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Short communication

Capillary zone electrophoresis separation of tryptophan and its metabolites, including quinolinic acid

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Abstract

Capillary zone electrophoresis with UV absorbance detection was used to separate tryptophan and ten of its metabolites. Run buffers of pH 4.0–10.0 were evaluated for their effect on resolution; a pH 9.6 buffer was found to give optimum separation of all components. Ethylenediaminetetraacetic acid (EDTA), which prevents complexation of some analytes with polyvalent cations, was included in the run buffer to insure good peak shape and reproducible mobilities. The resulting method was used to detect the presence of quinolinic acid in a urine sample. ©1997 Elsevier Science B.V.

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1. Introduction

The metabolites of the common amino acid tryptophan (Trp) play a wide variety of roles in the human body. Trp metabolism occurs by a number of pathways. One of the most important, the kynurenine pathway, involves cleavage of the indole ring to generate a series of mono- and bicyclic compounds (Fig. 1). Some of the members of this pathway are quite important for proper physiological function. For example, nicotinic acid (NA) supplies the key functionality of nicotinamide adenine dinucleotide (NAD), a central cofactor in intermediary metabolism. In human lenses, 3-hydroxykynurenine (3KN) acts as a UV filter; degradation of this substance may lead to formation of cataracts [1]. Kynurenic acid (KA) acts as an antagonist of a variety of excitatory amino acid receptors in the central nervous system of

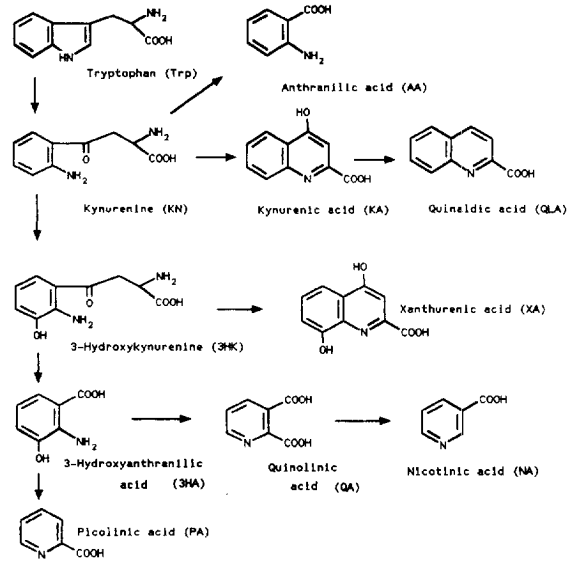

Fig. 1. Structures, names and abbreviations of tryptophan and metabolites of the kynurenine pathway.

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mammals [2]. Quinolinic acid (QA), like KA, is neuroactive, but is an agonist of N-methyl-D-aspartate receptors [3]. Changes in the levels of these Trp metabolites are associated with a variety of disease states. For example, increased QA concentration is detected in the cerebrospinal fluid of patients infected with the human immunodeficiency virus (HIV-1) [4]. Also, the amount of QA excreted in urine is one of the criteria for vitamin B6 deficiency [5].

Thus, there exists a need for analytical methods that permit the separation and quantitation of these compounds. Procedures utilizing gas chromatography [6,7] or liquid chromatography (LC) [8,9] have been developed, but are restricted to the analysis of single analytes or just a few metabolites of this pathway. A more recent method utilizes liquid chromatography with gradient elution to achieve separation of Trp and its metabolites [10], including QA, an important metabolite not generally included in other separation studies. However, no application of this method to the analysis of real samples, such as serum or urine, was given.

Capillary zone electrophoresis (CZE) is an instrumental method that is capable of highly efficient separations of aqueous ionic species. Trp and its metabolites possess multiple ionization sites and are thus prime candidates for separation by CZE. Indeed, CZE is particularly appropriate for the analysis of small negatively charged species such as QA, which may be expected to have relatively long migration times and to be well resolved from other analytes. This capability is particularly important in the analysis of biological samples, such as serum, urine or the extracellular fluid of the brain, since these types of samples will often contain larger and/or less negatively charged compounds such as peptides, proteins, carbohydrates and nucleic acids, which migrate quickly in CZE. This behavior is in contrast to that seen with reversed-phase LC, where QA elutes quickly from the column [10] and may co-elute with hydrophilic components of the sample matrix.

