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OPTIMIZED ISOCRATIC CONDITIONS FOR ANALYSIS OF CATECHOLAMINES BY HIGH-PERFORMANCE REVERSED-PHASE PAIRED-ION CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

We studied high-performance liquid chromatographic separation of the catecholamines epinephrine, norepinephrine, 3,4-dihydroxybenzylamine and dopamine utilizing an isocratic aqueous mobile phase flowing over a bonded octadecylsilane (μBondapak C_{18}) solid phase, with amperometric detection of the eluate. Separation was not dependent upon ionic strength of the mobile phase, while a definite dependence upon solvent pH was observed. Detector response showed a marked dependence upon both ionic strength and pH of the liquid phase, with optimum conditions for signal response occurring at pH 5.8 in an aqueous medium of 0.07-mole/l salt concentration. To achieve a baseline separation of the four catecholamines, inclusion of an aliphatic sulfonate as a paired ion was necessary. Heptanesulfonate was found to be the paired ion of choice. In the presence of the paired ion, a sensitivity was achieved which gave a peak more than ten times baseline noise when 100 fmoles of norepinephrine were injected on the column.

INTRODUCTION

The analysis of biological extracts for identification and quantitation of the catecholamines has been carried out by a variety of chemical techniques. In 1949 von Euler and Hamberg[1] introduced a colorimetric procedure which led to much research on the physiology of the neuron. At about the same time, the observation was made[2] that the adrenochrome identified by von Euler could be chemically modified to an adrenolutin with characteristic fluorescent properties. The fluorescence assay yielded the sensitivity necessary for study of the small amounts of adrenergic amines in tissue samples. This procedure has been widely used in the analysis of catecholamines[3].

In 1970, Engelman and Portnoy[4] introduced the concept of enzyme-catalyzed transfer of radiolabelled methyl groups to the catechol ring as a means for analyzing

very small quantities of individual catecholamines. This technique, while very useful in the research laboratory, has a major drawback in that it is very tedious, and requires considerable technical skill. For this reason the assay has not been popular in routine laboratories involved in day-to-day analysis of many biological specimens.

The advent of high-performance liquid chromatography (HPLC) has afforded the laboratory yet another approach to the analysis of catecholamines. Kissinger *et al.*[5–8] reported the use of an electrochemical detector with a cation-exchange liquid chromatograph to analyze catecholamines in the picogram range in the column effluent. Other workers[9,10] have used reverse-phase chromatography with an ultraviolet detector to analyze catecholamines. Preliminary studies in this laboratory with the cation-exchange system proposed by Kissinger *et al.*[5] as well as the reverse-phase system described by Molnár and Horváth[9] indicated that 3,4-dihydroxybenzylamine could not be included as an internal standard in the chromatogram as a separate, unfused peak. In each case, 3,4-dihydroxybenzylamine was found to co-chromatograph with epinephrine. In this paper we report the chromatographic behavior of catecholamines on a reversed-phase chromatographic medium coupled with a detector of the electrochemical type. The goal of this study was to determine the optimal conditions for performance of the electrochemical detector and separation of 3,4-dihydroxybenzylamine as a discrete entity in the chromatogram.

MATERIALS AND METHODS

Chemicals

The catechol standards norepinephrine (NE), epinephrine (E), and dopamine (DA) were purchased from Sigma (St. Louis, Mo., U.S.A.). The internal standard 3,4-dihydroxybenzylamine (DHBA) was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Aliphatic sulfonates, butanesulfonate, pentanesulfonate, hexanesulfonate, heptanesulfonate and octanesulfonate were from Eastern Organic Chemicals (Rochester, N.Y., U.S.A.). All reagents, including ethylenediaminetetraacetic acid (EDTA), were reagent grade from Fischer Chemicals (Chicago, Ill., U.S.A.).

