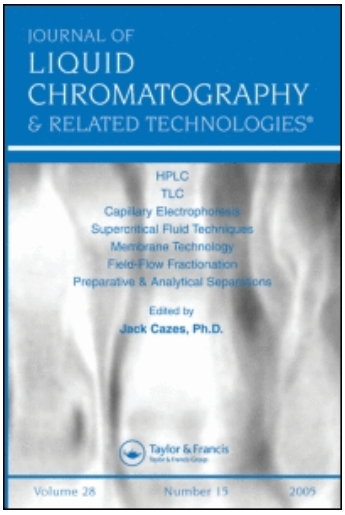

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Pseudo-Isotachophoresis Stacking: I. Investigating the Early Steps

Zak K. Shihabi[a]
[a] Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC, USA

To cite this Article Shihabi, Zak K.(2006) 'Pseudo-Isotachophoresis Stacking: I. Investigating the Early Steps', Journal of Liquid Chromatography & Related Technologies, 29: 2, 159 — 173
To link to this Article: DOI: 10.1080/10826070500416403
URL: http://dx.doi.org/10.1080/10826070500416403

Journal of Liquid Chromatography & Related Technologies®, 29: 159–173, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500416403

Pseudo-Isotachophoresis Stacking: I. Investigating the Early Steps

Zak K. Shihabi
Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC, USA

Abstract: Pseudo ITP (p-ITP) is a simple method for concentration directly on the capillary, especially for samples containing high levels of salts or proteins. By moving the sample closer to the window after injection, we investigated the early steps of stacking without the influence of the separation step (the separation buffer). About one third of the capillary was filled with the sample in the presence of salts and acetonitrile. Salts such as chromate ions moved fast as leading ions, not as a sharp peak but as a wide wave; one side of it having a high ultra-violet absorbency while the other side does not. The width of the wave is related to the salt concentration. Behind the salts, the anionic analytes moved rapidly forming sharp peak(s). As the concentration of the chromate ions are increased better stacking occurred. In the absence of salts or acetonitrile the wave did not form and the stacking was greatly diminished. Unexpectedly, the stacking was also accompanied by some separation, which was affected by the presence of buffers in the sample. This simple approach is useful for investigating other types of stacking.

Keywords: Stacking, Isotachophoresis, Pseudo-isotachophoresis, Sample concentration

INTRODUCTION

Stacking is very important for improving the poor detection limit of CE and to extend this method for routine applications.[1,2] Understanding the factors which affect the stacking is important for the theoretical as well as the

Address correspondence to Z. K. Shihabi, Ph.D., Department of Pathology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, USA. E-mail: zshihabi@wfubmc.edu

practical aspects. One stacking type which offers simplicity, high efficiency, and can be applied for practical analysis of many compounds is the pseudo-isotachophoresis (p-ITP).[3] This is a special stacking method used in capillary electrophoresis for concentrating samples based on the inclusion of both salts and organic solvents in the sample. It is similar to the isotachophoresis (ITP)[4–6] in principle. It employs a leading ion (salt), which offers the low field strength to slow one edge of the analyte band, but it does not employ a special terminating ion for the high field strength. Instead, it uses organic solvent (mostly acetonitrile), which is postulated to serve the same function of the terminating ion, i.e., supplying the high field strength necessary for the stacking step. Organic solvents eliminate the need for searching for an appropriate terminating ion. The sample becomes sandwiched between the two different fields of strength in order to concentrate. When ITP or p-ITP is induced in CE, this step occurs briefly (transient) before the separation step without reaching equilibrium.

It is difficult to study this technique in CE because the stacking step occurs briefly and, almost simultaneously, with the separation step. Here and in the next study, we developed two new different but complimentary approaches to follow this step in CE. After injecting a large volume, the sample is pushed under low pressure in the absence of voltage, close to the detection window. Later on, the voltage is turned on and the change in absorbance is recorded. This method removes most of the separation step from the stacking, allowing the follow up of the early steps of the concentration. In a second approach, the entire capillary is filled with sample and as it concentrates the changes in absorbance is recorded. Each of these two approaches has certain limitations as well as certain advantages; both being complementary to each other. Understanding the early events can lead to a better optimization of the stacking.

EXPERIMENTAL

Chemicals

Theophylline was obtained from Sigma Chemicals (Saint Louis, MO, USA); Iohexol from Winthrop Pharmaceuticals (New York, NY, USA); and phenytoin from Eastman Kodak (Rochester, NY, USA).

Instruments

A Beckman CE Model 2000 (Beckman Instruments, Fullerton, CA, USA) was set at 7 kV and 280 nm (or as specified). The capillary was 26 cm × 50 μm (I.D) untreated silica (Polymicro Technologies, Scottsdale, AZ, USA).

