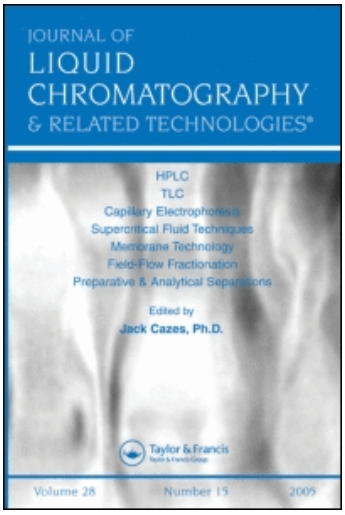

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Studies on the Mechanism of the Acetonitrile-Salt Stacking Method in Capillary Electrophoresis

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Studies on the Mechanism of the Acetonitrile-Salt Stacking Method in Capillary Electrophoresis

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Abstract: The acetonitrile-salt stacking (ASS) technique, as a newly developed stacking method, had great potential for analysis of low concentration compounds in bio samples, which contains high concentrations of salts. However, due to the mechanism being not very clear, the applications were limited. In this article, the experimental factors, related to the stacking effects of two symbol peptides, were carefully considered and studied. A reasonable hypothesis about the mechanism of ASS was proposed in order that the experimental parameters could be easily decided and optimized, as well as widening areas of applications.

Keywords: Capillary electrophoresis, Acetonitrile, Salts, Stacking, Mechanism

1. INTRODUCTION

Recently, capillary electrophoresis (CE) has been widely used in the bioanalysis area for its high efficiency, rapidity, and needing a small sample volume; it could solve all the problems that high performance liquid chromatography would encounter. But CE also has some disadvantages. A high limit of detection (LOD) was one of the biggest problems, which was due to the short detection optical length and small injection volume. This would be more serious for the analytes that were in a low concentration and lacked a strong chromophore.

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Many methods, such as preconcentration,[1] hyphenation technique,[2–4] etc., which were attempted to afford lower LOD, and stacking,[5–8] seemed to be the easiest and simplest way to enrich the concentration and didn't require complicated steps or special equipment. The most common method for stacking in CE is large volume sample stacking (LVSS).[9] It was widely used because it was simple and could be easily achieved by dissolving the sample in the same electrophoresis buffer, but at ten times lower ionic strength. However, as to biosamples, stacking phenomena could not occur easily in LVSS, due to the deleterious effects of salts. Normally, additional steps for desalting were need, which decreased the practicality of the method.

The acetonitrile-salt stacking method[10–14] has the advantage of stacking biosamples, which contains high concentration salts and (or) proteins and is always deproteined with acetonitrile. The method permits a larger volume injection of sample (about 10–30% of the capillary volume) than in the normal CZE mode (within 1% of the capillary volume). Shihabi et al. had used this method to determine three peptides,[10] insulin,[11] and anions.[12] In our previous works, ASS had been used to assay low concentration glutathione in diabetic nephropathy patient's plasma.[15]

All the applications showed that the ASS was a useful tool for assaying the low concentration compounds in biosamples; however, the applications were limited as the mechanism of ASS was not completely known. Few works were aimed on this area. Domen[16] et al. considered that the mechanism was like a LVSS procedure, however, the effect of salts was not mentioned. Shihabi had presumed that the ASS would be a transient pseudo-isotachophoresis procedure, which gave a detailed explanation on the function of NaCl in stacking.[13,14] The hypothesis is actually good, but not consummate. In this work, the mechanism of the ASS method was carefully considered and studied based on the experimental factors (buffer concentration, electric current, organic solvent, and salts kinds, etc.) using two peptides. Several additional data and new opinions were given, and a reasonable schematic drawing of the mechanism was finally achieved, as well as some experiment made to test the conclusion.

2. EXPERIMENTAL

2.1 Chemicals

Glutathione (both reduced and oxidized forms) were purchased from Sigma (St. Louis, MO, USA). Borate, sodium chloride (NaCl), and acetonitrile (ACN, HPLC-grade agents) were purchased from Xi'an Chemical Reagent Company (Xi'an, China). All the solutions and samples were prepared in redistilled water daily and filtered through a 0.22 μm filter before use.