Apparatus

The chromatographic system consisted of a 2-l glass solvent reservoir with a PTFE stir bar, a Model 6000A solvent delivery system, a Model U6K injector and a 4 mm × 30 cm μBondapak C_{18} column from Waters Assoc. (Milford, Mass., U.S.A.). The detection device was a Model LC-10 electrochemical detector from Bioanalytical Systems (West Lafayette, Ind., U.S.A.). The detector electrode consisted of a wax-impregnated carbon paste with flow cell defined by the 5 *M* (0.005 in) gasket. The electrode potential was maintained at 0.5 V *versus* a silver–silver chloride cell. The signal generated by the detector was converted by a Houston Omniscribe recorder to a chromatographic trace. All quantitation was performed by determination of peak heights. Response of peak height to concentration has been previously shown to be linear over a wide range of concentrations[5,8]. This was confirmed in our laboratory.

Solvents

Aqueous chromatographic solvents were prepared with double glass-distilled water. After salts were added and pH adjusted, solvents were filtered through a 0.3-

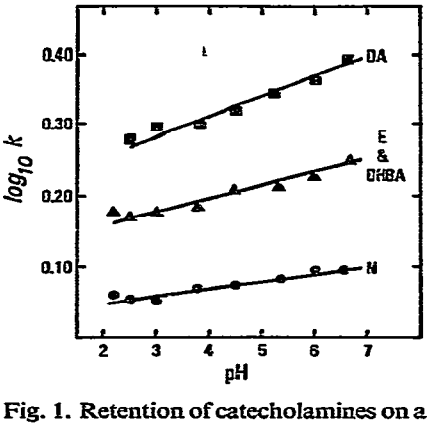
μm Millipore filter. Degassing of solvents was achieved by vacuum filtration and constant slow stirring of the solvent in the reservoir.

Standards

All stock catecholamine standards were prepared in 0.05 *M* $HClO_4$, 0.005 *M* $Na_2S_2O_5$ at a concentration of 100 μg/ml and stored at 4° C in the dark. Appropriate concentrations of the stock standards listed later in the text were diluted with 0.05 *M* H_3CCOOH, 0.005 *M* $Na_2S_2O_5$, and stored for no more than 12 h at room temperature. An injection volume of 25 μl, made with a Precision microsyringe with stainless-steel needle, was used throughout this text, except where noted.

RESULTS AND DISCUSSION

Separation of the catechol group (NE, E, DHBA, and DA) was directly affected by changes in the solvent pH (Fig. 1). These results are comparable to those previously demonstrated by Molnár and Horváth[9]. The theory for this phenomenon has been previously discussed[11]. The detector response, as measured by peak height, was also affected by change of pH (Fig. 2). Since the response is dependent upon oxidation of the catechol ring, which has been shown to be enhanced by increased pH[12], the trend indicated in Fig. 2 would be expected. As the pH rises from 2 to 6, the ring undergoes increased oxidation at the electrode surface. At a pH above 6, the catechol ring, in the presence of metal surfaces present in this chromatographic system, may undergo auto-oxidation prior to entering the detector. This is reflected by the disappearance of the signal observed in Fig. 2.



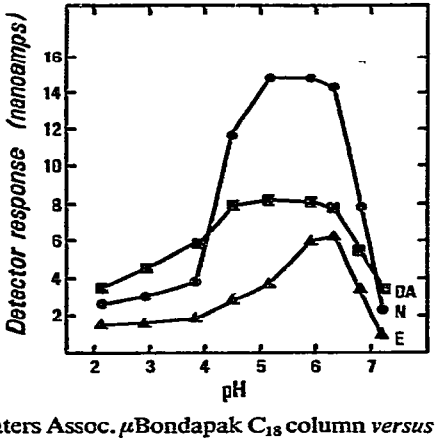


Fig. 1. Retention of catecholamines on a 4 mm × 30 cm Waters Assoc. μBondapak C_{18} column *versus* solvent pH. Solvent consisted of 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, adjusted to appropriate pH by titration with NaOH. Solvent flow-rate, 1.5 ml/min. The catecholamine mixture consisted of 1 μg/ml each of NE, E, DHBA and DA.

