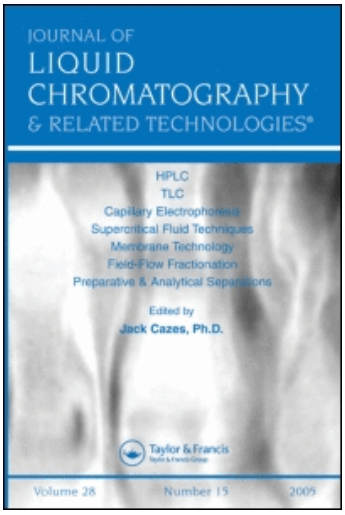

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A SIMPLE APPROACH TO ENHANCE DETECTION IN CAPILLARY ELECTROPHORESIS

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A SIMPLE APPROACH TO ENHANCE DETECTION IN CAPILLARY ELECTROPHORESIS

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□ *In spite of the fact that it offers several advantages, capillary electrophoresis (CE) continues to lag behind HPLC in routine use, mainly due to poor sensitivity of detection. In this paper, the sensitivity of detection is greatly improved through the combination of several simple steps: longer (50–100 cm, long), and wider (75–100 μm, i.d.) capillaries to hold extra sample volume and improve both the signal to noise ratio and provide better separation. Simultaneously, the analysis is performed under general stacking methods in order to decrease the band width. In order to utilize the wider capillaries, the separation was also performed with special buffers, which generate low current: zwitterionic, low ionic strength, or with added ethylene glycol. Based on these modifications, compounds such as procaineamide and mycophenolic acid were analyzed with detection limits in par with the HPLC, but with much better plate numbers and at much less operating costs. Furthermore, this approach was extended to the analysis of the uremic toxin indoxyl sulfate in serum, which represents a difficult analysis in a very complex matrix.*

Keywords capillary electrophoresis, detection, mycophenolic acid, procaineamide, stacking, wide capillaries

INTRODUCTION

Capillary electrophoresis (CE) has several advantages for routine analysis, especially the low cost of operation, the high plate number, and the use of small volumes of aqueous buffers in place of organic solvents. The affordability and disposal of the organic solvents used in HPLC is becoming increasingly difficult in many countries. In the pharmaceutical industry, CE is well suited for polar compound analysis and chiral separations. However, CE continues to be substantially less utilized than HPLC. One of the main problems that has kept this technique behind HPLC for widespread use is its poor detection limits.[1,2] This is due to the short

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light pathway inherent in the capillaries (~50 μm) compared to the long light path of the HPLC cells (50–100 mm). This leads to a large difference on the order of 30–80 fold in detection limits.[1,3–5] As the capillaries decreases further in diameter, this effect becomes more pronounced and thus many analytes falls below the detection limit of CE. Thus, HPLC remains the predominant separation technique.

Narrow capillaries in CE offer better resolution and better theoretical plate numbers due to the better heat dissipation and also provide a shorter analysis time.[3] As result of this, most of the separations at the present time are performed in 50 μm internal diameter (i.d.) capillaries with very few tests performed in 75 μm. Because of the poor heat dissipation and poor resolution, the 100 μm i.d. capillaries are no longer used in CE; although these capillaries offer 4 folds improvement in detection limits compared to the 50 μm.

Several approaches have been used in the past to enhance the detection in CE such as sample solvent extraction,[6–8] better sensitive detectors,[9] special (bubble) cells,[10] and stacking.[2,11–16] These approaches have their own inherent problems and, separately by themselves, do not enhance the sensitivity of detection enough to be in par with the HPLC. Another approach is labeling the analytes with sensitive labels.[17,18] These methods complicate the procedure. This paper presents a different but simple approach, based on the combination of a few simple strategies, which improves the detection limits of CE to HPLC and provides much better theoretical plate numbers (N). The simple steps involve first using wide capillaries (75–100 μm). The second step is increasing the capillary length (about double). These two steps increase the signal by about 4–8 folds. The third step is the use of a simple stacking method, either the high field[16] or acetonitrile stacking,[16] which decreases the band width and concentrates the sample directly on the capillary. Stacking is a simple virtue of CE. The previous two types of stacking can improve the detection limits by a factor of 5–15 fold. None of these steps, by itself, is adequate enough to improve the sensitivity to that of HPLC. However, the combination of these 3 steps can yield 40–100 fold in detection improvement.