2.2 Instrumentation

Experiments were performed on a Beckman P/ACE^{TM} MDQ system (Beckman, Fullerton, CA. USA) equipped with a UV-Vis detector, an autosampler, and a temperature controller (15–60 $\pm$ 0.1°C). Instrument control and data analysis were carried out by Beckman P/ACE system software (Ver. 2.3) on a personal computer. For pH measurements, a pH meter (pHS-25, Weiye, Shanghai, China) calibrated with a precision of 0.01 pH unit was employed.

2.3 Preparation of Standard Samples

Standard stock solutions of GSH (3.6 mmol/L) and GSSG (1.9 mmol/L) were prepared quantitatively with water and stored at 4°C until use. The working standard solutions were used after the dilution of stock solutions, and finally contained 20 mmol/L pH 8.0 borate buffer, 45 mmol/L NaCl, and 70% (v/v) ACN (or as specified).

2.4 Electrophoresis Procedure

All the separations were performed on a fused silica capillary (75 μm I.D., effective length of 21 cm, total length of 31 cm, Yongnian photoconductive fibre factory, Hebei China), using a 300 mmol/L borate buffer (pH 8.0, the counter ion was sodium ion) as the stacking and separation buffer (Buffer), at 20°C, with a constant voltage of +5 kV. The samples were injected in pressure mode at inlet (0.5 psi for 30 s or as specified). Detection wavelength was set to 200 nm. Before each run, the capillary was rinsed with Buffer at 20 psi for 1 min to equilibrate the capillary inner wall.

3. RESULTS AND DISCUSSION

3.1 Influence of Matrix Viscosity on Stacking

As the viscosity (μ) of the sample zone (SZ) was important for stacking, in this section, the ACN ($\mu = 0.3$) was replaced by several organic solvents such as acetone ($\mu = 0.34$), methanol ($\mu = 0.534$), ethanol ($\mu = 1.08$), and isopropanol ($\mu = 1.9$). The relationship between viscosity and stacking effect (theory plate number, N) was shown in Figure 1. As the viscosity of the organic solvent decreased, the N of both GSH and GSSG decreased. The reason would be that the lower the viscosity of the organic solvent, the faster the analytes moved in SZ, which resulted in better stacking effects. In this point of view, the mechanism of the ASS was likely to be LVSS, which obtained

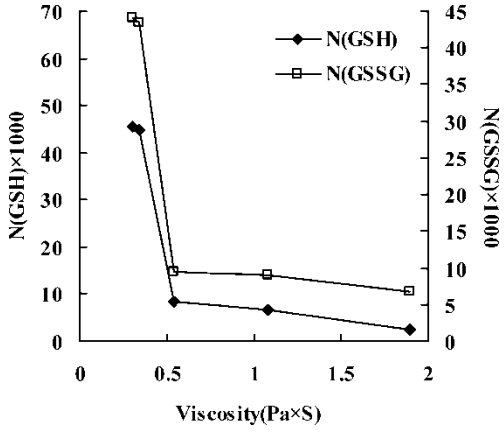

Figure 1. Influence of the solvent viscosity on theory plate number under ASS mode. Conditions: 100 mmol/L pH 8.0 borate buffer as the BGS, at 200 nm, 25°C, injection time was set to 12 s at 0.5 psi. Sample contained 70% ACN, 45 mmol/L NaCl, 10 mmol/L BGS and glutathione.

stacking phenomena based on the velocity difference of analytes between SZ and background solution (BGS). In order to further confirm this point, the influence of buffer concentration on the stacking effects of GSH under LVSS (Figure 2A) and ASS (Figure 2C) modes were shown in Figure 2 (the results of GSSG was similar to that of GSH). The N of both ASS and LVSS increased as the buffer concentration increased, and the shapes of the N curve were similar as expected.

Moreover, ASS seemed to be more effective than LVSS, for the N of ASS was always larger than that of the LVSS under the same conditions. The presence of ACN and (or) NaCl in SZ would be the reason. Figure 2B showed the N curve of the sample that only contained ACN. Comparing B with A and C, we found that the presence of ACN (B) caused better stacking than LVSS (A) and NaCl (C) could increase the stacking effects of (B). These results indicated that the ASS procedure was like LVSS, but has an additional effect on stacking than does LVSS.

