10–150 pg/ml
Control subjects under stress (n = 2)	900–1200 pg/ml	10–70 pg/ml	10–50 pg/ml
Essential hypertension (n = 3)	1.6–1.8 ng/ml	40–80 pg/ml	200–250 pg/ml
Pheochromocytoma (n = 3)	4.0–14.5 ng/ml	80–250 pg/ml	65–470 pg/ml

CONCLUSIONS

In this study, we attempted to formulate the effect of ionic strength, pH and a secondary ion-pairing reagent retention of CAS. The developed separation method was applied to the analysis of CAs in plasma samples from control subjects and patients with hypertension and pheochromocytoma. This method appears to be ideally suited to routine clinical investigations of short-term CA responses of the sympathetic nervous system.

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