Basically, in this paper, the amount of the loaded sample on the capillary was increased through an increase in the volume of the capillary. Both the width and the length of the capillary were increased in order to hold more sample and also to provide adequate room for the separation. However, increasing the capillary volume by itself was not useful, since it can lead to sample overloading, unless it is combined with a stacking method. Increasing the width and the length of the capillary also has side effects. Increasing the width caused an increase in Joule heating with an increase in band width. Increasing the length caused a slower analysis time, unless the voltage was increased, which necessitated using special buffers. Zwitterionic buffers

and organic solvent modifiers in the buffer decreased the current and indirectly helped to speed the analysis. The performance as theoretical plate numbers and resolution in the wide capillaries was improved through the use of stacking. This decreased the band width and avoided sample overloading. There are several types of stacking,[2,6,11–16] most of them utilize clever ideas, but they are very complicated or only applicable to a very limited number of compounds.[2,6] Here, two simple but general types of stacking methods were utilized: 1) high field stacking,[6,15] which is applicable for most compounds; and 2) acetonitrile,[16] which operates when acetonitrile and salts are present in the sample, concentrating many types of compounds based on the principle of pseudo-transient isotachophoresis.[19] The result of the combination of the stacking, an increase in the total capillary volume, and the use of special buffers was that the CE sensitivity became similar to that of the HPLC without using expensive columns or large amounts of organic solvents.

EXPERIMENTAL

Chemicals

Procaineamide, mycophenolic acid, hippuric acid, and boric acid were obtained from Sigma Chemicals, St. Louis, Mo, USA. Ethylene glycol was obtained from Mallinckrodt Baker (Philipsburg, NJ, USA).

Instrument

Two untreated capillaries 45 cm × 50 μm (i.d.) and the other 45 cm × 100 μm (i.d.) were used in a Quanta 4000 CE instrument (Waters, Milford, MA, USA). The instrument was set at 0.29 V/cm, and 214 nm (or as specified) and electrophoresed for 10 min (or as specified).

Separation Buffer

Boric acid, 280 mmol/L, pH 7.5 was used as the separation buffer. Ethylene glycol at different concentrations was added to the buffer as specified later.

Reagents

A mixture of standards of procaineamide 300, mg/L, hippuric acid, 170 mg/L, and mycophenolic acid 17 mg/L was diluted further as specified later.

Acetonitrile Stacking

Serum (or standard) 100 μL was vortex-mixed with 200 μL of acetonitrile in a 500 μL microcentrifuge tube and centrifuged for 15 s at 13000 × g. The supernatant was injected on the capillary.[20]

HPLC

The indoxyl sulfate standard was injected on the CN column 150 mm × 4.6 Microsorb MB, 5 μm column (Varian Analytcial Instruments, Palo Alto, CA). It was eluted with phosphoric acid, 15 mmol/L, at a flow rate of 1 mL/min with the detection at 214 nm (Thermo Separations, Riviera Beach, FL).