In this paper we report the use of CZE with UV absorbance detection for the separation of Trp and ten of its metabolites. The effects of run buffers of various pH on separation efficiency and resolution were determined. The final method was then applied to the determination of QA in a urine sample.

2. Experimental

2.1. Apparatus

Two capillary electrophoresis (CE) systems were used in the studies. An ISCO Model 3140 (ISCO, Lincoln, NE, USA) was employed for the study of the effect of buffer pH on the resolution of the metabolites. Other studies, including analysis of a urine sample, were performed using a modular unit constructed in-house. In this system, electrophoresis was driven by an in-house constructed power supply (University of Kansas Instrument Design Lab) connected to Pt electrodes. The anodic high-voltage end of the capillary was isolated in a Plexiglas box fitted with an interlock for operator safety. The system utilized an ISCO CV4 capillary electrophoresis detector. All experiments were performed at ambient temperature (ca. 22–25°C), and samples were introduced by a laboratory-built pressure injection system.

2.2. Reagents

Tryptophan and its metabolites were purchased from Sigma (St. Louis, MO, USA). All solutions were prepared in NANOpure water (Sybron-Barnstead, Boston, MA, USA). Dowex 1X8-400 ion-exchange resin (Aldrich Chemical Co., Milwaukee, WI, USA) was converted to the formate form by washing 45 g of resin in a glass column with 400 ml of 6 *M* HCl, 400 ml of water, 1600 ml of 3 *M* sodium formate, 400 ml of water, 800 ml of 3 *M* formic acid and 1200 ml of water.

2.3. Capillary electrophoresis

The ISCO Model 3140 system employed a 100 cm (75 cm to window)×50 μm I.D., 360 μm O.D. column operated at 30 kV. Samples were injected for 20 kPa/s. The column was washed as follows between runs: 2.0 min, 100 m*M* NaOH; 2.0 min, 5 m*M* EDTA; 2.0 min, water; 5.0 min, buffer.

Conditions for the in-house system were the same except that a 83 cm (57 cm to window) column

operated at 25 kV was used and samples were injected for 145 kPa/s (or 414 kPa/s for analysis of urine). Washing of the column was also identical except that the EDTA wash was omitted since EDTA was included in the run buffer.

Run buffers used in the study of buffer pH on separation were 20 m*M* of the following: sodium acetate, pH 4.0 and 5.0; sodium phosphate, pH 6.0, 7.0 and 8.0; and sodium borate, pH 9.0 and 10.0.

2.4. Urine sample preparation

The procedure described is a modification of a previously published method [11]. A 1.5 cm (I.D.) column was filled to a packed height of 5.2 cm. The resin-filled column was washed with 34 ml of 3 *M* sodium formate in 3 *M* formic acid and then 103 ml of water. 30 ml of fresh human urine from a normal subject was then applied and the column was eluted successively with 10 ml of water, 50 ml of 0.08 *M* HCl and 98 ml of 0.15 *M* HCl. Flow-rates for all elutions were approximately 4 s/drop. The 0.15 *M* HCl fraction was evaporated to dryness at 50°C. The residue was re-dissolved in 12 ml of water and evaporated again to aid in removal of residual HCl. The final residue was dissolved in 0.4 ml of water. A 0.050-ml aliquot of the solution was removed and mixed with 0.120 ml of 0.10 *M* NaOH to neutralize the remaining HCl.

3. Results and discussion

3.1. Effect of buffer pH on CZE separation

Trp and its metabolites contain ionizable carboxyl and amine groups. Phenolic groups of some of the compounds provide additional sites for ionization. Since the extent of ionization of an analyte and, thus, its electrophoretic mobility are functions of the pH of the run buffer, a study of the effect of buffer pH on the CZE separation was made.

