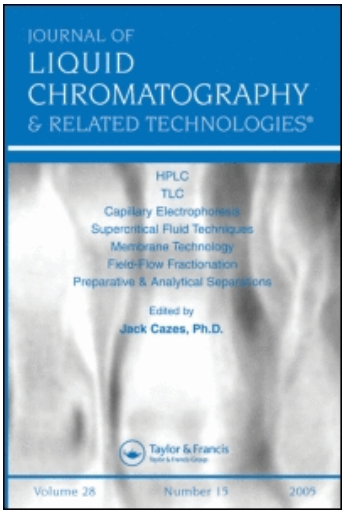

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CAPILLARY ZONE ELECTROPHORETIC SEPARATION OF HOMOVANILLIC AND VANILLYLMANDELIC ACIDS

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ABSTRACT

Capillary zone electrophoresis with untreated fused-silica capillaries and acetate buffer was evaluated for the separation and analysis of catecholamine metabolites. Homovanillic acid and vanillylmandelic acid, which are excreted in abnormally elevated levels in the urine of patients with neuroblastoma, were separated from other possible catecholamine metabolities, using a 200 mM acetate buffer at pH = 4.10. The high buffer concentration was necessary to minimize the peak tailing resulting from analyte-capillary wall interactions. The concentration detection limits of the injected sample at 214 nm were 36 mg/L for vanillylmandelic acid and 64 mg/L for homovanillic acid.

INTRODUCTION

Homovanillic (4-hydroxy-3-methoxyphenylacetic) acid (HVA) and vanillylmandelic (4-hydroxy-3-methoxymandelic) acid (VMA) are catabolic products of catecholamines that are excreted in abnormally elevated amounts in the urine of patients with neuroblastoma (1,2), pheochromocytoma (3), psychosis (4) and hepatic encephalopathy (5). In the case of neuroblastoma, detection of these metabolites is critical. Infants diagnosed at an early stage have a much higher

survival rate than children above the age of 1 year in whom neuroblastoma is diagnosed at an advanced stage (6).

The simultaneous quantification of VMA and HVA has become a routine mass screening test for diagnosis and follow-up of children with neuroblastoma (6,7-10). Numerous methods have been advanced for the extraction, separation and quantification of these acids (6-12). The high pressure liquid chromatography (HPLC) method introduced by Takeda and co-workers (12) is the most widely used for mass screening purposes.

The recent development of capillary zone electrophoresis (CZE) has not only presented researchers with the potential for achieving rapid high-resolution of macromolecules, but it has also opened the way for the application of electrophoresis to the separation of small molecules such as inorganic and organic ions and ionizable molecules (13-16). Although CZE is often compared to high pressure liquid chromatography (HPLC), yet as a separation technique it has its own specific features. With CZE, separation is achieved by differential migration of charged solutes in a semi-conducting buffer under the influence of an electric field gradient (13-17). Thus, CZE provides a valuable technique that is orthogonal to HPLC and that may be used as alternate or complementary technique for further characterization of analytes (17).

The objective of this work is to investigate the potential of CZE for the separation of catecholamine metabolites and for the analysis of VMA and HVA in infant urine samples.

EXPERIMENTAL

A Beckman CZE system (Model P/ACE) equipped with a UV detector, an automatic injector, a column cartridge (50 cm x 75 μm i.d. surrounded by coolant), and a System Gold data station was used in this study. VMA, HVA and the other catecholamine metabolites were purchased from Aldrich Chemical Company, Milwaukee, WI. All runs were carried out at 25°C. Injections were made using the pressure mode for 2 seconds at 0.5 psi. Buffer solutions were degassed and filtered through 0.2 μm Nylon 66 filters prior to use.

A 10 mL sample of urine was acidified to a pH of <1 with 5 M HCl, 1.5 g of NaCl was added and the solution was first extracted with 6 mL of ethyl acetate then with 6 mL of diethyl ether. The combined organic layer was evaporated under nitrogen until it dried and redissolved in 100 μL of water, prior to CZE analysis.

