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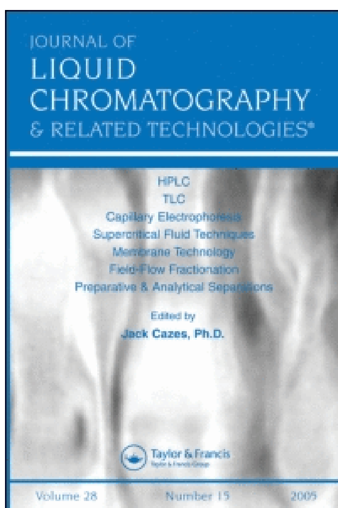

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Effect of Different Experimental Variables on the Separation of Catecholamine Metabolites by Ioninteraction RP-HPLC

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EFFECT OF DIFFERENT EXPERIMENTAL VARIABLES ON THE SEPARATION OF CATECHOLAMINE METABOLITES BY ION-INTERACTION RP-HPLC

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ABSTRACT

The liquid chromatographic separation of catecholamine acidic metabolites using tetrabutylammonium bromide as ion interaction agent is investigated. The effect of several experimental variables, such as mobile phase pH, organic modifier concentration, ion interaction agent concentration, and temperature is analyzed. The mobile phase composition that produces the best chromatographic resolution for the analysis of the compounds of interest is determined. The hydrodynamic voltammogram of the analytes showed that good selectivity can be achieved with electrochemical detection, allowing for the analysis of different compounds at different applied potentials. The Signal-to-Noise ratio is studied as a function of the applied potential.

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Based on this study, an optimum applied potential for the analysis of the catecholamine acidic metabolites is determined. A limit-of-detection of approximately 500 fmol can be calculated based on a Signal-to-Noise ratio of 3. The calibration curves of the compounds are linear over a wide concentration range.

INTRODUCTION

Catecholamines are compounds of particular importance for the regulation of the healthy functioning of the body. As neurotransmitters and information carriers, they regulate, among other responses, the adaptation of the body to stress situations. Their over- and under-production in disease states results in the breakdown of the balanced state of the body.

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Dopamine (DA), noradrenaline (NA), adrenaline (A) and 5-hydroxytryptamine (5-HT) serve as chemical neurotransmitters in the Central Nervous System (CNS). The concentration of their metabolites in the different body fluids has diagnostic importance in clinical chemistry, pharmacology and neurochemistry. For example, the concentration of vanilmandelic acid (VMA), homovanillic acid (HVA) and 3,4-dihydroxymandelic acid (DOMA) in urine is important in the assessment of hormonally active tumors of the chromaffin tissue.[1] NA is predominantly metabolized to VMA, whose urinary excretion can be used to assess the turnover of NA in the sympathetic nervous system.[2] 3,4-dihydroxy-phenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenyllactic acid (VLA), and vanillic acid (VA), in conjunction with HVA, can be used in the diagnosis of neuroblastoma.[3] In human, HVA is the main dopamine metabolite. Hence, changes in the activity of the dopaminergic systems are paralleled by changes in their HVA production.[4] Thus, the determination of catecholamines and their metabolites in the various body fluids has great importance.

Various techniques have been used to determine catecholamines and their metabolites in urine, including spectrophotometry,[5,6] paper chromatography,[1] gas chromatography,[7] and gas chromatography-mass spectrometry.[8] However, the introduction of "high performance liquid chromatography" with electrochemical detection (LC-EC) has provided the scientist with a major tool to determine the levels of these compounds in the peripheral and central nervous systems. Catecholamines and their metabolites can be detected by their oxidation at a carbon based electrode following separation on an appropriate chromatographic column. This method provides a rapid, inexpensive and very sensitive assay for those compounds in brain, plasma, and urine samples.

Moreover, lower detection limits have been informed for the amperometric, as compared to the fluorometric, detection of VMA in urine samples from neuropsychiatric disturbed and normal controls subjects.[9]

Catecholamines and their metabolites can be separated by ion exchange, reverse-phase, and secondary chemical equilibrium chromatography (ion-pair or ion-interaction chromatography).[10] However, ion exchange separations have been almost completely replaced by the reverse-phase methods.

These methods have gained great popularity[11] because of their broad scope regarding the polarity range of the solutes to be separated, their low cost regarding mobile phase components, and the possibility of separating ionic or ionizable compounds using the secondary chemical equilibrium methodology.[12]

Secondary chemical equilibrium liquid chromatography (ion-pair or ion-interaction) uses water/organic mobile phases, a buffer to control pH, and an ion pairing agent.[13] The separation is carried out in reverse-phase columns (C_8 or C_{18}).