3.2 Influence of Salts on Stacking

Since the salts seemed to have additional effects on stacking, in this section, $NaNO_2$ (B), CH_3COONa (C), Na_2SO_4 (D), Na_2CO_3 (E), $CaCl_2$ (F), NH_4Cl (G), and KCl (H) were used to replace the NaCl (A), and their effects on the N values were shown in Figure 3. For salts A, G, and H, they had different cations (anions were same), but the N values were similar. This indicated that the difference of the cations seemed to have no relationship

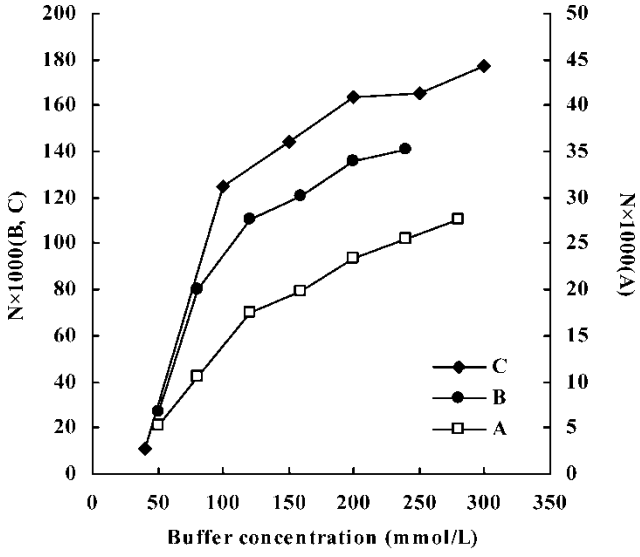

Figure 2. Influence of the buffer concentration on theory plate number under LVSS (A and B) and ASS (C) mode. Conditions: pH 8.0 borate buffer as the BGS, injection time was set to 10 s at 0.5 psi. Sample (A) contained 10 mmol/L BGS and glutathione. Sample (B) contained 70% ACN, 10 mmol/L BGS and glutathione. Sample (C) contained 70% ACN, 45 mmol/L NaCl, 10 mmol/L BGS and glutathione. Other conditions see Figure 1.

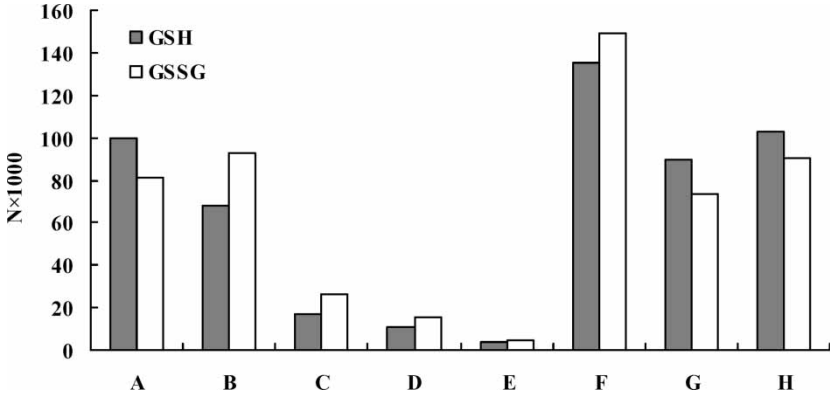

Figure 3. Influence of the salts on theory plate number under ASS mode. A–H was presented for NaCl, $NaNO_2$, CH_3COONa, Na_2SO_4, Na_2CO_3, $CaCl_2$, NH_4Cl, and KCl, respectively. Conditions: 300 mmol/L borate buffer as BGS, sample contained 70% ACN, 25 mmol/L salt, 30 mmol/L borate buffer and glutathione. Other conditions see Figure 1.

to the stacking effects of glutathione (GSH and GSSG were negative charged at the experimental conditions). On the contrary, for the salts A, B, C, D, and E, different anions presented for different N values. Compared with the results with the anions' mobilities ($Br^- > Cl^-(I^-) > NO_2 > SO_4^{2-} > CH_3COO^- > CO_3^{2-}$),[17,18] it was found that the stacking effects increased as the mobilities increased. This was similar to the mechanism of the NaCl-induced isotachophoresis process (ITP); the anions in high concentration and with high mobilities moved ahead and all the analytes acted as "leading ions".[19,20] This also was supported by Shihabi.[13,14] In the experimental conditions selected, Cl^- (A) And NO_2^- (B) seemed to have better effects than the others, for their mobilities were larger than that of GSH and GSSG. While the mobilities of SO_4^{2-} (D) and CH_3COO^- (C) were lower than GSH and GSSG, they possibly acted less as "leading ions"; as a result, the N values were just like that in LVSS (Figure 2A).