Fig. 2. Signal response generated by the electrochemical detector *versus* solvent pH. The solvent consisted of 0.1 *M* NaH_2PO_4, 0.1 m*M* EDTA, adjusted to the appropriate pH by titration with NaOH. Solvent flow-rate, 1.5 ml/min. 25 μl of the catecholamine mixture, consisting of 1.0 μg/ml each of NE, E, DHBA and DA were injected at each different pH.

Molnár and Horváth[9] reported the effect of ionic strength on retention of catechol compounds on a μBondapak C_{18} column. They concluded that ionic interactions beteeen the aliphatic solid phase and the charged species of the solute are minimal. Our observations (Fig. 3) support those of Molnár and Horváth[9] and are shown here as confirmation of that work. However, the signal output of the electrochemical detector in our studies showed a marked dependence on ionic strength (Fig. 4). The electrochemical reaction occurring in the detector involves the interaction of the solute molecule with the surface of the carbon paste electrode. Thus, at finite current, the electrochemical measurement within the flow cell is limited by mass transport, defined by Fick's first law of diffusion:

$$J = -D\left(\frac{\partial c}{\partial x}\right)$$

where J is the flux, D the diffusion coefficient, and $\partial c/\partial x$ is the partial derivative of the distance (x) the solute molecule (c) must travel to contact the electrode. Thus the signal generated by the detector was anticipated to be dependent on the flow-rate which is regulated by the dimensions of the flow cell, as well as the diffusion rate. Under the conditions of this experiment, the dimensions of the flow cell were constant. Alteration of the solvent flow-rate produced the anticipated effect, *i.e.* at increased flow-rates, the relative signal response was observed to decrease. Under conditions indicated for Fig. 4, the flow-rate was constant. Thus, the only variable was the diffusion coefficient, D, defined by the Nernst equation:

$$D = \frac{RT}{F^2}\frac{\lambda}{|Z_i|}$$

where R is the Rydberg gas constant, T the temperature in °K, F the Faraday constant, λ the equivalent ion conductivity, and Z_i the number of ions in solution. The Nernst

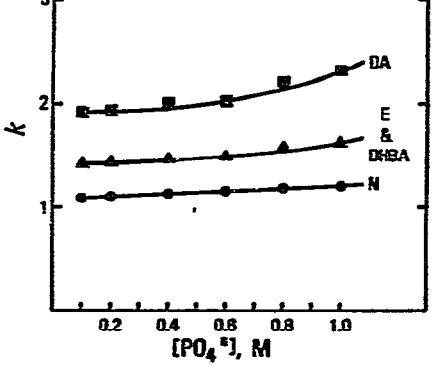


Fig. 3. Retention of catecholamines on a 4 mm × 30 cm Waters Assoc. μBondapak C_{18} column *versus* ionic strength of the solvent. The solvent consisted of varying concentrations of NaH_2PO_4 (pH 4.0) each with 0.1 m*M* EDTA. The catecholamine mixture consisted of 1 μg/ml each of NE, E, DHBA and DA.

equation was initially intended for study of a single ion species, and therefore application to the problem presented in Fig. 4 would be a gross oversimplification. It is introduced here only to promote the interpretation that the diffusion of an ion through an aqueous solution should be inversely proportional, in some manner, to the number of charged species in solution (Z_i). This is generally suggested by the trend in Fig. 4 at a phosphate concentration greater than 0.07 *M*.