A mixture of Trp and its metabolites was combined with the internal standard 2-pyridylacetic acid and the neutral marker acetophenone. This mixture was analyzed by multiple CZE runs using buffers from pH 4.0 to 10.0 in 1.0 unit increments. Some of the components analyzed, particularly Trp, KN, XA and QA, complex readily with polyvalent cations, resulting in poor, irreproducible peak shapes and altered mobilities. EDTA was added to the between-run wash cycle to help prevent this undesirable interaction. A plot of electrophoretic mobilities versus buffer pH for each analyte is given in Fig. 2.

The profiles exhibited by each analyte (Fig. 2) are those expected from the pK_a value of the analytes. For example, data for xanthurenic acid (XA) taken from Fig. 2 are replotted in Fig. 3 and the pK_a is estimated to be 7.5, close to the reported value of 7.3 for the 8-hydroxy group [12]. Information in Fig. 2 was used to help predict a buffer pH that might be used to separate all analytes. Vertical distances between profiles at a given pH indicate the relative separation to be expected for a group of analytes. Since Trp, kynurenine (KN) and 3-hydroxkynurenine (3HK) are not separated at pH values lower than 7, and 3-hydroxyanthranilic acid (3HA) and pyridylacetic acid (I.S.) coelute at pH 8.0, buffers with pH values between 9.0 and 10.0 were examined. A buffer of pH 9.6 was found to give good separation of all components (Fig. 4). For these separations, EDTA was actually included in the borate buffer rather than in the between-run washes as in previous runs. It was found that when 5 m*M* EDTA was included as a component of the run buffer, better separation efficiency was obtained, as seen in Fig. 4. This separation is similar to that reported previously by Malone et al. [13] for Trp and six of its metabolites using CE with amperometric detection. However, in that study, the important metabolites KA and QA were not included since they lack the requisite electrochemical activity.

Limits of detection (LOD) under the conditions utilized in Fig. 4 are in the low μ*M* range, approximately 2 μ*M* for 3HK, KN and XA, 5 μ*M* for QA and 8 μ*M* for NA ($S/N=3$). The LOD for a given analyte can be easily lowered by using a detection wavelength corresponding to the absorption maximum for that analyte. For example, our studies showed that for QA, the LOD at 254 nm is reduced by 20% when 270 nm, the secondary absorption maximum for QA in pH 9.6 buffer, is used. Indeed,