Procedure

Theophylline (250 mg/L), Iohexol (500 mg/L), and phenytoin (80 mg/L), dissolved in 0.5% NaCl, were separated using a buffer composed of boric acid, 180 mmol/L, pH 8.9. The samples were injected for 90 s, filling 38% of the capillary volume (to the detector). The sample was pushed under low pressure to the detector window. As soon as the signal starts to increase (indicating that the sample is residing at the detector window) the voltage was turned on.

RESULTS

Moving the Sample to the Detector Window

As long as the sample size is small, $<2\%$ of the capillary volume, and the voltage is turned on immediately after the injection, while the segment is at the inlet of the capillary (normal CE conditions of separation), a good resolution and plate number is obtained for the drug theophylline (T) and the contrast agent iohexol (I), regardless if the sample contains salts, acetonitrile, or the combination of the two, Fig. 1. Unfortunately, the sensitivity is too poor to be useful in practical analysis. To improve the detection, most of the CE separations are usually performed under some type of stacking (mostly high-field stacking). Here we use transient p-ITP[2] for stacking, which offers better concentration. When the sample size is increased to 38% of the capillary, under non-stacking conditions, sample overloading occurs and the separation is lost, especially, if the sample contains salts, or acetonitrile in the absence of salts, Fig. 2C and 2D. However, in presence of both acetonitrile together with salts, a good peak height and resolution is obtained due to stacking, Fig. 2A and 2B. In this case, the overall separation is the result of the two steps: the stacking and the separation. The stacking efficiency[7] in this case was 27.5 (note the improvement in signal to noise). Sodium chloride, the major small anion in physiological fluids, can be replaced with other salts such as potassium chromate, Fig. 2B. The chromate, for its high UV absorbance, is more useful in following the role of salts in stacking in this study.

The resolution, peak height, and overall separation in CE are the result of the two simultaneous steps; the stacking and the separation in the buffer. It is difficult to separate these two steps in order to investigate and optimize each. In order to study the stacking events with minimum contribution of the separation step, we moved the sample closer to the window (by pushing it under low pressure); afterwards, the voltage was turned on. Initially a blank sample containing only water (without acetonitrile, salts or the analytes) was injected. A flat baseline is observed, Fig. 3A. Later on, we added the two compounds I &

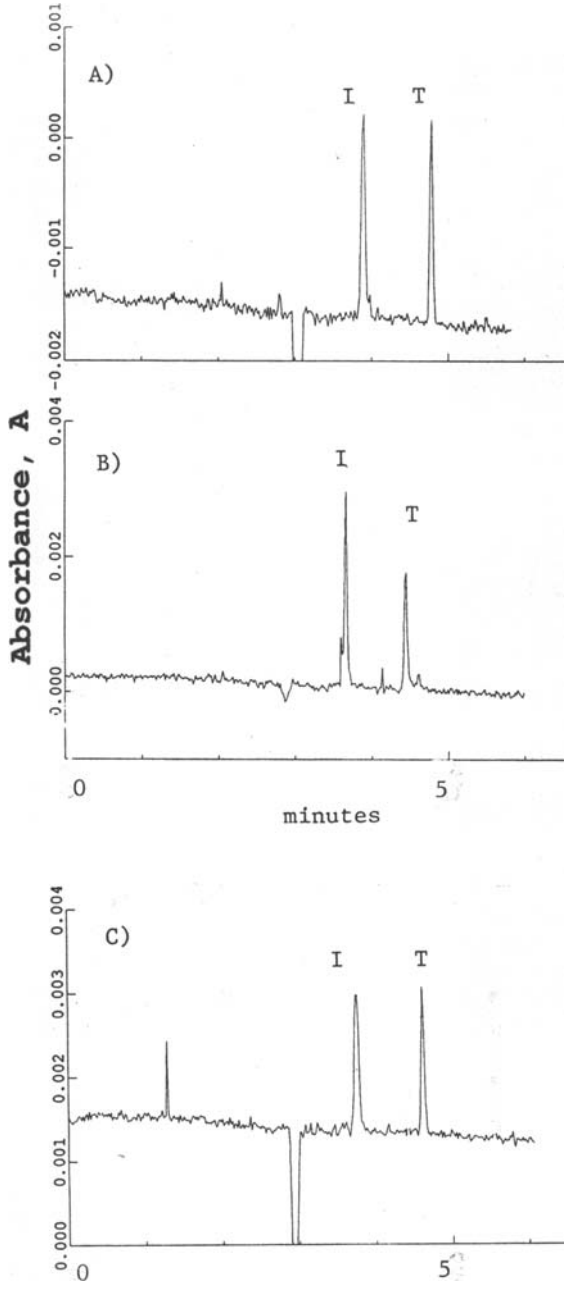

Figure 1. Separation of iohexol 160 mg/L (I), and theophylline (T) 80 mg/L. Injection 1.8% of the capillary volume with sample dissolved in: (A) two volumes of acetonitrile and one volume of 1% NaCl; (B) two volumes of water and one volume of 1% NaCl; and (C) two volumes of acetonitrile and one volume of water.