RESULTS AND DISCUSSION

At the present time, most analyses in CE are performed in capillaries of 50 μm i.d. This internal diameter offers good separation with a good resolution significantly better than that obtained in the 100 μm i.d. capillaries, as is evident in Figure 1. In this analysis, the current was low, only 18 μA for the 50 μm capillary; however, it was high, 73 μA for the 100 μm capillary. Because of the low current generated in the small diameter capillary, the voltage can be increased so the separation can be achieved faster. However, the absorbency of the analytes for the 50 μm capillary is approximately four times less than that of the 100 μA, Figure 1. In this example, the separation was carried out under non-stacking conditions (sample was dissolved in the same electrophoresis buffer) with injection of a large volume of sample (5% of the capillary). This, of course, led to sample overloading with poor resolution especially for the wide capillary as seen in Figure 1-bottom. These conditions were chosen in order to illustrate how such poor separation obtained in the 100 μm capillary can be turned around and later improved, as illustrated in the subsequent experiments.

The main problems in the wide capillaries are the high generated current and the poor heat dissipation. Both led to an increase in band broadening with poor peak resolution. Because of the excess heat in these capillaries, the separation voltages were limited to low levels, which resulted in a long analysis time. On the other hand, the heat dissipation in these capillaries can be decreased by using: zwitterionic buffers, adding organic solvents, or low ionic strength buffers for the electrophoresis step. Adding an organic solvent to the separation buffer decreased the high current and improved the overall separation. Organic solvents were added often to the buffers in Micellar electrokinetic chromatography (MEKC) to improve the separation selectivity.[21–24] In MEKC, all binding constants

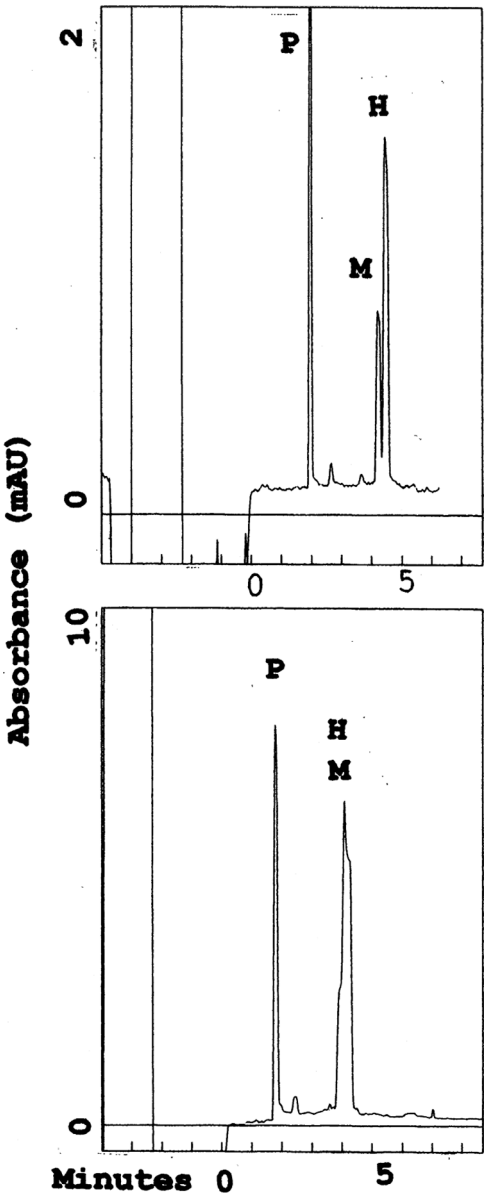

FIGURE 1 Comparison of the separation in: Top 45 cm × 50 μm (i.d.) column with that in Bottom; 45 cm × 100 μm (i.d.) capillary. The capillaries are filled to 5% of their volume with sample dissolved in the same separation buffer (non-stacking conditions). P = Procaineamide 30, mg/L, H = hippuric acid, 17 mg/L, and M = mycophenolic acid 1.7 mg/L.

were decreased.[23] The migration time was also decreased and the critical micelle concentration was shifted to higher values.[24] Ethylene glycol, added to the separation buffer, Figure 2, at 5 and 10%, improved the