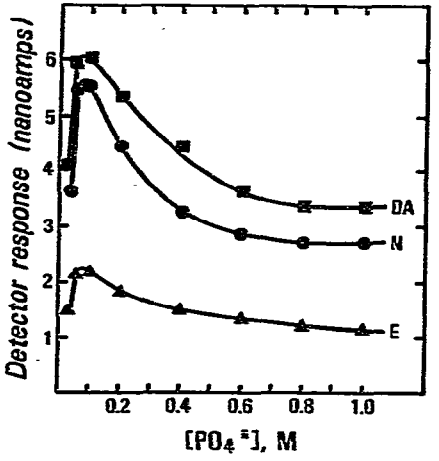
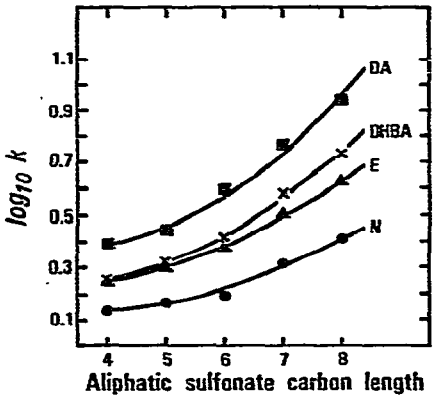


Fig. 4. Signal response generated by the electrochemical detector *versus* solvent ionic strength. The solvent consisted of varying concentrations of NaH_2PO_4 (pH 4.0) with 0.1 m*M* EDTA. 25 μl of the catecholamine mixture, 1.0 μg/ml each of NE, E, DHBA and DA, were injected at each different ionic strength indicated in the figure.

The diminished detector response at low ionic strength indicated in Fig. 4 must be explained by an entirely different mechanism. The detector was composed of a two-loop circuit with amplification. One loop served as a reference; the second loop (working electrode) provided an electron sump at which oxidation occurred, with concurrent generation of a signal voltage proportional to current at the working electrode. To complete the circuit, the reference and working loops must make electrical contact via an ionic medium. Under the constraints of diminished ionic strength, resistance to very small current flow (nA) was increased, thereby decreasing the relative response of the detector.

To enhance the resolution of catecholamines, and particularly to effect a baseline separation between E and DHBA, a useful internal standard, the technique of ion pairing was studied. Coupled with reverse-phase HPLC, ion pairing with aliphatic counter ions provided a very powerful tool for positioning peaks in a chromatogram where desired. This technique promoted the best features of reversed-phase columns (high resolution and column stability) with the strong separation characteristics of ion-exchange chromatography. In view of the success that Knox and Jurand[13] have had with soap chromatography in the separation of biogenic amines, a variety of aliphatic sulfonates were studied as possible ion-pairing agents to achieve resolution between E and DHBA. The results of adding paired ions of varying straight carbon chain length are shown in Fig. 5. In this study, ionic strength of both the buffer and

aliphatic sulfonate were constant. The only physical factor that was changed that could affect resolution was the number of carbons in the aliphatic portion of the pairing species. No change in relative detector response was observed. From Fig. 5, it was deemed that the most practical paired ion would be either heptanesulfonate or octanesulfonate. The effect of heptanesulfonate concentration on retention (k) is shown in Fig. 6. The trend in Fig. 6 closely resembles that seen by Knox and Jurand[13].



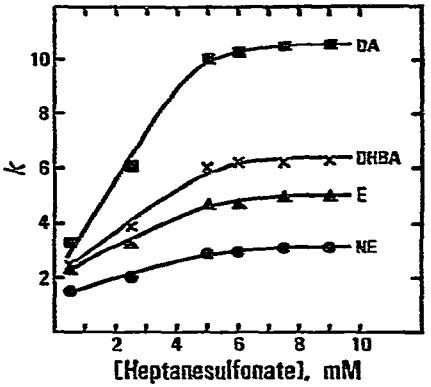


Fig. 5. Retention of catecholamines on a 4 mm × 30 cm Waters Assoc. μBondapak C_{18} column as affected by the carbon chain length of aliphatic sodium sulfonate paired ions. The solvent (1.5 ml/min) consisted of 0.1 M NaH_2PO_4 (pH 5.0), 0.1 mM EDTA with 210 mM aliphatic sulfonate as implied in the figure. The sulfonates were butanesulfonate, pentanesulfonate, hexanesulfonate, heptanesulfonate, or octanesulfonate. The catecholamine mixture consisted of 100 ng/ml each of NE, E, DHBA and DA.