In addition, the stacking effect of $CaCl_2$ was approximately one time larger than that of the NaCl, which was due to the concentration of the Cl^-.[15]

From all of the above, we could conclude that anions were the important factors for stacking glutathione (negative charged), and that the effect of the salt was similar to that of the ITP.

3.3 Stacking Phenomena of Eight Samples

In this section, eight samples were stacked under the same condition. The composition of the eight samples and their injection volumes were shown in Table 1 and the electropherograms were shown in Figure 4. Figure 4a showed that the GSH and GSSG could not be detected in the typical CZE mode because the concentrations of the analytes were lower than the LODs. In the LVSS mode (Figure 4b and 4c), as the injection volume increased, GSH and GSSG were detected. But the resolution between GSH and GSSG

Table 1. Composition of the eight samples and their injection volumes (μL)

	Injection volume	GSH	GSSG	Buffer	H_2O	ACN	NaCl
a	0.3 psi, 3 s	5	5	—	90	—	—
b	0.5 psi, 24 s	5	5	—	90	—	—
c	0.5 psi, 24 s	5	5	10	80	—	—
d	0.5 psi, 24 s	5	5	90	—	—	—
e	0.5 psi, 24 s	5	5	80	—	—	10
f	0.5 psi, 24 s	5	5	—	20	70	—
g	0.5 psi, 24 s	5	5	—	80	—	10
h	0.5 psi, 24 s	5	5	—	10	70	10

Note: The total sample volume is 100 μL.

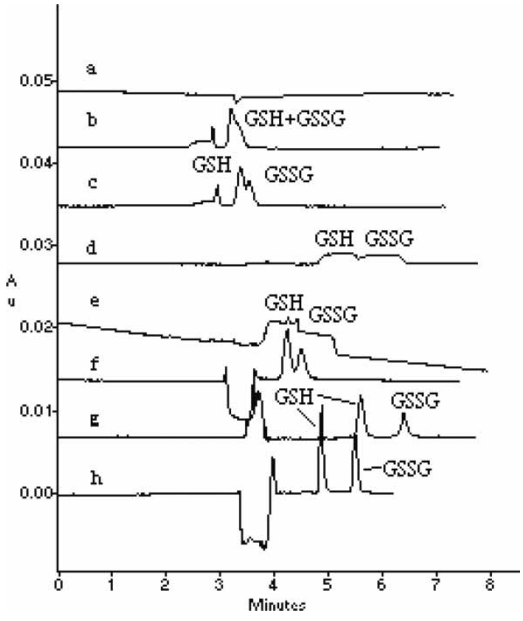

Figure 4. Comparison of eight electropherograms in same CE condition: 300 mmol/L pH 8.0 borate buffer as the BGS. Other conditions see Figure 1 and Table 1.

was too small so that the two peaks overlapped. All this showed that LVSS has little stacking effect in the selected conditions.

When the sample contained ACN (Figure 4f), the stacking phenomena really occurred in the LVSS mode. As ACN has lower viscosity than water, analytes move faster in SZ (f) than in SZ (b), which results in a better stacking effect (see Section 3.1). Comparing Figure 4 g with 4 h, we could also draw the same conclusion.

In comparing Figure 4b with 4 g, we found that the presence of salts seemed to make stacking better. This was confirmed by comparing Figure 4f with 4 h. However, in samples d and e, the presence of the salts could not assist SZ to obtain the stacking phenomena. This might indicate that salts could not act as the assistor unless in the presence of a special sample zone, which has a lower conduction and lower viscosity than that of the BGS. The higher resistant SZ provided a higher electric field and the lower viscosity provided a faster migration velocity of the analytes. In the absence of the special SZ, stacking phenomena would not occur properly. As it was stated in the section above, this is similar to the mechanism of the transient-ITP method. Salts acted as "leading ions", while the ACN would act as "pseudo terminating ions".

Based on the results in this section, it was found that ACN likely could help the stacking in the ASS mode by itself (like that in LVSS) and

probably act as an assistor of salts in stacking, as well as the fact that the salts could not make the stacking phenomena occurred by themselves. The way that the salts affected stacking was also considered to be like the ITP step.