Various models have been proposed in an attempt to explain the way in which ion interaction agents influence chromatographic separation.[14] Each of these mechanisms can define the retention behavior of samples under specific conditions; and possibly the mechanism that best describes the separation is a combination of several of the above mentioned proposals.

In our laboratory we are interested in the HPLC analysis of catecholamine acidic metabolites, e.g. VMA, iso-vanilmandelic acid (i-VMA), DOPAC, VLA, VA, and HVA in rat urine, and the correlation of their urinary concentration with stress. In this work we present a study of the effect of pH, cationic ion-interaction agent concentration, percent of organic modifier, and temperature on the chromatographic resolution of these compounds.

Hydrodynamic voltammograms (HDVs) of the above mentioned compounds, performed in flow experiments, showed either a current "plateau" in the potential range from 0.6 V to 0.78 V, or a current maximum at a potential of approximately 0.75 V. The signal decreased sharply at potentials above 0.8 V.

We also studied the signal-to-noise ratio (S/N) as a function of the working potential. Maximum S/N ratios were obtained at a potential range from 0,73 V to 0.77 V.

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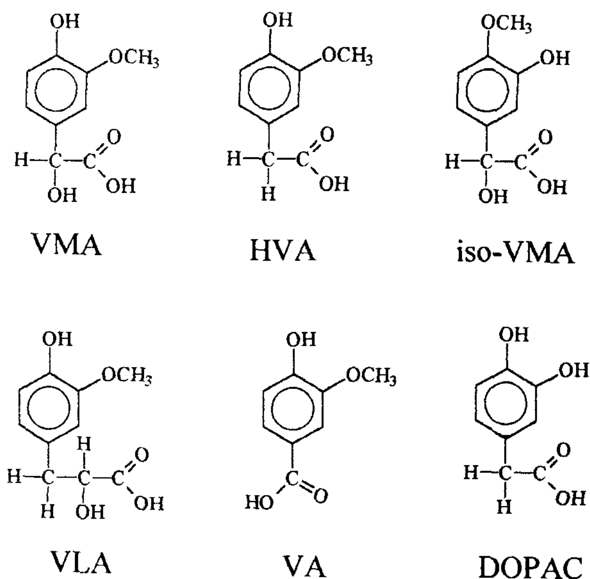

Scheme 1

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MATERIALS AND METHODS

Equipment

The chromatographic system consisted of a Gilson 307 solvent delivery module (Gilson, France), a Rheodyne 7125 injection valve with a 20 μL loop (Rheodyne, USA), and a Phenomenex IB-Sil column of 100x4.6 mm, packed with 5 μm particles (Phenomenex, USA) and coupled with a guardcolumn with the same stationary phase.

A home made potentiostat was used as an amperometric detector. The electrochemical signal was fed to a PC compatible computer equipped with Peak Simple data processing software (SRI, USA). The electrochemical flow cell for the liquid chromatographic experiments consisted of a Bioanalytical Systems model MF-1000 glassy carbon working electrode (Bioanalytical Systems, USA), a stainless steel auxiliary electrode and a Saturated Calomel Electrode (SCE) as the reference electrode. The dead volume of the thin layer flow cell was approximately 5 μL.

An Orion model 720A bench top pH/ISE meter was used in the pH measurements. The pH meter was calibrated at pH 4.00 and pH 7.00 before its use.

Reagents

All catecholamine metabolites used in this study (see Scheme 1) were analytical grade reagents (Sigma Chemical Company, USA) used without further purification. The mobile phase was prepared with acetic and phosphoric acids (Merck, Argentina) 0.05 M each, and tetrabutylammonium bromide (TBABr) (Sigma Chemical Company, USA) of the required concentration. The final pH of 4.5 was obtained by the addition of NaOH (Merck, Argentina).

Methanol (Sintorgan, Argentina) was added before fixing the pH for the chromatographic analysis. All reagents were analytical grade and the solutions were prepared with HPLC grade water obtained from a WaterPro Mobile System (Labconco, USA).

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RESULTS AND DISCUSSION

A preliminary optimization of the mobile phase composition was performed prior to the study of the effect of experimental variables on the chromatographic resolution of catecholamine metabolites. A mobile phase of approximate composition 15% methanol, pH 4, and $2x10^{-3}$ M TBABr gave capacity factors in the range 2-12 and it was taken as a starting composition.

Effect of pH

A study of the effect of pH on the chromatographic resolution was performed to further optimize the separation. (Figure 1). The chromatographic resolution was determined by the following equation:

$$R_s = \frac{(t_2 - t_1)}{(1/2)(t_{w1} + t_{w2})} \qquad (1)$$

where t_1 and t_2 are the retention times for two adjacent bands ($t_2 > t_1$), and t_{w1} and t_{w2} are their base line widths, respectively.