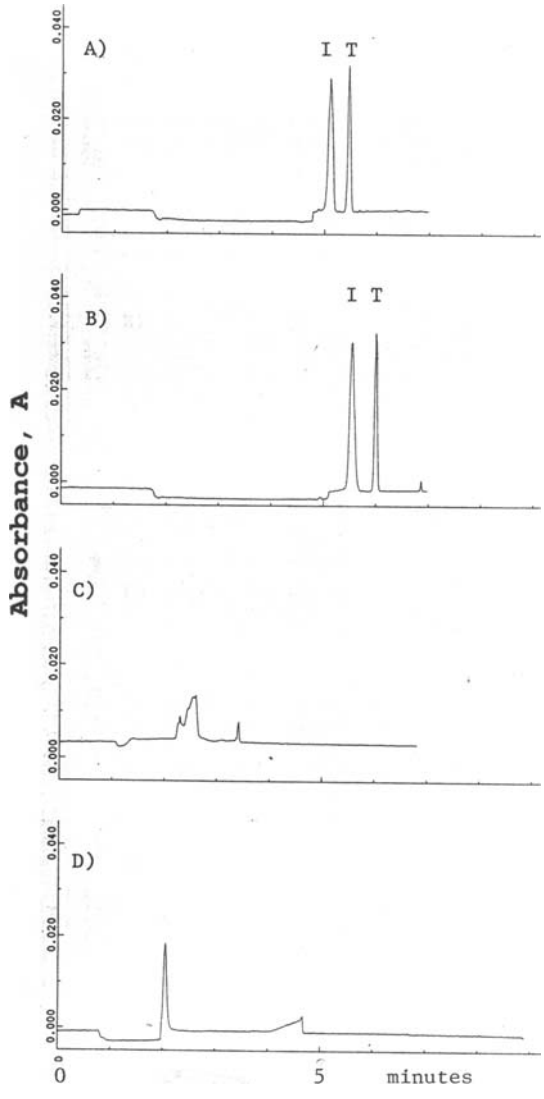

Figure 2. Separation of I (160 mg/L)) and T (80 mg/L). Injection 38% of the capillary volume with sample dissolved in: (A) two volumes of acetonitrile and one volume of 1% NaCl; (B) two volumes of acetonitrile and one volume of 1% potassium chromate, (C) two volumes of water and one volume of 1% NaCl; and (D) two volumes of acetonitrile and one volume of water.

T. These two compounds concentrated as one peak at the anodic edge of the sample plug, Fig. 3B. The concentration or the stacking here is due to the high field strength in the sample.[8,9] The same pattern is observed if the sample contained only acetonitrile in the absence of salts, Fig. 3C and 3D. The