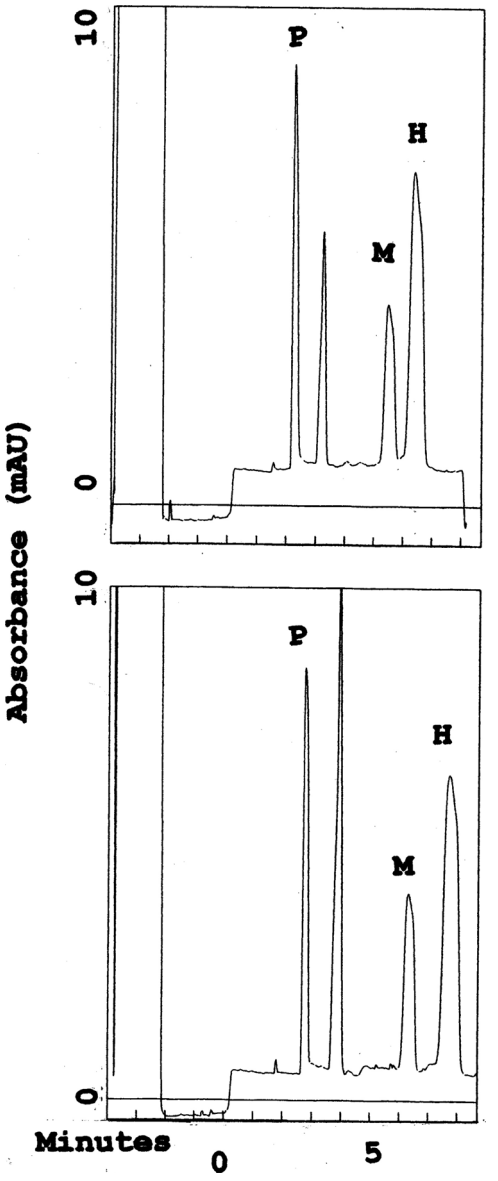

FIGURE 2 Effect of ethylene glycol concentration in the separation buffer at resolution in the wide capillary: Top −5% and Bottom −10%. The capillary is filled to 5% of its volume with sample dissolved in the same separation buffer (non-stacking conditions). P = Procaineamide 30, mg/L, H = hippuric acid, 17 mg/L, and M = mycophenolic acid 1.7 mg/L.

resolution greatly for the wide capillary compared to Figure 1-Bottom. However, the peaks remained wide, exhibiting sample overloading. The ethylene glycol affected the separation through different mechanisms. It disrupted the ordered structure of water molecules decreasing the current conductance.

It decreased the EOF. It also affected the ionization of analytes and improved their solubility. It is interesting that the ethylene glycol did not have to be added to the buffer. It can be added to the capillary wash solutions (sodium hydroxide), data not shown. This indicated that the ethylene glycol also modified the charge on the capillary surface. The affect on the separation was more pronounced when the ethylene glycol was added to the buffer rather than to the wash solution. In this case both the charges on the surface of the capillary, as well as the ionization and solubility of analytes, were affected. As illustrated in Figure 3, several parameters of the separation, such as the current, migration time, resolution, and plate number, were all affected by the addition of ethylene glycol to the separation buffer.

In order to decrease the large band width in the 100 μm i.d. capillaries, stacking was utilized. Many stacking methods have been described. The majority of such methods are applicable only to a few compounds. However, the most common and simplest type of stacking, which is applicable to a wide range of compounds, is the high field stacking; where the sample is simply prepared in the same buffer but at a lower ionic strength, or in water. Figure 4 shows how this simple step is effective in decreasing the band width in the buffer in the absence and in the presence of 5% and 10% ethylene glycol, compared to Figure 2.