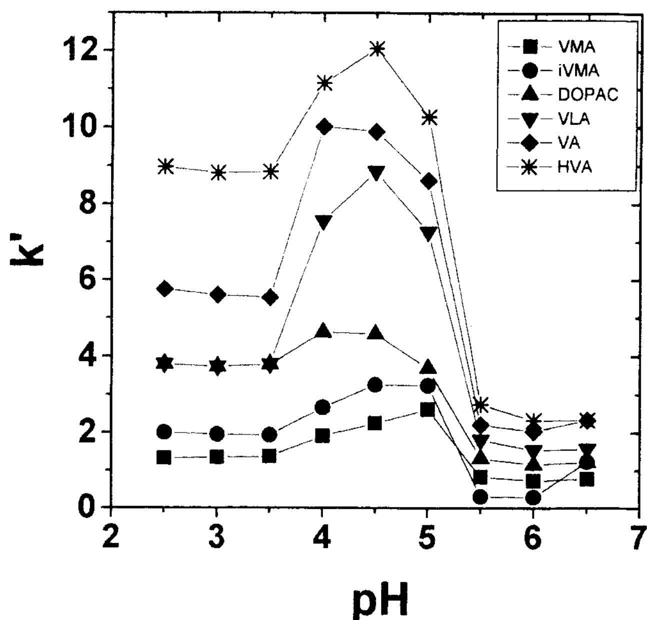

Figure 1. Effect of the mobile phase pH on the capacity factor of various catecholamine acidic metabolites. Mobile phase composition: 2 mM tetrabutylammonium bromide, 15% methanol. Temperature: 293 K, working electrode potential: 0.75 V.

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The pH range studied was from 2.5 to 6.5 because the coelution of several compounds was observed at pHs over 6.5. k's between 1 and 9 were obtained at pH 2.5. However, only five out of six compounds were detected; DOPAC and VLA coeluted at this pH. Similar results were obtained at pH 3 and 3.5 (see Figure 1). A good separation of the six analytes was obtained at the pH range between 4 and 5. Coelution of the analytes was observed at pH > 5.5, the k' range obtained at these pHs was considered inappropriate for the analysis.[13]

Selectivity in chromatography arises from differences in the partition coefficients of the analytes.[15] On the other side, the resolution factor (R_S) of two adjacent bands takes into account both the difference in retention and the average peak width of the peaks. It has been calculated that a resolution factor of 1 indicates a fairly good separation of the two bands, and that only 2% of one band overlaps with the other.[16] In our case, the selectivity and resolution obtained at pH 4.5 for the closest eluting pair of peaks (VMA and i-VMA) were approximately 1.5 and 3.2, respectively.

A selectivity of 1.4 and a resolution of 2.1 were obtained at pH 4 for the same pair of compounds. Thus, pH 4.5 was considered the optimum value for the analysis of the catecholamine metabolites.

Maxima in k' vs. pH plots were observed in our case at a pH range from 4 to 5. Maxima in the k' vs. pH plots were also observed by other authors in ion interaction chromatography.[17,18] Gennaro et al.[17] studied the role of the mobile phase pH on the separation of amines, diamines, amides, and other functionalities, using o-phosphate and octylammonium as ion-pairing agent. They proposed that the higher capacity of the stationary phase could be found at low pH values, and that retention for weak acids was maximum at pH values close to pK_a. Kong et al.[18] studied the combined effects of pH and surface-active ion concentration (octylamine hydrochloride) on the separation of various weak acids and bases. They explained the foldover of k' values considering that the concentration of octylammonium ion decreased at high pH. In our case, the maxima observed in the k' vs. pH plots may be explained taking into account two facts: i- the weak acid functionalities of the analytes are completely in the anionic form at pH values over 5, and ii- the relatively small size of the tetra-butyl ammonium ion. It is possible that the hydrophobicity of the tetra-butyl ammonium ion is not enough to maintain the retention of the analytes. Also, it should be considered that retention in reversed phase ion-interaction chromatography in the pH range close to the pK_a of the analytes involves a complex mechanism accounting for acid-base as well as ion-interaction equilibria.

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Effect of Methanol Concentration

The dependence of the capacity factors (k') of the analytes was studied as a function of the percentage of organic modifier (Figure 2). Retention of compounds on the C_{18} reverse-phase column in the "ion-interaction" chromatographic mode is dependent upon a variety of forces.[19] The analytes interact with the mobile and stationary phases, and with the "ion-interaction" reagent either dissolved in the mobile phase or adsorbed in the stationary phase. The total interaction is dependent on Coulombic, and hydrophobic/hydrophilic forces.