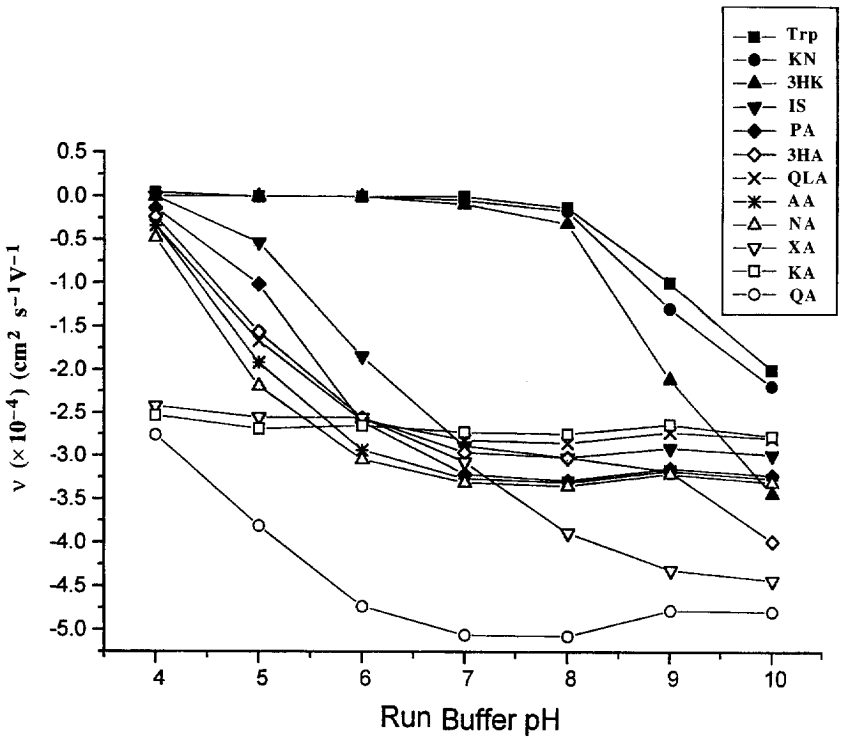

Fig. 2. Electrophoretic mobility of Trp, metabolites and an internal standard (2-pyridylacetic acid, IS) as a function of pH. For metabolite identification, see Fig. 1.

the LOD at 254 nm is lowered by 75% when using a wavelength of 204 nm, the primary maximum, for detection. However, the lack of selectivity encountered when using 204 nm detection for real samples is likely to offset the benefit of a lower LOD.

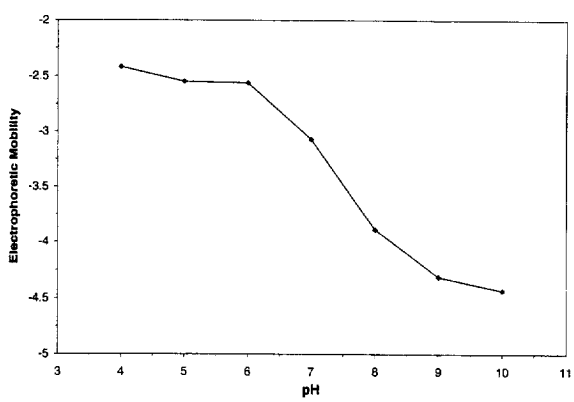

Fig. 3. The effect of buffer pH on electrophoretic mobility of xanthurenic acid (taken from Fig. 2). From the plot, the pK_a is estimated to be ~7.5.

3.2. Determination of quinolinic acid in urine

This CE method was used to identify QA in a urine sample from a normal subject. The sample was prepared as described in Section 2.4 and analyzed by CE using the pH 9.6 buffer (Fig. 5A). A peak corresponding to QA was identified by spiking another aliquot of the sample with standard QA and comparing migration times (Fig. 5B). Note that the detector was set at 270 nm in order to increase slightly the sensitivity and selectivity of the method for QA. Based on the relative peak areas of QA in the two electropherograms, the concentration of QA in the unspiked sample is 20 μM. This corresponds to 0.9 μM QA in the original urine sample. Comparison of this result to literature values is difficult since urinary QA amounts are reported in units of