RESULTS AND DISCUSSION

The separation of VMA and HVA was first investigated using a phosphate buffer at neutral and basic pH. At pH = 7, the two acids are ionized and negatively charged, however, it appears that the difference in their charge-to-size ratio is not significant enough to affect their separation in free-zone capillary electrophoresis. Using a 50 mM phosphate buffer at an applied voltage of 15 kV, the two solutes co-eluted as a rather broad peak with a migration time of 18 minutes (data not shown). As the pH of the phosphate buffer was increased to 8.5, the migration time increased and the peak shape deteriorated without any appreciable improvement in separation. This led to the conclusion that neutral and basic buffers are inappropriate for the separation of these two acidic compounds. In an effort to improve the CZE separation of catecholamines and their metabolites, Kaneta et al. chose to control the electrophoretic mobility by complex formation with borate ion, and to reverse the electroosmotic flow by modifying the buffer with a cationic surfactant having a long alkyl chain (18). Under these conditions the migration time of VMA (Ref. 18, Fig. 4) exceeded 50 minutes, which renders this approach inappropriate for mass screening programs where short analysis time is an important attribute. To avoid the need for elaborate procedures that require buffer modifications with complexing agents, surfactants or both, it was decided to see if the use of low pH buffers, near the dissociation constants of the solutes of interest would accomplish the separation on the basis of subtle differences in charge-to-size ratios. As the pH is decreased the ionization of both acids, VMA and HVA, is suppressed, however, since VMA is a weaker acid (because of the presence of the electron-releasing OH^-

group on the α carbon located next to the carboxylate ion), its negative charge is diminished to a larger extent in comparison to HVA. Hence, VMA migrates more rapidly and moves closer to the neutral marker while HVA migrates relatively slower because of its larger negative charge.

First, a 50 mM phosphate buffer in the pH range 2.5-4.5 was used for separation and analysis. VMA and HVA were widely separated in this buffer, however, the analysis time was long and the slower migrating HVA peak was broad. Attempts at improving peak shape by varying the pH and ionic strength of the buffer were not successful in eliminating solute-capillary wall interactions which are the main cause of peak broadening and tailing. Increasing the ionic strength of the buffer by increasing the buffer concentration and/or addition of ionic salts can, in some cases, minimize solute-capillary wall interactions, however, phosphate buffers generate excessively high currents under such extreme conditions.

Acetate, on the other hand, is a much "cooler" buffer (19), thus, the use of high acetate buffer concentration is feasible. For example, Figure 1 shows the separation of VMA and HVA using a 200 mM acetate buffer at pH = 3.75. The current was limited to 52.5 μA at an applied voltage of 25 kV. Under these conditions, VMA and HVA were widely separated and the VMA peak was symmetrical and sharp. It is to be noted that the current generated by a 200 mM acetate buffer at 25 kV increases exponentially with increasing pH in the pH range studied, as shown in Table I, which also lists the migration times of VMA, HVA and the neutral marker mesityl oxide. Electroosmotic flow generally increases with increasing pH. However, the data presented in Table I indicates that, for this particular system, the electroosmotic flow (as measured by the migration time of mesityl oxide), decreases with increasing pH. This is attributed mainly to the accelerated ionization of acetic acid as the pH approaches its pK value. The resulting increase in the ionic strength of the buffer decreases the zeta potential and consequently slows down the electroosmotic flow.

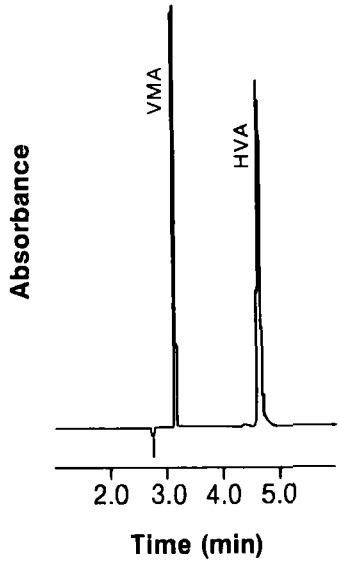

FIGURE 1. Electropherogram of the separation of VMA and HVA. Instrument: Beckman Model P/ACE System 2000; Column: 50 cm x 75 μm fused silica; Injection: pressure mode for 2 sec. at 0.5 psi; Buffer: 200 mM acetate; pH = 3.75; Applied Voltage: 25 kV; Current: 52.5 μA; Detection: UV 214 nm.

Table I: Migration times and measured current at different pH values using 200 mM acetate buffer at an operating voltage of 25 kV

	Migration time (min)			
pH	Mesityl oxide	VMA	HVA	Current μA
3.15	2.21	2.31	2.70	21.4
3.55	2.52	2.67	2.53	34.1
3.75	2.78	3.14	4.65	52.5
4.10	3.88	5.67	11.81	117