In ion-interaction chromatography, as well as in reversed phase liquid chromatography (RPLC), the lower the polarity of the mobile phase the higher its eluting properties. The degree of reduction of a solute's retention is related to the volume fraction (Φ) of the organic modifier in the mobile phase by Equation 2:

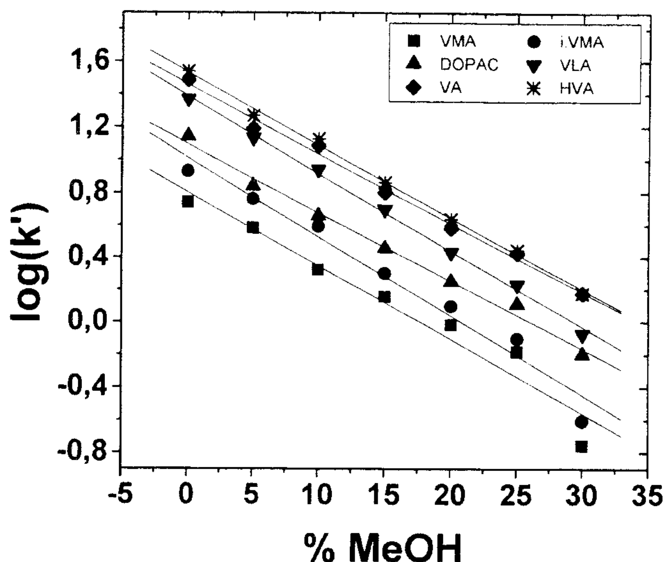

Figure 2. Effect of the percentage of methanol on the capacity factors of various catecholamine acidic metabolites. Mobile phase compostion: 2 mM tetrabutylammonium bromide, pH 4.5. Temperature: 293 K, working electrode potential: 0.75 V.

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$$\log(k') = \log(k')_w - S\Phi \qquad (2)$$

where $(k')_w$ is the capacity factor of the solute with a completely aqueous mobile phase and S is the slope coefficient which defines a solvent strength parameter. Good correlation coefficients were obtained ($r \geq 0.99$) for the logarithmic plots in Figure 2. The slope coefficient was approximately the same for the six solutes analyzed. It is possible that in ion-interaction chromatography, as well as in RPLC with hydroorganic mobile phases, the S values of different compounds depend on their molecular weights and contact area with the stationary phase.[20,21] The similarities of the chemical structures between the solutes analyzed (see Scheme 1) should also be considered.

Capacity factors in the range from 1 to 4.5 were observed at a methanol percentage of 20%. A poor resolution of the analytes was obtained at this methanol percentage. Conversely, capacity factors in the range from 1.5 to 7.6, and from 2.1 to 13.5 were observed at methanol concentrations of 15% and

10%, respectively. Larger capacity factors (longer analysis time) were obtained at lower methanol concentrations. Thus, a methanol concentration of 15% was used for the subsequent experiments.

Effect of the Ion-Interaction Reagent Concentration

The effect of tetrabutyl ammonium ion concentration on the chromatographic resolution was studied at pH 6.5 and 4.5, and 15% methanol in both experiments (Figure 3, A and B). The ion-pairing agent concentration range studied was from 0 to 50 mM. pH 6.5 was selected because all the catecholamine metabolites are mostly ionized at this pH value. Also, pH 4.5 was used because of the good resolution observed at this pH value.

The capacity factor of the analytes increased with the concentration of the ion-interaction reagent at pH 6.5 (Figure 3, A). The k' values obtained at this pH and at the TBABr concentration range studied were between 1 and 5.5, making quantitation difficult. Also, HVA and VA coelute at TBABr concentrations smaller than 5 mM, and i-VMA and DOPAC coeluted in the whole TBABr concentration range studied. Thus, only four or five (instead of six) chromatographic peaks were detected.

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Capacity factors also increased with the TBABr concentration at pH 4.5 (Figure 3, B). The k' values of VMA, i-VMA, and DOPAC increased with TBABr in the concentration range studied. The k' value for VLA increased between 1 mM and 5 mM, and remained almost constant thereafter. The k' values for VA and HVA slightly decreased at the concentration range between 1 mM and 20 mM, and increased at a TBABr concentration of 50 mM. The k' values obtained at this pH were higher than those obtained at pH 6.5. Good chromatographic resolution of the analytes was observed at all the TBABr concentrations studied, but 2 mM TBABr gave the best results and was considered as the optimum ion pairing agent concentration. The results obtained with this study are rather limited and no definitive conclusion can be drawn. However, they show the complexity of the retention mechanism involved in the liquid chromatographic separation of the analytes, where ion pair formation and acid-base equilibria must be taken into account.