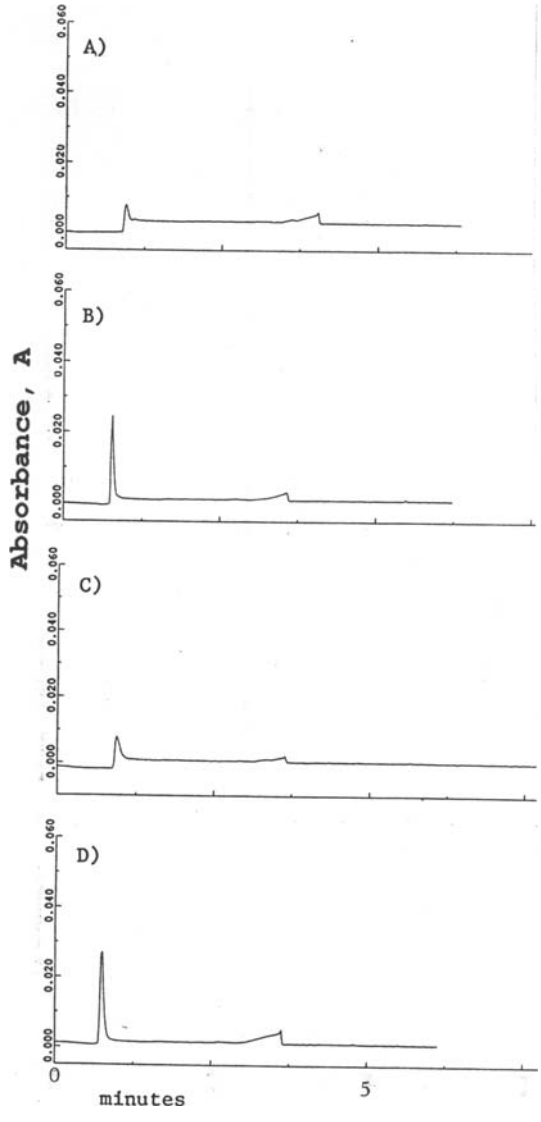

Figure 3. Stacking of iohexol (I) and theophylline (T). The sample was pushed to the detector window under low pressure, after that the voltage is turned on; (A) blank, water; (B) as in A but also contained I and T; (C) blank, two volumes of acetonitrile and one volume of water; (D) as in C but contained I and T.

concentration is also due to the same factor. A sample containing only chromate shows a wave-like change in absorbance where one side has a low and the other a high absorbance, Fig. 4B. As the chromate ions are increased they migrate not as a sharp band but as wide wave, with one side

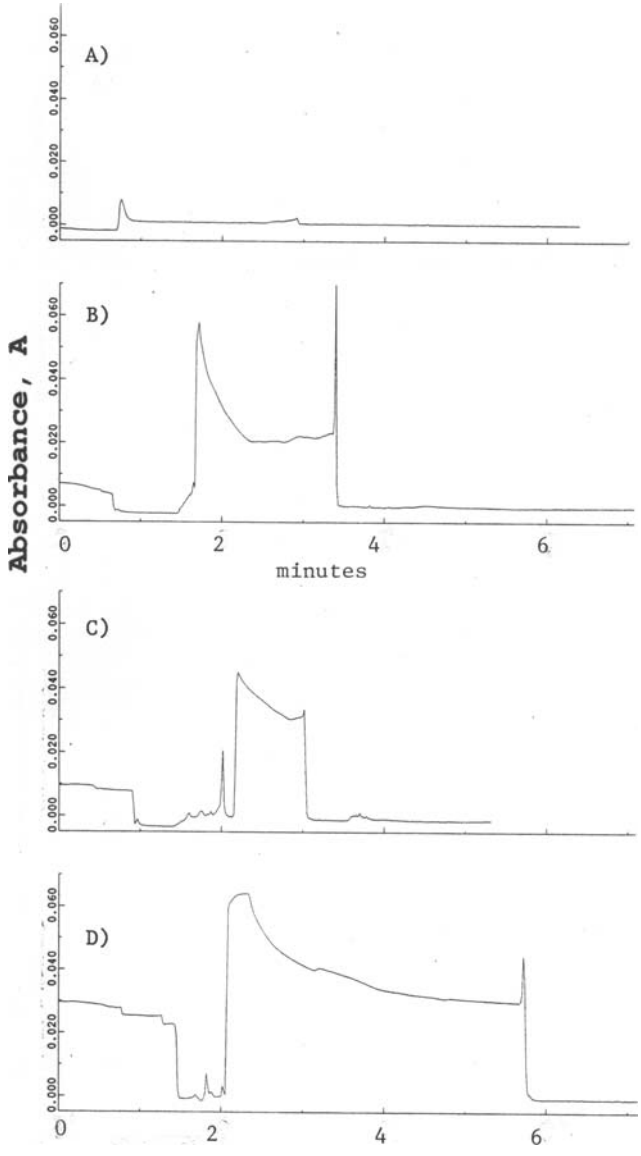

Figure 4. Migration of the chromate ions. The sample contained two volumes of acetonitrile and one volume of potassium chromate and is pushed to the detector window: (A) 0% chromate, (B) 0.2% chromate, (C) 0.4% chromate, and (D) 1% chromate.

having higher absorbance than the other, Fig. 4C and 4D. Addition of the two analytes I and T to the blank shows up as a new single peak that forms behind the chromate wave. Initially, a single peak not well separated behind the chromium ions wave is observed, Fig. 5D. Afterwards, two peaks are

rapidly formed, Fig. 5C. With time, a better separation of the two peaks is observed, Fig. 5B.