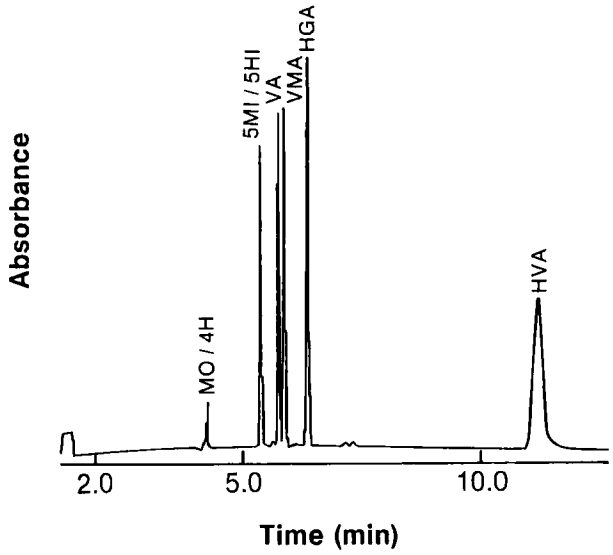

FIGURE 2. Electropherogram of a standard mixture of catecholamine metabolites. Buffer: 200 mM acetate; pH = 4.10; Applied Voltage: 25 kV; Current: 115 μA; Other Experimental Parameters: As in Figure 1. Solutes: MO = Mesityl oxide; 4H = 4-hydroxy-3-methoxyphenyl-glycol; 5 Mi = 5-methoxyindole-3-acetic acid; 5 Hi = 5-hydroxyindole-3-acetic acid; VA = 4-hydroxy-3-methoxy benzoic acid; VMA = vanillylmandelic acid; HGA = homogentisic acid; and HVA = homovanillic acid.

As shown in Table I and Figure 1, VMA and HVA are easily separated in less than 5 minutes, however, under these conditions both peaks will overlap with other metabolites present in a complex sample such as urine. Improved separation from other possible catecholamine metabolites is accomplished at pH 4.10 as shown in Figure 2. Increasing the pH beyond 4.10 results in improved separation at the expense of increased analysis time and excessive Joule heating.

Figure 3A shows an electropherogram of an untreated infant urine sample and Figure 3B shows an electropherogram of the same sample spiked with 0.5 mg/mL each of VMA and HVA. The separation and quantification of VMA and HVA are feasible under these conditions, since several infant urine samples were tested and there were no major peaks that interfered with the analysis.

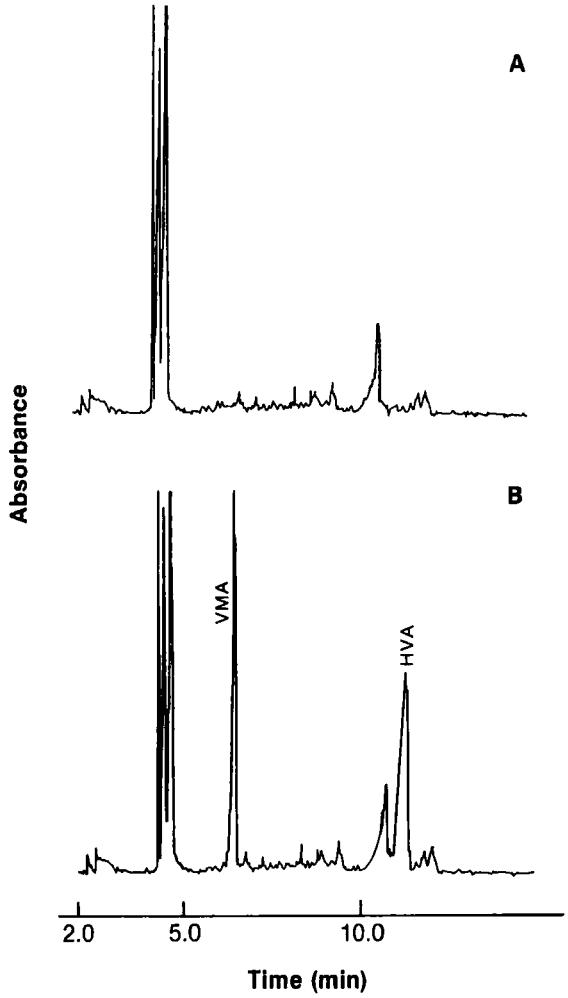

FIGURE 3. Electropherograms of normal infant urine: (A) Untreated normal infant urine, (B) Normal infant urine spiked with vanillylmandelic acid and homovanillic acid. Experimental Conditions: As in Figure 2.

A semi-qualitative comparison of VMA and HVA molar concentrations to peak heights was conducted and it was determined that the lower concentration detection limit at 214 nm was 36 mg/L for VMA and 64 mg/L for HVA. Since the concentration of VMA and HVA in the urine of normal infants is less than 5 mg/L (11), a concentration step was necessary before samples could be analyzed by CZE using UV detection at 214 nm. Several extraction procedures were reported in the literature (6-12). In this work, a 5 mg/L sample of both VMA and HVA was easily detected after concentration using the procedure reported by Tuchman et al. (9) as described in the Experimental.

In conclusion, the CZE procedure described here is simple, rapid and highly suitable for mass screening of both HVA and VMA.

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