A preliminary study of the effect of the ion pairing agent alkyl chain length was performed using cetyltrimethylammonium chloride (CTAC). CTAC concentrations ranged from 0.05 mM to 0.2 mM. The results showed a decrease of k' values with incremental CTAC concentrations. This behavior is characteristic of Micellar Liquid Chromatography (MLC), in which an increase

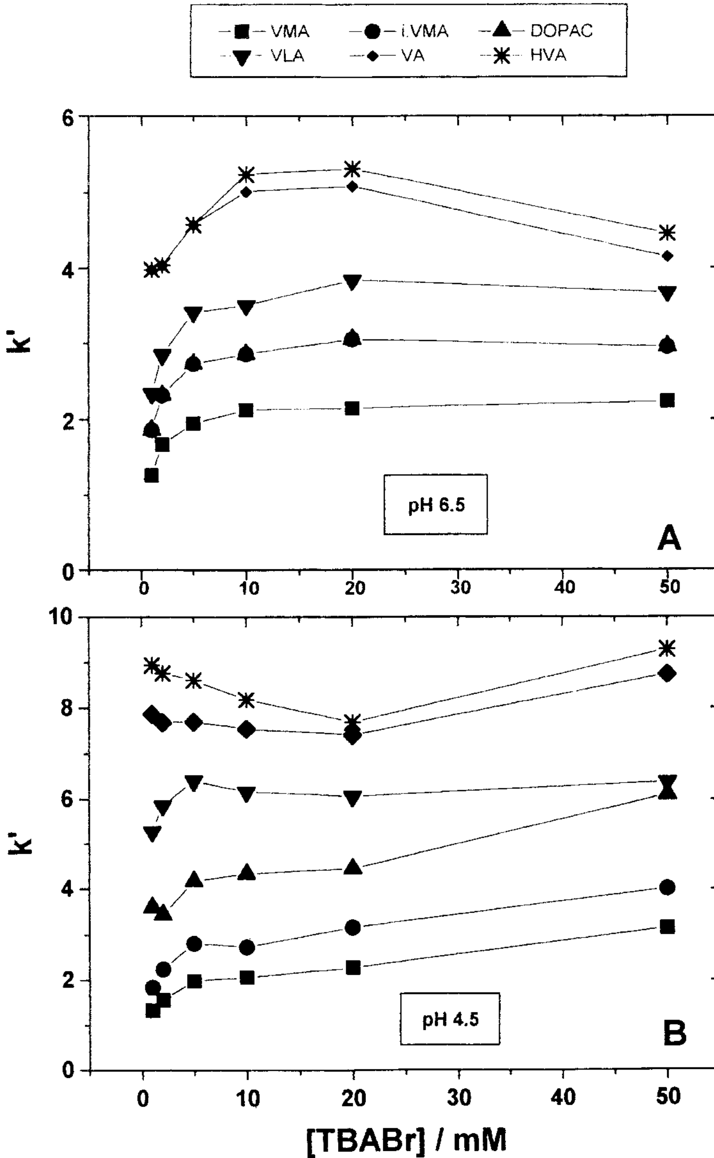

Figure 3. Effect of the concentration of tetrabutylammonim bromide on the capacity factors of various catecholamine acidic metabolites. Mobile phase composition: 15% methanol, pH 4.5. Temperature: 293 K, working electrode potential: 0.75 V.

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of surfactant concentration leads to a decrease in retention and an increase in elution strength.[20] Although the critical micellar concentration of CTAC in water is approximately 1.4×10^{-3} M,[22] much lower values have been informed for CTAC solutions containing phenolic compounds.[23] Lower values of the critical micellar concentration were also found in solutions containing hexadecyltrimethylammonium bromide and anionic substrates.[24] Thus, it is possible that our experiments were performed at a CTAC concentration range where micelles aggregates are formed and the results obtained might correspond to MLC instead of reversed phase ion-interaction liquid chromatography. More experimentation is being conducted in our laboratory to further understand the effect of the ion-interaction agent alkyl chain length on the k' value.