The single peak, formed in water or pure acetonitrile in absence of salts, represents the two compounds I and T. It has almost less than half of the height of that formed in the acetonitrile and water. The shape of the wave in the

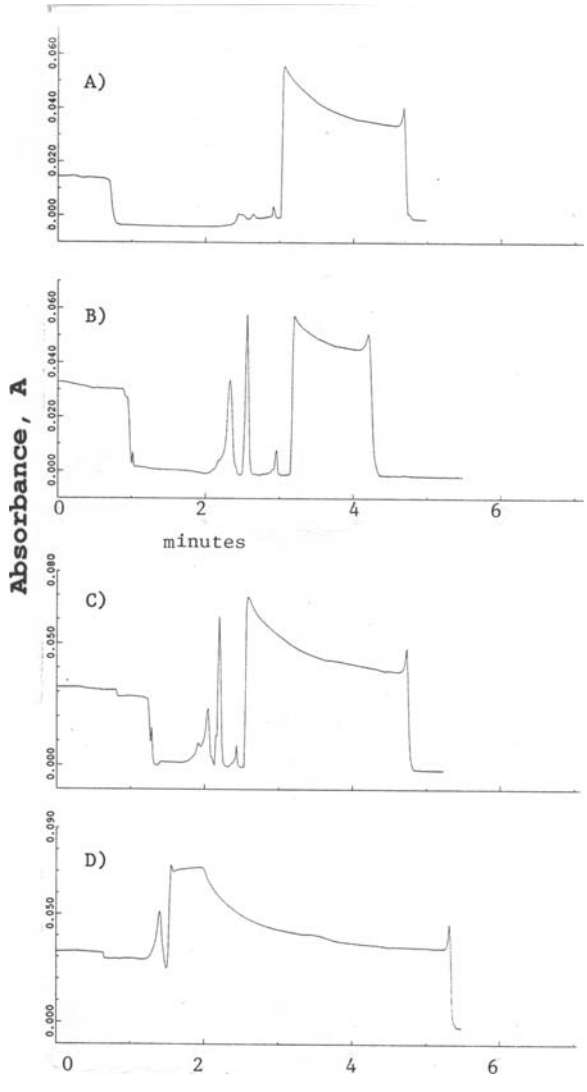

Figure 5. Stacking of iohexol and theophylline in two volumes of acetonitrile and one volume of 1% potassium chromate. The sample is pushed to the window: (A) blank sample; (B) as in A but with added iohexol and theophylline; (C) the sample as B but is pushed for extra 10 s; (D) the sample as in B but it is pushed for extra 20 s.

sample zone, Fig. 5, indicates that there are two areas with huge differences in absorbance and, consequently, in the field strength. The area with a high absorbance, i.e., high chromium ions, has a low field strength suppressing the mobility of the analytes I and T present in that region of the sample plug. On the other hand, the segment of the wave with lower absorbance reflects the acetonitrile area devoid of chromate ions, and with a high field strength driving the compounds I and T in that region of the plug to migrate rapidly. Thus, the two edges of a long sample plug move at different speeds due to a difference in the field strength, which eventually leads to the concentration.

In addition to the concentration, observing a separation of I and T while the sample is residing in the injection zone just before entering the separation buffer, was to a certain extent, a surprise. Of course, many compounds only stack and do not separate unless they move into the separation buffer. For example, in another experiment, phenytoin was added to the theophylline and iohexol sample. When the sample is injected and separated without moving it close to the window a good resolution ($R = 2.5$) between the theophylline and phenytoin peaks was obtained, but much less than that obtained between iohexol and theophylline ($R = 6.5$), Fig. 6A. However, when the sample is pushed to the window we did not see any separation for the phenytoin and the theophylline, Fig. 6B.

Low concentrations of salts can dissipate fast in the buffer without much effect on stacking. Higher salt concentrations cause the wide wave and cause the anionic analytes to move further from the neutral molecules and closer towards the anode, leading to a slight increase in the migration time. Similar to what happens in ITP, as the leading ion concentration here is increased the overall velocity is decreased. More importantly, the salts at optimum concentration, improve the overall resolution, Fig. 7. The stacking, as peak height, and the resolution are improved, Fig. 7A–C. During the stacking step (i.e., the sample at the window), as the salt (chromate) concentration increases the peak(s) migration is slightly slowed down but a better separation is observed, Fig. 8. Probably, the presence of salts together with acetonitrile, effects the ionization and solubility of some compounds differently (e.g., I and T), leading to different migration, i.e., better separation during the stacking step. This is reminiscent of the "salting effect" in organic solvent extraction. Thus, the separation of some compounds starts early while they are residing in the sample segment. Thus, the presence of the acetonitrile together with salts in the sample can improve not only the peak height but the separation too. It can lead to separation early on before the sample enters the electrophoresis buffer.