The acetonitrile stacking is a more practical method in routine work especially for samples containing a high concentration of proteins. This stacking is applicable also in the absence and presence of ethylene glycol in the buffer. This stacking occurs when the sample is dissolved in acetonitrile (66% in 1% sodium chloride) or simply deproteinized with two volumes

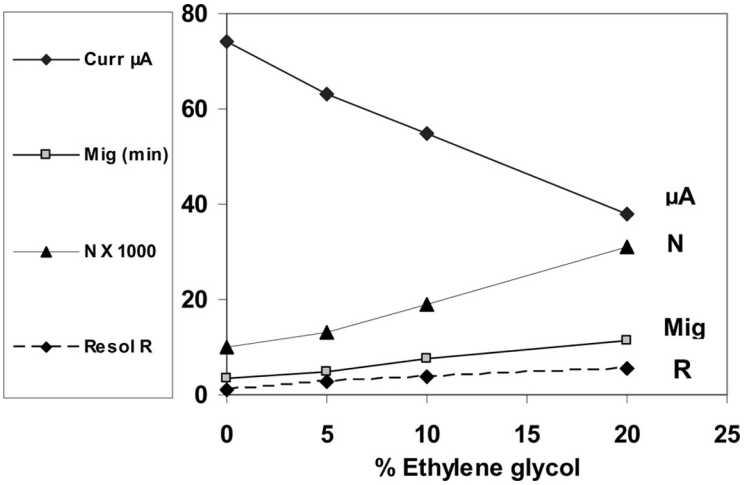

FIGURE 3 Effect of ethylene glycol (under high field stacking), on current μA, N = theoretical plate number, R = resolution, migration time, and peak height on the mycophenolic acid peak.

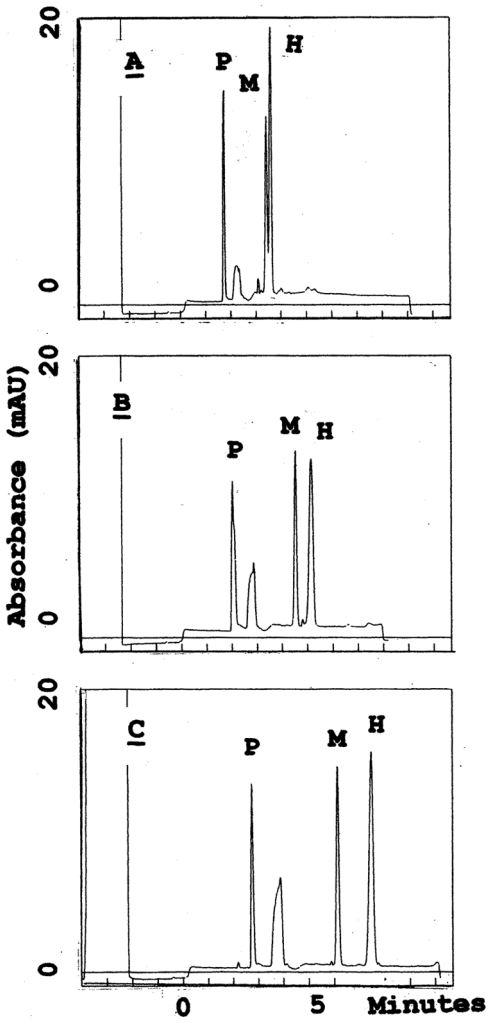

FIGURE 4 Effect of high field stacking on the separation in the wide capillary at: [A] 0%, [B] 5%, and [C] 10% ethylene glycol in the buffer. The sample dissolved in the same electrophoresis which is diluted 10 times. P = Procaineamide 30, mg/L, H = hippuric acid, 17 mg/L, and M = mycophenolic acid 1.7 mg/L.

of acetonitrile. It is based on transient pseudo isotachophoresis.[19] The separation is slightly different from the high field especially for the cationic compounds, which stack in the acetonitrile method much better when zwitterionic buffers are used. The acetonitrile effect becomes more evident when the sample size increases over 10%, Figure 5. When loading 15% of the capillary volume, the acetonitrile gave significantly better stacking than that of the high field, Figure 5. This treatment has other advantages such as removing proteins, which can ruin the capillary or interfere with the