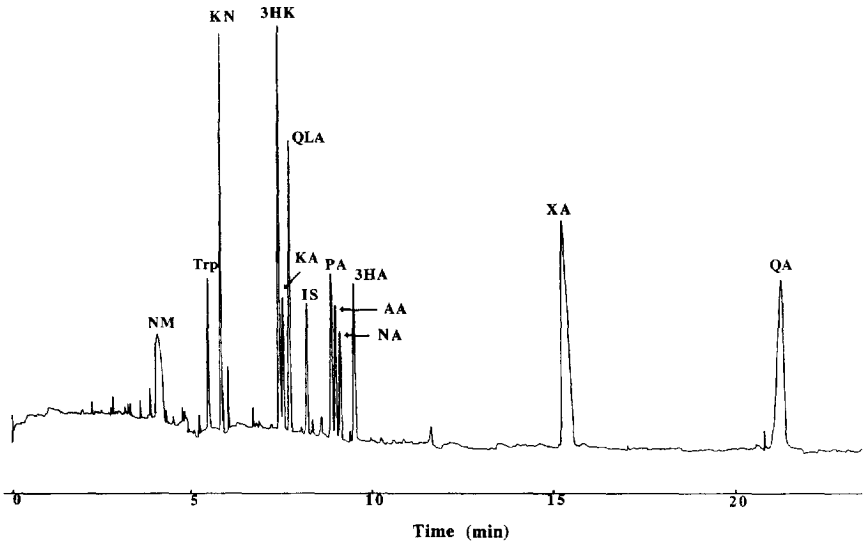

Fig. 4. Electropherogram of a standard mixture of Trp and metabolites containing 38 μ*M* of NA and XA and 77 μ*M* of all others. Also present are 77 μ*M* each of neutral marker (NM) acetophenone and internal standard (IS) 2-pyridylacetic acid. Separation conditions: 20 m*M* sodium borate with 5 m*M* EDTA, pH 9.6; detection is at 254 nm. For peak identifications, see Fig. 1. Other conditions given in Section 2.3.

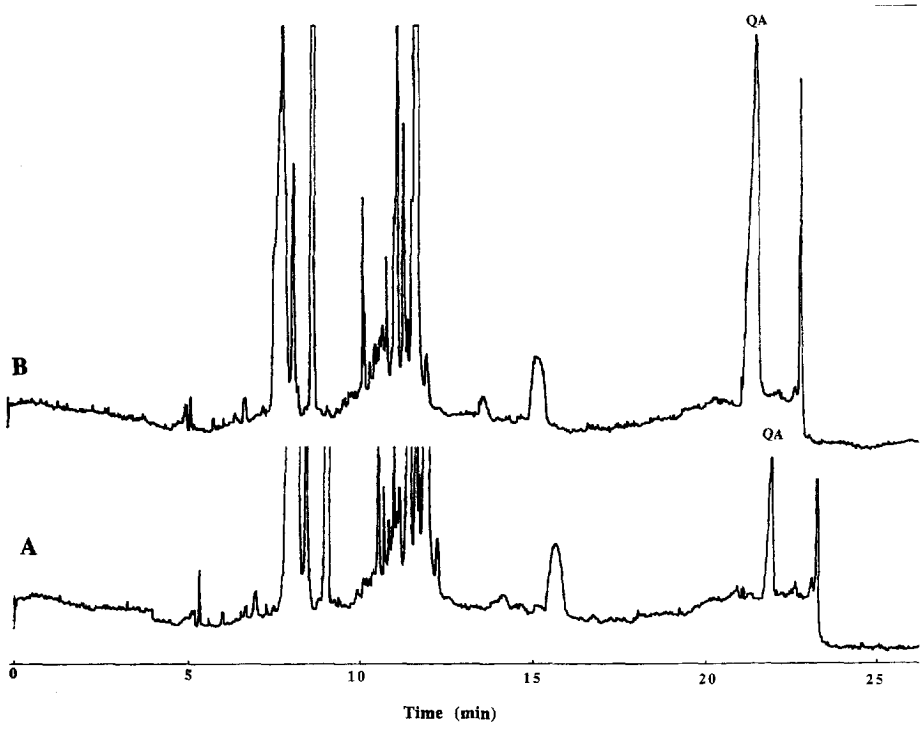

Fig. 5. Electropherogram of a urine sample prepared from a normal subject (A) without and (B) with spiking of QA (70 μ*M*). Separation conditions are the same as in Fig. 4, but detection is at 270 nm.

μM/day [14,15] and a full day's sample was not collected in this instance.

4. Conclusion

It has been shown that by proper choice of run buffer pH, CZE with UV detection can be used to separate Trp and ten of its metabolites, including QA. The method was applied to the determination of QA in the urine of a normal subject. In this study, the limits of detection were in the low micromolar range, which was adequate for the urine sample studied. However, further increases in sensitivity will be needed in order to extend the method to a broader range of biological samples, such as brain microdialysates. Toward this end, continuing experiments involve the use of postcolumn photochemical derivatization followed by electrochemical detection to make possible the sensitive detection of QA in such samples.

Acknowledgments

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