3.4 Current Curves During ASS, LVSS, and CZE Processes

In Figure 5, the current of CZE, LVSS, and ASS mode were shown. In CZE mode, the current was approximate to a straight line, because the contribution of the SZ was same the BGS; the whole capillary was filled with one buffer. Unlike the current under CZE mode, there were four areas in the current curve of a typical ASS procedure (Figure 5A–5D). (C) was a decreased (an increased) area, B and D both were approximate lines. The possible reason could be that, at the initial status, the SZ had low viscosity and conduction due to the 70% ACN, and the salts in the SZ adjusted the conduction to a high level; as the voltage is added, the current should be high. Once the voltage is added, the ions in the SZ would move quickly out in the presence of CAN; as a result, the conduction of the SZ would decrease, and the current decreased correspondingly. That the ions in BGS moved slower than ions in SZ made the ion stress of SZ decrease in the short period of time (Figure 5A).

When the moving-in and moving-out action of the ions between SZ and BGS reached the equilibrium, the current became stable (area B). All the fluid

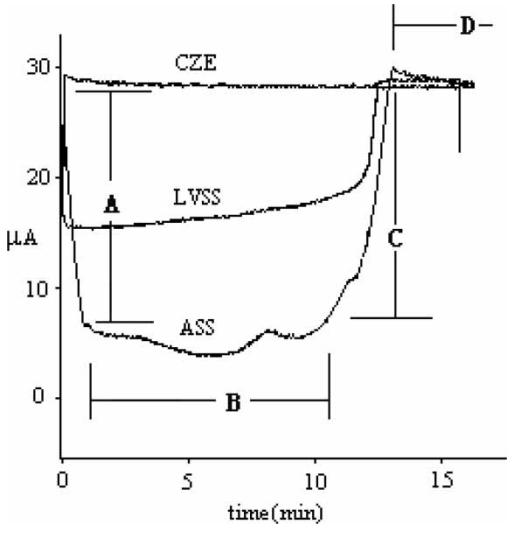

Figure 5. Comparison of current curves in CZE, LVSS, and ASS mode. Conditions: CZE, LVSS, and ASS were refered to the sample a, b, and h in Table 1. Other conditions see Figure 4.

in the capillary moved towards the outlet (anion node) under the drive of electroosmotic flow (EOF). Once the SZ began to move out of the capillary, the conduction of the fluid in the capillary decreased, and the current increased (as area C) correspondingly. When all the SZ moved out of the capillary, the current became stable again, as in the CZE mode.

Compared with the current of LVSS with ASS, it was found that the "decreasing area" was not intense in LVSS. The reason was that the velocities of the ions were lower than that of ASS; as a result, either the resistance of the SZ or the current increased slowly.

Based on the facts, we could conclude that the presence of ACN, which performed a high electric field and low viscosity, are one of the main forces for stacking, and that the stacking phenomena would take place in a short period of time (in area A or early area B). Therefore, the mechanism would be a quick LVSS procedure.

3.5 Fast Pull-Out Experiments

As stated in the sections above, stacking phenomena may occur in a short period of time, and the influence of the stacking time was studied. In this section, all the fluid inside the capillary was pulled out under high pressure (20 psi) after a different stacking time. The electropherograms were shown in Figure 6. A-H in Figure 6 was presented for 0.5, 1, 2, 4, 5, 6, 8, and 10 min of stacking, respectively. It was found that the peak width at 0.5 min was similar to that of 10 min, as well as the height at 0.5 min also similar to at 10 min. This indicated that the concentrating procedure should be a quick step. Within 0.5 min (or less than 0.5 min), the sample would stack between SZ and BGS for different velocities in each zone. Based on these facts, it was further confirmed that the ASS stacking procedure was a quick step.

3.6 Possible Mechanism of ASS

From all the above, a possible scheme of the mechanism was achieved and shown in Figure 7. The whole procedure could be divided into four parts, part I (step A and B), part II (step C), part III (step D–F), and part IV (step G).