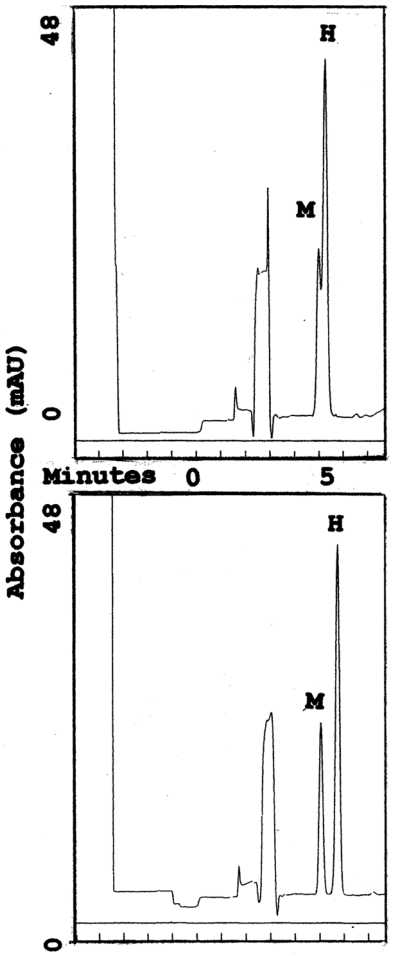

FIGURE 5 Comparison of large sample injection (15% of the capillary volume) on separation by both: (Top) high filed and (Bottom) acetonitrile stacking.

absorbance of the compound of interest. It also frees molecules from their binding proteins. For example, folic acid binds tightly to milk proteins. This vitamin, in the presence of milk proteins, is not detected by direct injection of samples containing milk. However, this can be detected if the sample is treated with acetonitrile (data not shown).

In general, the acetonitrile works much better than the high field stacking for samples with complex matrix such as serum. As an example, the quantification of indoxyl sulfate, a toxin which accumulates up to 50 times in the serum of patients with renal failure, was adapted to the wide capillary. This analysis was chosen to illustrate that wide capillaries can be utilized efficiently for analysis of compounds present at low concentrations