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Effect of Temperature

The effect of temperature on the secondary chemical equilibrium was investigated at a temperature range from 303 K to 333 K. Typical results are shown in Figure 4 as Van't Hoff plots. The elution time for the different compounds decreased with incremental temperature. Good correlation ($r > 0.99$) between ln(k') and 1/T was observed for all the analytes studied. This behavior was predicted for both reversed-phase ion-interaction liquid chromatography[25] and micellar liquid chromatography.[26]

Stranahan and Deming[25] proposed a thermodynamic model for reverse-phase ion-interaction liquid chromatography. They suggested that the distribution constant for a component I (K_i) in a dilute solution is given by:

$$K_i = \exp\left(\ln\frac{V_l^o}{V_a^o} + \ln\gamma_{il} + \frac{(\sigma - \sigma_i^o)s_i}{RT} - \frac{\omega_{ij}\chi_{ja}}{RT} \right) \qquad (3)$$

where V_a^0 and V_l^0 are the average molar volume of the adsorbed and liquid phases, respectively, γ_{il} is the activity coefficient of I in the bulk liquid phase; s is the interfacial tension of the system and σ_i^0 is the interfacial tension of pure I in equilibrium with the solid adsorbent; ω_{ij} is the energy of interaction of the i and j species; and c_{ja} is the molar fraction of j in the adsorbed phase. A linear relationship between ln(k') and 1/T is expected from equation (3) if s, σ_i^0, ω_{ij}, and χ_{ja} remain constant at the temperature range under study. Tomasella and Cline Love studied the thermodynamic properties in Micellar Liquid Chromatography based on the three-phase equilibrium model.[26] They proposed

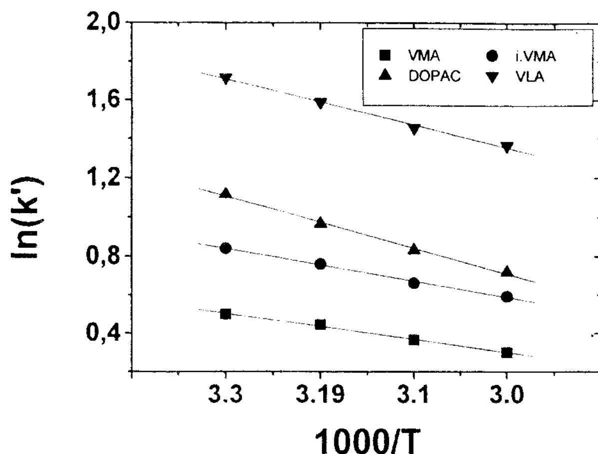

Figure 4. Effect of the temperature on the capacity factors of various catecholamine acidic metabolites. Mobile phase compostion: 2 mM tetrabutylammonium bromide, pH 4.5, 15% methanol. Working electrode potential: 0.75 V.

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that the transfer of the solute from the bulk aqueous phase to the stationary phase should be described by the following Van't Hoff equation:

$$\ln k'(1+K_2[M_m]) = \frac{-\Delta H_1^o}{RT} + \frac{\Delta S_1^o}{R} + \ln\phi[L_s] \tag{4}$$

where K_2 is the equilibrium constant for the distribution of the solute between the bulk solvent and the micelle in the flowing solution, $[M_m]$ is the concentration of surfactant in the micelle in the mobile phase, ϕ is the chromatographic phase ratio and L_s the stationary phase sites. If ΔH_i^0 and ΔS_i^0 are independent of temperature, then the resulting Van't Hoff plots will be linear. Moreover, Melander et al. applied the Van't Hoff equation to reverse-phase chromatography:[27]

$$\ln k' = \frac{-\Delta H^o}{RT} + \frac{\Delta S^o}{R} + \ln\phi \tag{5}$$

where the chromatographic retention is measured by the capacity factor k', as related to the equilibrium constant given as k' = ϕK. Once again, a linear relationship between ln k' and 1/T will be observed if both ΔH^o and ΔS^o are independent of temperature.

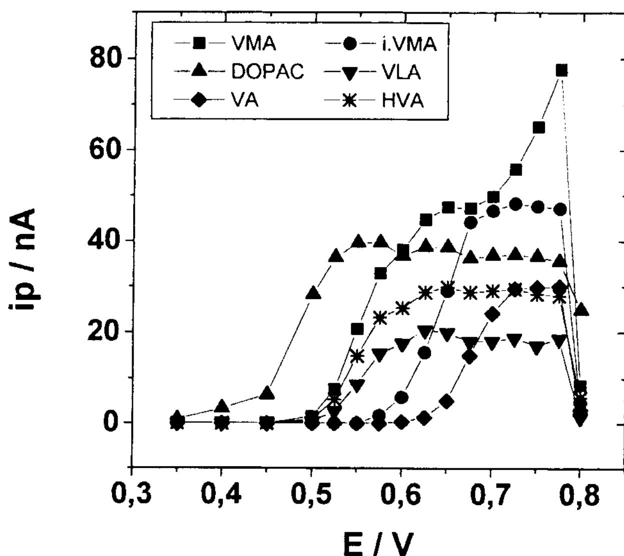

Figure 5. Hydrodynamic voltammogram of various catecholamine acidic metabolites. Mobile phase composition: 2 mM tetrabutylammonium bromide, pH 4.5, 15% methanol. Temperature 293 K.