In part I, step A, a sample zone was introduced into the capillary under pressure (0.5 psi) from the left side, then a separation voltage of 5 kV was added as described in the Figure 7A (positive node at inlet). As a result, the direction of the EOF was from left to right. Once the voltage was added (in step B), the ions (both anions and cations) in the SZ would migrate faster (anions toward left side and cations toward right side) than that in BGS, for the conductivities of the SZ was lower than the BGS. When these ions moved through the SZ and reached the BGS, they were stacked and concentrated (step B) as their migration velocity reduced. These ions moved so

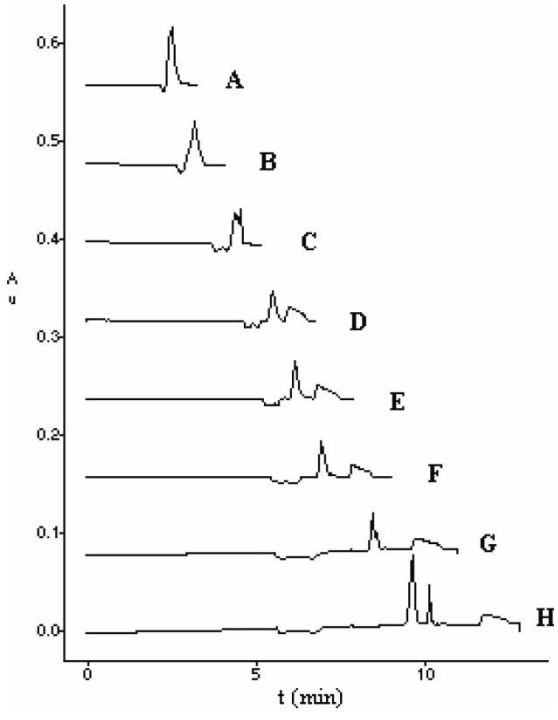

Figure 6. Influence of stacking time on stacking. Conditions see Figure 4.

quickly that part I occurred in a short period of time (see section above). Furthermore, the current during this part decreased quickly because of the reason described in a previous section (this part corresponded to part A of Figure 5). In other words, the mechanism of part I was similar to LVSS. The ACN was the main cause of the velocity difference and stacking, so that this step could be named as ACN-caused "quick LVSS step, q-LVSS".

In part II, the stacked ions between the sample zone and BGS, were arranged in the order of their mobilities. For example, at the left interface, the anions were Cl^-, GSSG, GSH, borate ions from left to right (step C). This was similar to the NaCl-induced isotachophoresis stacking method,[19,20] in which the Cl^- acted as "leading ion" for its high mobility and concentration. The ACN, which provided a high electric field, in the sample zone might act as the "pseudo terminating ions".[13,14] The stacked zone would be further condensed in this step. As the ACN just moved as the EOF, the isotachophoresis process would finish when the stacked zone left the SZ. This step was also a short time process, therefore, it would probably be called a "transient isotachophoresis, t-ITP" procedure.

Once the stacked ions zone left the SZ, a CZE process, the most time consuming step in the whole procedure, began (part III). The well stacked

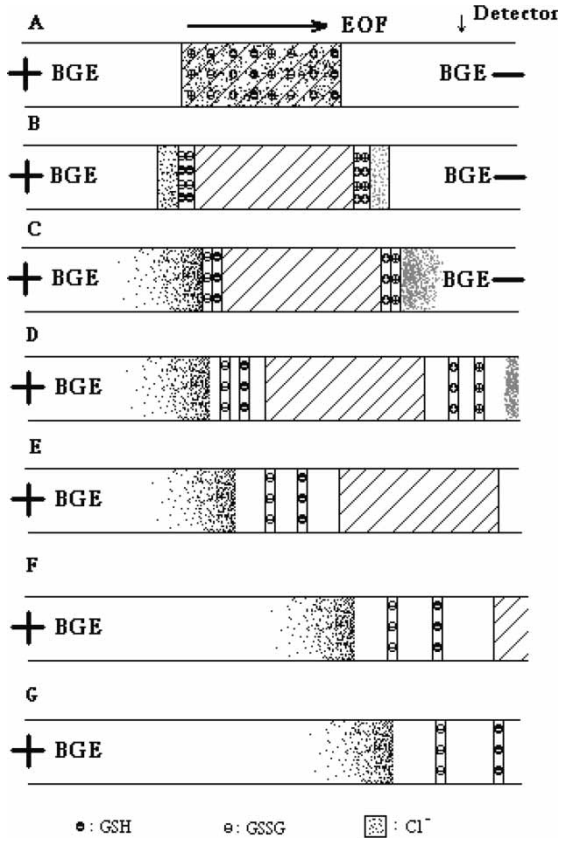

Figure 7. Schematic drawing of the possible mechanism.

ions were separated with their different mobilities and pushed towards the outlet of the capillary by EOF. When they passed the detection window, they were recorded (steps D–F). During step D and E, the current was stable and the conduction of all the fluid in the capillary was steady (part B of Figure 5). In step F, as the SZ began to move out of the capillary, the conduction of the fluid decreased and the current increased until all the SZ was replaced with BGS (part C in Figure 5). Moreover, as the current increased, more voltage was added onto BGS, and the CZE separation process and velocities of the ions in BGS would be accelerated.