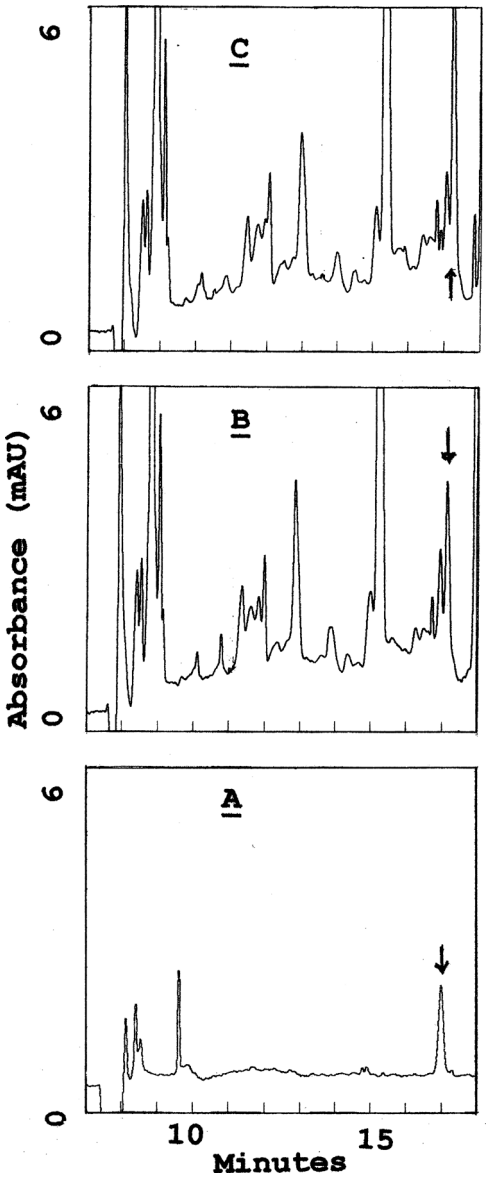

FIGURE 6 Analysis of serum indoxyl sulfate by CE. [A] Standard of indoxyl sulfate 500 ug/L, [B] serum from a healthy person (830 μg/L), and [C] Same sample in B but with an added 500 μg/L indoxyl sulfate standard. The serum (or standard), 100 μL was deproteinized with 200 μL acetonitrile with sample injection 10.6% of total capillary volume. (Capillary 100 cm × 100 μm i.d.; separation buffer 1.35 mol/L boric acid at pH 8.0, containing 5% ethylene glycol with detection at 214 nm, electrophoresed at 23 kV. (Arrow = indoxyl sulfate migration).

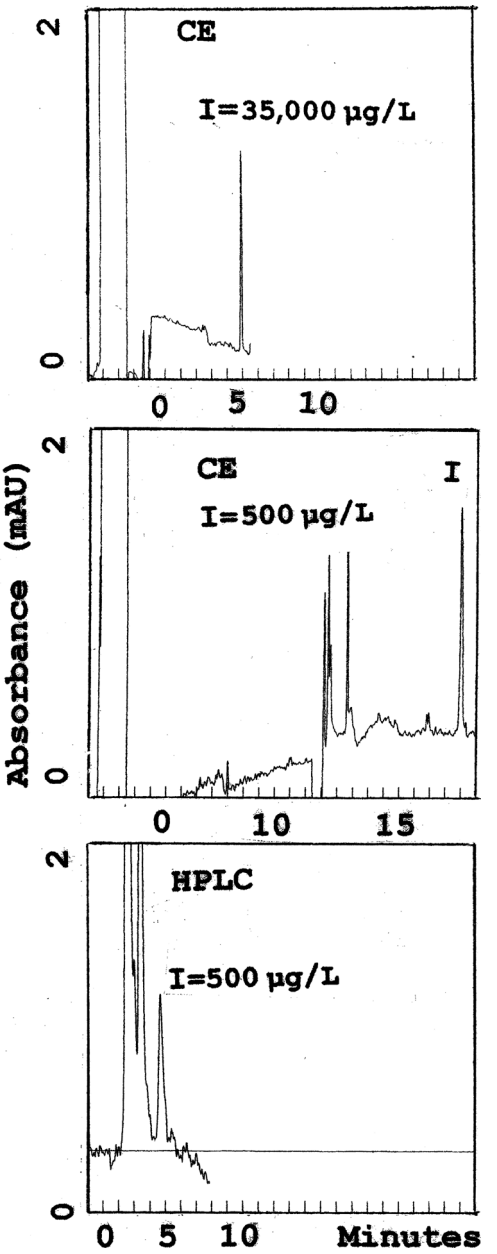

FIGURE 7 Comparison of indoxyl sulfate analysis (I) on: (Top) narrow capillary (40 cm × 50 µm i.d.), under non stacking conditions. Note standard = 35,000 µg/L dissolved in the separation buffer and injected for 20 s (<1% capillary volume), (Middle) A wide capillary (100 cm × 100 µm i.d.), standard (500 µg/L) stacking conditions with 66% acetonitrile filling 10.6% of the capillary volume, and (Bottom) standard 500 µg/L by HPLC. (N = A: 58,000, B: 165,000, and C: 3,800).