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The linear relationship between ln k' and 1/T obtained in our study is in agreement with any of the above mentioned models, and no further conclusion can be drawn from this study. A stronger dependence of k' upon temperature would be expected for an ion-exchange as well as for an ion-pair mechanisms.

The results obtained reflect the complexity of the separation mechanism. As expected, the increase in temperature improved the column efficiency and pressure drop.

Hydrodynamic Voltammograms and S/N Analysis

The hydrodynamic voltammogram and the S/N ratio for the oxidation of the catecholamine metabolites were studied to analyze the electrode's selectivity and to determine the "best" working potential for the liquid chromatographic analysis. Typical responses are shown in Figure 5 and Figure 6. The initial working potential was 0.35 V.

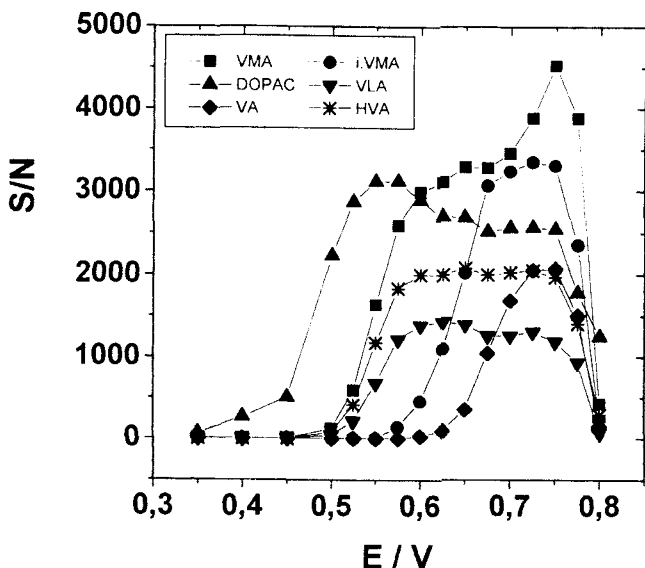

Figure 6. Signal-to-noise ratio (S/N) analysis of various catecholamine acidic metabolites at different working electrode potentials. Mobile phase composition: 2 mM tetrabutylammonium bromide, pH 4.5, 15% methanol 293 K'.

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After obtaining a stable, drift free baseline at each set potential, the baseline noise was measured at the detector's highest sensitivity, and then five separate injections of the catecholamine metabolites mixture were analyzed. The working potential was then increased by increments of c.a. 0.025 V, and the analysis repeated.

Each catecholamine metabolite behaved in a different way in the hydrodynamic voltammetric study (see Figure 5). Only DOPAC showed appreciable oxidative currents at potentials below 0.50 V. A current peak was obtained at a potential range from 0.55 V to 0.58 V, then the current remained essentially constant at a potential range from 0.60 V to 0.78 V, and sharply decreased at potentials above 0.80 V. VMA, HVA and VLA are oxidized at potentials above 0.50 V. The oxidative current of VMA increased in the potential range from 0.50 V to 0.63 V, reached a plateau at a potential range from 0.63 V to 0.70 V, a current peak was obtained at approximately 0.78 V, and finally the current decreased at potentials above 0.80 V. The oxidative current behavior of HVA and VLA was similar, it increased at a potential range from 0.50 V to 0.63 V and then remained constant up to 0.78 V, finally it

decreased at potentials above 0.80 V. Oxidation of i-VMA began at approximately 0.58 V, a current plateau was obtained at a potential range from 0.65 V to 0.78 V, and the oxidative current decreased above 0.80 V. Finally, VA was only oxidized at potentials above 0.63 V. Its oxidative current increased between 0.63 V and 0.70 V, reached a plateau at a potential range from 0.73 V to 0.78 V, and sharply decreased at potentials above 0.80 V.

The above mentioned hydrodynamic voltammetric behavior of the catecholamine metabolites is indicative of the selectivity that can be achieved with the electrochemical detector. Only DOPAC can be detected by setting the working electrode potential at approximately 0.50 V. DOPAC, VMA, HVA, and VLA can be detected at a working electrode potential of approximately 0.58 V. Finally, all six compounds are detected at a working electrode potential of approximately 0.75 V. This detector's selectivity in conjunction with the resolution power of the chromatographic column make LC-EC the technique of choice for the analysis of catecholamine acidic metabolites.