Filling the Entire Capillary

In another approach, the whole capillary was flushed completely with the sample. Thus, the sample volume is 100% of the capillary. Interpretation of

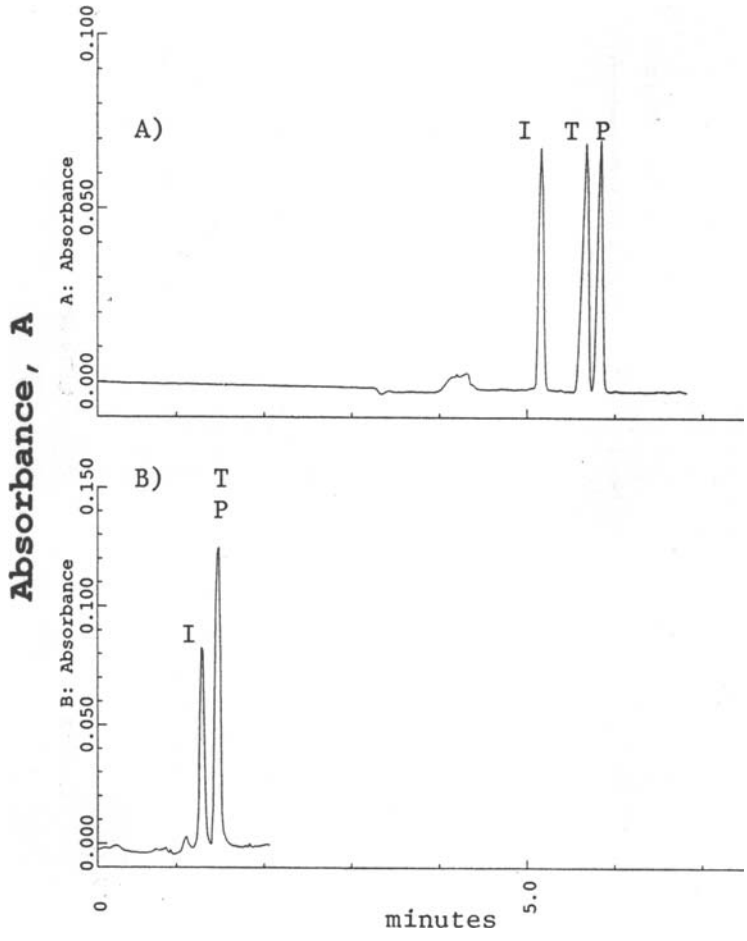

Figure 6. Separation of iohexol, (I) 160 mg/L, theophylline (T), 80 mg/L and phenytoin, (P) 80 mg/L prepared in two volumes of acetonitrile and one volume of 1% NaCl filling 17% of the capillary volume (214 nm): (A) the voltage was tuned on immediately with the sample at the anode; and (B) the voltage was turned on later after the sample was pushed to the detector window.

the data from the whole capillary is not simple or straightforward because the sample can stack after the detection window; thus, nothing can be detected or some part of the sample migrates through the window to the anode. Also, because of the absence of a strong buffer, slight changes in the pH or salts can cause large changes in the migration. Impurities can stack and show as large peaks. Thus, it is important to keep the sample simple and to avoid multi-components or complex samples in this approach. However, when the capillary was flushed completely with sample containing sodium chloride and acetonitrile (Blank) few minor peaks are observed, Fig. 9A. The same

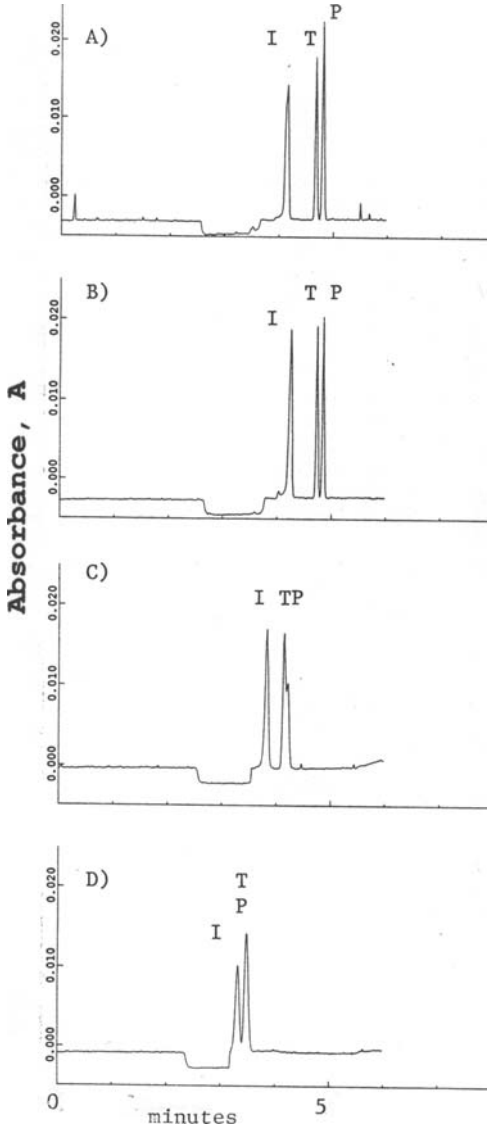

Figure 7. Effect of the salts as potassium dichromate on the separation of iohexol (I), theophylline (T), and phenytoin (P) as in Fig. 6A; potassium chromate concentration: (A) 2%; (B) 1%; (C) 0.5%; and (D) 0.2%.