in a complex matrix such as serum. The separation is performed in a capillary 100 μm (i.d) wide × 100 cm long, filling 10.6% of the capillary volume with sample. Figure 6 shows the detection of indoxyl sulfate in serum on the wide capillary. It can be seen that serum values much less than 1 mg/L can be detected easily in this wide capillary. The reference range based on analysis of 40 normal individuals is 0.5–11 mg/L, mean 2.8 mg/L. The recovery of 10 mg/L from serum was 98%. Hippuric acid at 2 mg/L can be added to the acetonitrile as an internal standard, migrating slightly after the indoxyl sulfate peak. The RSD by peak height was ~7% (n = 10). No interference from drugs or endogenous substances was encountered. Addition of different amounts of serum to standards in a dialysis cell showed that this compound binds tightly to serum proteins, especially albumin. The indoxyl sulfate standard was compared for sensitivity of detection here by both HPLC and CE on the narrow and also on the wide capillaries, Figure 7. The detection in the wide capillary, under stacking conditions, is about 70 times better than that by the narrow and short capillary (50 μm i.d.) under non-stacking conditions. Note, in Figure 7, the standard concentration used in the 50 μm i.d. capillary had to be increased 70 folds to give a signal similar to that on the wide capillary. It is also evident in Figure 7 that sensitivity in the wide capillary is very close to that of HPLC (both with detection limit ~50 μg/L).

The observed high sensitivity of the assay by CE was the results of not one factor, but the combination of several factors together: increase in length, increase in width, addition of ethylene glycol, and at the same time stacking. More important, the theoretical plate number N is much better by the CE than that by HPLC. For CE narrow capillary, (Figure 7-A) N = 58,000, for CE wide capillary, (Figure 7-B), N = 165,000 and for HPLC (Figure 7-C), N = 3,800. This improvement in sensitivity and plate numbers can be observed for other compounds such as mycophenolic acid,[20] and procaineamide (figures not shown). Procaineamide (and other basic drugs) stack much better in organic zwitterionic buffers.[25,26] These buffers also have the advantage of generating much lower current that enables using higher voltages for rapid analysis.

CONCLUDING REMARKS

Most of the analyses in CE are chosen simply with the aim of speed and resolution without attention to the sensitivity of detection. Here, the emphasis in CE analysis was on enhancing the sensitivity of detection. Simple maneuvers which brings along the detection limits of the CE to be in par with that of HPLC were chosen. A long capillary offers two important points: larger volume for the sample and a longer path for the bands to separate

from each other. The wider capillary offered much better signal to noise ratio. The volume of the capillary is defined by $= \pi \times radius^2 \times length$. Increasing both the length and radius increases the overall volume of the capillary. However, the band widths, in such columns unfortunately are not desirable leading to low theoretical plate numbers. In order to decrease the band width in these capillaries, simple but common stacking methods are vital.

As illustrated here, the combination of stacking long and wide capillaries greatly improved the detection in CE. None of these, by itself, offersed high enough sample concentration to match the detection limits of the HPLC. The wide capillaries posed problems stemming from generating excess heat, which did not dissipate fast enough. Simple maneuvers were shown to overcome this heat while keeping the voltages relatively high enough to bring along a reasonable speed of separation. However, it is important to keep in mind that several factors contribute to the sensitivity of the assay or the minimum detection limits in both HPLC and CE such as capacity factor/migration time, N, solvent/buffers, and electronic design of the detector.

It is important to choose the correct dimensions of capillary for a particular analysis. Narrow and short capillaries are very useful during setting up the method to seek the optimum separation conditions rapidly. However, after that for routine analysis of industrial or biological samples which are present at low concentration, a longer and wider capillary becomes important than the speed for better detection limits. The 75 μm i.d. capillary offers a medium choice between the speed and the sensitivity. However, the final choice of the capillary dimensions is a compromise between speed and sensitivity depending greatly on the particular analysis and the presence of interfering substances. Also, wide capillaries from a practical point of view are less subject to clogging from small particles. These capillaries can be washed and loaded with large volumes of sample for stacking much more rapidly than narrower capillaries.

Comparing the signal of several compounds such as mycophenolic acid, indoxyl sulfate, and procaineamide, it is clear that the detection limits of the CE can match those of the HPLC with much better plate numbers. As a result of this, many compounds which are out reach of the detection of the CE can now easily performed on the CE without the need for large amounts of organic solvents, laborious extraction steps, or expensive columns.

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