Fig. 6. Retention of catecholamines on a 4 mm × 30 cm Waters Assoc. μBondapak C_{18} column as affected by the concentration of the paired ion, heptanesulfonate. The solvent (1.5 ml/min) was 0.1 M NaH_2PO_4 (pH 5.0), 0.1 mM EDTA containing varying amounts of heptanesulfonate. The catecholamine mixture consisted of 100 ng/ml each of NE, E, DHBA and DA.

CONCLUSION

Determination of solute concentration after separation by a chromatographic method requires application of an optical or chemical method to the eluate. Two factors influence the sensitivity of a chromatographic technique. They are (1) the sensitivity of the detection mechanism for concentration of the solute of interest in the column eluate, and (2) the amount of solute spreading that occurs during the separation procedure. By diminishing the solute dilution that occurs during chromatography, the concentration per unit volume of the solute in the eluate may be increased, which effectively increases the sensitivity of the entire system.

HPLC, using a solid support of small, uniform particle size offers sharp solute resolution. When this type of separation is coupled with a sensitive, specific method of detection, such as amperometry, the system becomes a powerful tool for analysis of a very low concentration of the solute of interest. This is evidenced by the chromatographic trace shown in Fig. 7. Trace A shows the results when 25 μl of a catecholamine standard mixture containing 100 ng/ml each of NE, E, DHBA and DA were introduced

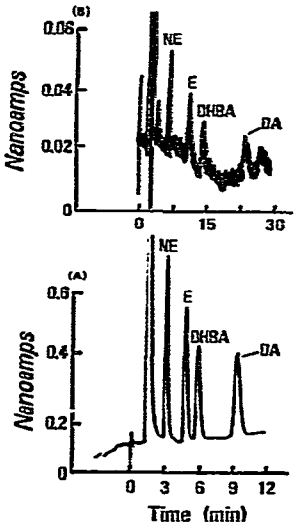


Fig. 7. Chromatographic trace generated by the electrochemical detector. A, 25 μl of a mixture containing 100 ng/ml each of NE, E, DHBA and DA; the solvent (2.0 ml/min) was 0.1 *M* NaH_2PO_4 (pH 5.5), 0.1 m*M* EDTA, 5.0 m*M* heptanesulfonate. B, 5 μl of a mixture containing 5 ng/ml each of NE, E, DHBA and DA; the same solvent as in A flowed at 0.8 ml/min.

into the system. Trace B shows the sensitivity of the method. The peaks in trace B represent 20 pg (*ca.* 100 fmoles) of each of the catecholamine standards.

To accomplish the degree of sensitivity indicated in Fig. 7, the pH and ionic strength must be controlled. Maximum detector response was observed at an ionic strength of 0.07 *M*, pH 5.5–5.8. Under these constraints, it is necessary to add an aliphatic counter ion to the separation solvent to achieve an isocratic separation that gives baseline resolution between the catecholamines of interest. The obvious advantage of the system described here is that a paired ion may be added or removed to alter resolution between peaks of interest and interfering peaks that may appear during the analysis of biological specimens.

Classical approaches to handling of biological specimens for analysis of catecholamines have incorporated a preliminary clean-up step in which the catecholamines are adsorbed onto aluminum oxide under mildly alkaline conditions, followed by elution with acid. We have found this to be necessary prior to analysis using the system described here. Our experience with the aluminum oxide clean-up step has convinced us of the need to include an internal standard with elution characteristics similar to the endogenous catecholamines. Recovery of the catecholamines from biological specimens by adsorption on aluminum oxide under strictly controlled conditions was observed to vary by up to 20%. For this reason, we feel it is essential that an internal standard be incorporated into the procedure. Inclusion of DHBA, a synthetic catecholamine, throughout the clean-up and HPLC steps provides a correction factor to account for recovery of sample during the preliminary clean-up step, a feature not available previously[1–4]. This is a significant contribution toward the faster, more sensitive method for accurate analysis of catecholamines presented here.

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