observation is obtained when the capillary was run under continuous buffer (non-stacking), Fig. 9B. However, when the sample contained the two compounds, I and T in addition to the acetonitrile and salt, stacking without any separation is observed, Fig. 9C. If the sodium chloride was buffered

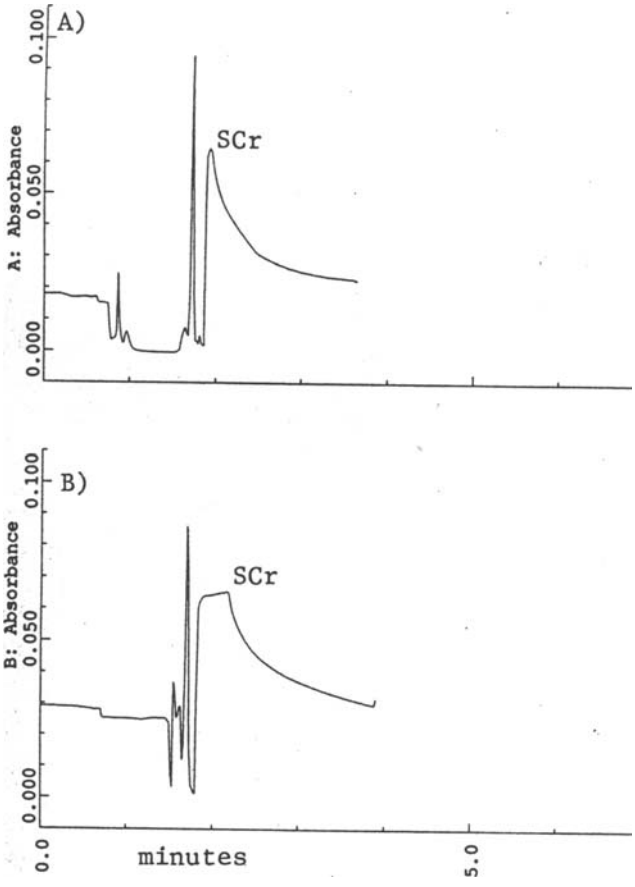

Figure 8. Effect of the salts (potassium dichromate SCr) on the separation of iohexol (I), theophylline (T), and phenytoin (P). Conditions are as in Fig. 6B. The sample was pushed to the window before the voltage is turned on: (A) chromate 0.2% and (B) chromate 1%.

with 60 mM of borate at pH 9.5, and again the capillary is flushed with sample, both a stacking and some partial resolution between the theophylline and the iohexol is observed, Fig. 9D. However, if the acetonitrile is removed and replaced by water the separation is lost and the stacking is reduced too. If the borate buffer was lowered to pH 8.4 there is stacking without separation. A lesser degree of concentration (without separation) is obtained when the sample is dissolved in water without buffers compared to that for the acetonitrile.