Once the SZ moved completely out of the capillary (part IV), the current restored to the level of CZE (part D in Figure 5).

In other words, we could conclude that the mechanism of the ASS would be an ACN caused quick LVSS and salts induced transient-isotachophoresis procedure (q-LVSS-t-ITP), coupled with CZE separation step.

3.7 Test of the ASS Mechanism

The mechanism in the section above also indicated that cations could be stacked at the other side (right side in Figure 7) of the SZ, and the cations and anions could be easily stacked and analyzed in a single procedure without interfering with each other. Diltiazem hydrochloride, metoclopramide, cysteine, and uric acid were selected to test the obtained mechanism. The four analytes could be divided into two groups under the selected experimental conditions. One group was positively charged, such as diltiazem hydrochloride, metoclopramide, and the other was negatively charged. The concentrations of the four analytes were both lower than their LODs, so that they could not be detected under normal CZE mode (as shown in Figure 8b, no peak was found). However, when it came to q-LVSS-t-ITP, the case changed. Both cations and anions were well stacked simultaneously as the injection volume was increased (Figure 8a), and as expected, the cations were stacked at the outlet side of the sample zone and detected earlier than

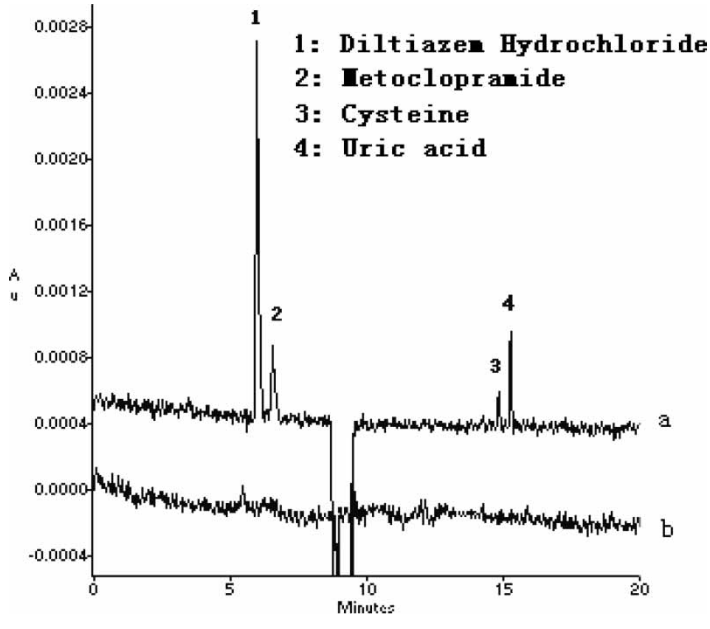

Figure 8. Stacking of anions and cations with q-LVSS-t-ITP simultaneously. Figure 8a was under the ASS mode with an injection volume of 12 s at 0.5 psi, Figure 8b was under CZE mode with a typical injection volume of 3 s at 0.3 psi. Conditions: 100 mmol/L pH6.5 phosphate buffer as the BGE, at 200 nm, 25°C. Sample contained 70% ACN, 45 mmol/L NaCl, 10 mmol/L pH6.5 phosphate buffer and the four analytes. The negative peak in Figure 8 was presented for the sample zone which contained 70% ACN.

the sample zone, while the anions at the inlet side appeared after the sample zone. This further proved the obtained mechanism.

4. CONCLUSION

The mechanism of the ASS technique was proven to be a q-LVSS-t-ITP process, coupled with a CZE separation step. The ACN was the main force for the quick LVSS step and could act as "pseudo terminating ions" in the t-ITP procedure, where the salts acted as "leading ions". The obtained mechanism also showed the possibility of stacking cations and anions simultaneously.

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