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The signal-to-noise ratio was analyzed in the potential region from 0.35 V to 0.80 V in order to determine the "best" working potential for the analysis of all six compounds (Figure 6). The trend of the S/N ratio curves for the different catecholamine metabolites closely resembled that of the HDV. Only the S/N ratio of DOPAC was appreciable below 0.50 V. DOPAC, VMA, HVA, and VLA showed high S/N at a potential range from 0.53 V to 0.60 V. Finally, all six compounds showed high S/N ratios at a potential range from 0.73 V to 0.75 V. Maxima in the S/N ratio values occurred because in the potential range from 0.50 V to 0.75 V, the signal increased while the background noise remained nearly constant. On the other hand, the signal either remained constant or decreaseed in the potential range from 0.75 V to 0.80 V, while the background noise increased at the same potential range lowering the S/N ratio. According to K. Stulik et al.[28] the working electrode potential giving the highest S/N ratio should be taken as the "optimum" potential for the LC-EC analysis. Thus, a working electrode potential of 0.75 V was selected as the "optimum" for the chromatographic analysis.

Calibration Curves

The calibration plots of the catecholamine acidic metabolites were linear over the concentration range studied (ca. from $3.0\text{x}10^{-7}$ M to $1.0\text{x}10^{-5}$ M). Good correlation coefficients were obtained for all the analytes in this study ($r > 0.99$). Taking into account the linearity of the calibration plots, and the S/N

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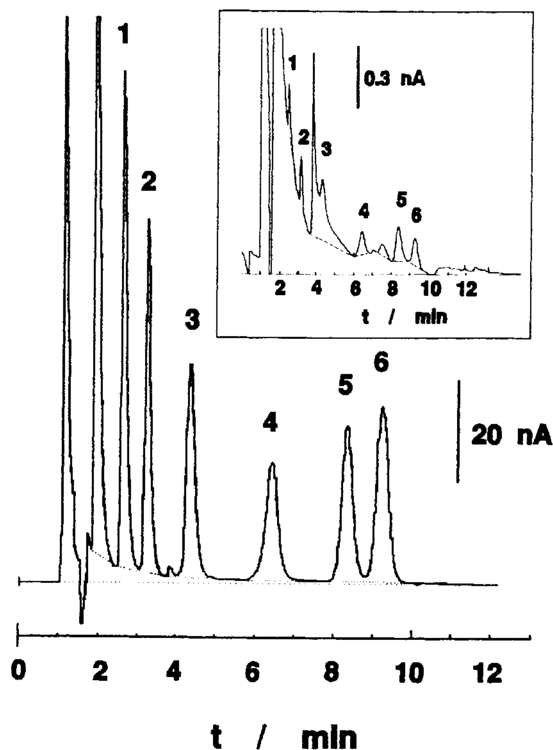

Figure 7. Typical chromatogram attained by applying the working conditions obtained in this study. Injection of a 20 μL mixture of (1) VMA, (2) i-VMA, (3) DOPAC, (4) VLA, (5) VA, (6) HVA; 1.0×10^{-5} M each. Mobile phase composition: 2 mM tetrabutylammonium bromide, pH 4.5, 15% methanol. Flow rate: 1.0 mL/min. Temperature: 293, working electrode potential: 0.75 V. Inset: high sensitive analysis of catecholamine acidic metabolites. Same mixture and conditions as before, but at a concentration of 3×10^{-9} M each metabolite.

ratio analysis, a limit of detection of approximately 500 fmol VMA (20 μL injection of a 2.5×10^{-8} M solution) can be extrapolated for a S/N ratio of 3. A typical chromatogram of a mixture of the analytes is shown in Figure 7. The effectiveness of the chromatographic separation is indicated by the high resolution obtained at a flow rate of 1.0 mL/min. The inset in Figure 7 shows the high sensitivity achieved with the LC-EC method using the "optimum" experimental conditions obtained in this study. These results show the usefulness of the LC-EC method for the analysis of catecholamine acidic metabolites.

CONCLUSIONS

The analysis of six catecholamine acidic metabolites was performed by ion-interaction reverse-phase liquid chromatography using tetrabutylammonium bromide as ion-interaction agent. The effect of various experimental parameters on the chromatographic resolution was assessed. The best separation was obtained with a mobile phase composition of 15% methanol, pH 4.5, and 2 mM tetrabutylammonium bromide, at a flow rate of 1.0 mL/min. Maxima were observed in the k' vs. pH plots, and pH 4.5 gave the highest chromatographic resolution. No major effects were observed in the resolution vs. temperature study. The calibration plots were linear, and high sensitivity was achieved when the "best" experimental conditions obtained in this study were applied.

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