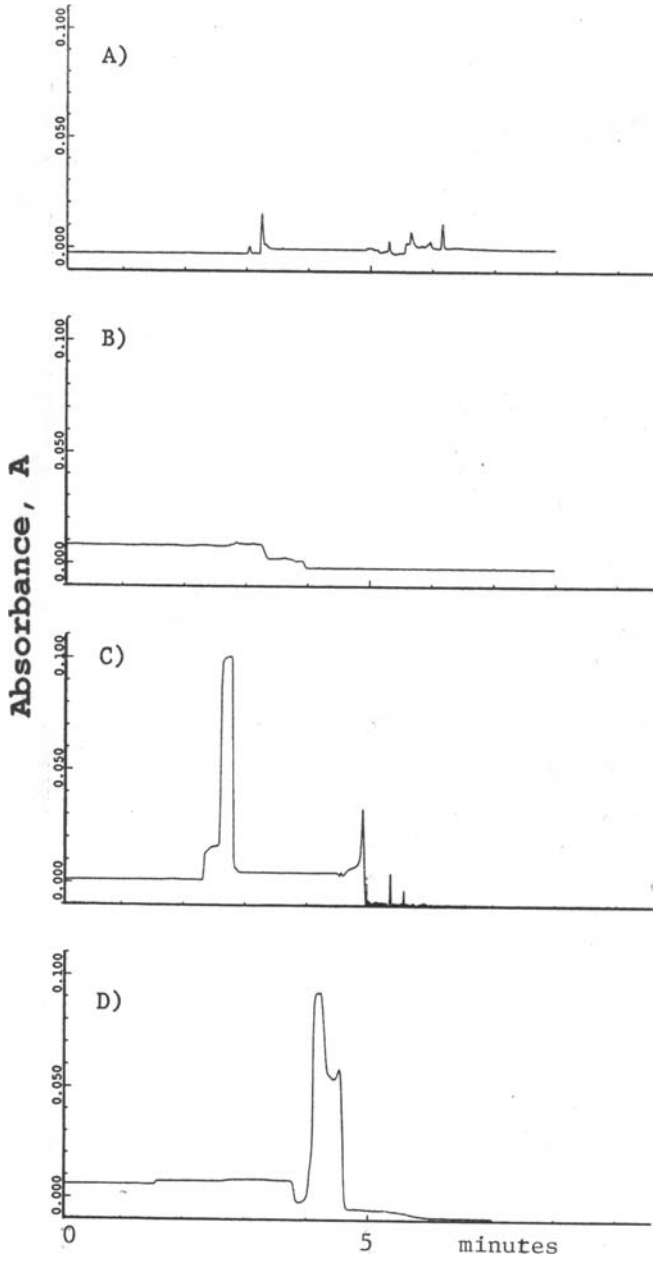

Figure 9. Filling the entire capillary with sample: (A) blank sample containing only two volumes of acetonitrile and one volume of 1% NaCl; (B) sample containing I and T dissolved in the same separation buffer (continuous buffer, non-stacking); (C) as in A but contains I and T; and (D) as in C but with 60 mmol/L borate pH 9.4 added.

CONCLUSIONS

By moving the sample to the detector window, and also by filling the whole capillary with sample, the early events of stacking of p-ITP in absence of the separation buffer are investigated. Early on, as soon as the voltage is turned on, the leading ions (chromate) migrated rapidly ahead of the analytes as a wide wave, rather than a sharp peak, hindering the migration of the analytes at the cathodic side of sample plug. The anodic side of the sample plug had a decreased absorbance, indicating an area of acetonitrile devoid of salts but with high field strength. Initially, a sharp and tall peak emerged being adjacent to the chromate; however, later on two peaks can be detected. The acetonitrile must have contributed to this rapid movement of the analytes through the high field strength, pushing the analytes towards the cathodic side. When the acetonitrile was omitted a smaller peak appeared near the anodic side.

The similarity of p-ITP to the traditional ITP is evident here. Both methods lead to a high degree of sample concentration. Both require a leading ion. In both methods, peak height is enhanced by increasing the salts concentration (to limited extent). In absence of a leading ion (chromate or chloride ion) the stacking is diminished greatly. The same is true for the terminating ion in ITP. However, in p-ITP the acetonitrile or alcohol can take the same role of terminating ion, avoiding the need for selecting a proper terminating ion.

Surprisingly, compounds did not just concentrate only during the stacking step, but some also separated. The presence of buffer in addition to salts in the sample affected the stacking and the separation. This simple approach should be useful for investigating and optimizing other types of stacking.

REFERENCES

1. Quirino, J.P.; Terabe, S. Sample stacking of cationic and anionic analytes in capillary electrophoresis. J. Chromatogr. A **2000**, *902*, 119–135.
2. Shihabi, Z.K. Stacking in capillary zone electrophoresis. J. Chromatogr. A **2000**, *902*, 107–117.
3. Shihabi, Z.K. Transient pseudo-isotachophoresis for sample concentration in capillary electrophoresis. Electrophoresis **2002**, *23*, 1612–1617.
4. Gebauer, P.; Bocek, P. Theory of zone separation in isotachophoresis: a diffusional approach. Electrophoresis **1995**, *16*, 1999–2007.
5. Krivankova, L.; Bocek, P. Synergism of capillary isotachophoresis and capillary zone electrophoresis. J. Chromatogr. B **1997**, *689*, 13–34.
6. Krivankova, L.; Pantukova, P.; Bocek, P. Isotachophoresis in zone electrophoresis. J. Chromatogr. A **1999**, *838*, 55–70.
7. Zhang, C.X.; Thormann, W. Head-column field-amplified sample stacking in binary system capillary electrophoresis. 2. Optimization with a preinjection plug and application to micellar electrokinetic chromatography. Anal. Chem. **1998**, *70*, 540–548.
8. Chien, R.-L.; Helmer, C.J. Electroosmotic properties and peak broadening in field-amplified capillary electrophoresis. Anal. Chem. **1991**, *63*, 1354–1361.

9. Burgi, D.S.; Chien, R.-L. Optimization in sample stacking for high-performance capillary electrophoresis. Anal. Chem. **1991**, *63*, 2042–2047.

Received August 15, 2005
Accepted September 20, 2005
Manuscript 6699