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Organic solvent high-field amplified stacking for basic compounds in capillary electrophoresis

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Abstract

Many water-miscible organic solvents, especially acetonitrile and acetone, bring along significant degrees (~30 times) of stacking by electroinjection through high-field amplified injection for the basic compounds compared to that for aqueous buffers or water. The relative stacking of different compounds in acetonitrile or acetone is different compared to that for water. Stacking by electroinjection in organic solvents is less stringent and easier to accomplish in practice. Acids and salts, in aqueous solutions, can ruin the stacking for both organic and aqueous solvents; however, this effect can be better tolerated by diluting the sample in acetonitrile. Thus, this stacking is termed "organic solvent high-field amplified injection". This stacking by electroinjection is enhanced by increasing the electrophoresis buffer concentration and can be better than that by pressure injection. From the practical aspects, some cationic drugs present in serum such as amiodarone can be detected at the therapeutic levels by electroinjection on the capillary after protein precipitation by acetonitrile.

Keywords: Stacking; Basic drugs; High-field; Electromigration; Electroinjection; Pressure injection; Amiodarone; Quinidine

1. Introduction

It remains to be a common practice in CE to dissolve the sample in aqueous buffers, especially in a dilution of the same separation buffer, or just in plain distilled water [1,2] and inject it hydrodynamically on the capillary. This approach is simple and gives satisfactory separation in the majority of analyses. However, for compounds present at low concentration, it may not be a good choice for a sensitive detection. Previously, we have shown that anionic compounds dissolved in acetonitrile and salts and injected hydrodynamically concentrate 10–30 folds on the capillary (stacking) leading to improved sensitivity [3]. The acetonitrile has the added advantage of removing the excess of proteins present in the sample [4,5]. The basic mechanism behind this type of stacking is pseudo-transient isotachophoresis [5]. Stacking of basic compounds is more difficult than that for anionic ones [6]. Many of these compounds are hardly water-soluble and also they tend to adsorb to the capillary walls distorting their stacking. Many of the pharmacologically active drugs are basic compounds such as; amiodarone, morphine, codeine, oxycodone, tricyclics and catecholamines. These compounds are present in low concentration. Practical methods for measuring these compounds from biological tissues by CE are greatly needed.

Electroinjection (electromigration) is not used as commonly as the hydrodynamic injection for sample introduction in CE because it is subject to many variables, which can lead ultimately to concentration bias. On the other hand, Palmer et al. [7] as well as we [8] have shown that the electroinjection has the ability to concentrate the sample on the capillary far more than can be achieved by the hydrodynamic injection [7]. In theory, the capillary can be loaded very rapidly with sample at the inlet, beyond its full size; yet it remains to provide a good separation and good theoretical plate number [7,8]. This concentration is greatly needed to improve the poor sensitivity of the CE. Unfortunately, the electroinjection is greatly affected by salts and acids in the sample. Often

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cationic compounds are dissolved in dilute acids in order to solubilize them or to convey the positive charge. Excess of acids can arise also during protein precipitation especially in biological fluids such as plasma. Neutralization of acids can be difficult since it results in an excess of salts, which again ruins the separation.

In order to improve the detection limits of the basic compounds in CE, the combination of electroinjection and organic solvent field amplified stacking is investigated in this work. Here, we investigate the effect of diluent (aqueous versus organic) on the stacking by the electromigration, i.e. under field-amplified injection on the analysis. We demonstrate that electroinjection, in conjunction with dissolving the sample in acetonitrile, yields far better stacking than that obtained by pressure injection in conjunction with aqueous solvents. We study the effects of acids and salts on both types of injections. Furthermore, we attempt to extend these studies to the analysis of drugs in serum. Since stacking under electroinjection from acetonitrile or organic solvents is different from that of aqueous solutions it is suggested to be termed as "organic solvent high-field amplified injection".

2. Materials and method

2.1. Chemicals

Tyramine HCl, propranolol HCl, quinidine sulfate dihydrate, quinidine anhydrous and amiodarone HCl were obtained from Sigma Chemicals (Saint Louis, MO, USA); Quinine dihydrate from Aldrich Chemicals, Milwaukee, WI, USA); and amitriptyline HCl from USP Inc, Rockville, MD, USA.

2.2. Stock solution

Stock solution (300 mg/l in 20% methanol in water) was prepared of the following compounds: tyramine, amitriptyline, propranolol, and quinine sulfate. This stock solution was diluted 20 fold either in water, or acetonitrile. Amiodarone 100 mg/l was dissolved in 50% methanol. Quinidine anhydrous and quinidine sulfate (500 mg/l) were prepared in water.

2.3. Instrument

A Model 2000 CE instrument (Beckman, Fullerton, CA, USA) with a short capillary 30 cm × 50 μm (I.D.) (Polymicro Technologies, Scottsdale, AZ, USA) was set at 8 kV, 214 nm with hydrodynamic sample injection at 10 s or by electroinjection at 2 kV for 3 s.

2.4. Separation buffer

The separation buffer was phosphate, 60 mmol/l, pH 6.2.

3. Results and discussion

Electroinjection from water or low ionic aqueous buffers leads to a high degree of sample concentration on the capillary due to the high-field strength [2,9,10]. Here, two model basic drugs propranolol and quinine were dissolved in several diluents and the sample was introduced by electroinjection. The peak height of these two compounds in acetonitrile was about 40 times higher compared to that in distilled water or for that prepared in the diluted electrophoresis buffer. This indicates that a better stacking is taking place in many of these organic solvents relative to that for water. The mechanism for stacking in organic solvents, to some extent, is similar to that for water, i.e. high-field strength injection. However, it is also different since it is occurring in organic solvents which can affect the field strength, ionization and solubility of the analytes and also of the co-ions differently from aqueous solvents. Other organic solvents such as ethanol, isopropanol and acetone brought also similar stacking (Fig. 1). However, the degree of stacking and the relative ratio of the peaks heights are different. For example the ratio of propranolol to quinine peaks are 10, 3, and 2 in acetone, acetonitrile and water, respectively. Organic solvents also have the advantage that small ions and salts which can ruin the separation [10], have limited solubility in these solvents. For this reason, this type of stacking can be specifically termed "organic solvent high-field amplified injection".

In the second experiment, we studied the stacking efficiency (ES) [11] of quinidine as a sulfate and as anhydrous, where

$$\mathrm{ES} = \frac{\text{concentration in the sample zone}}{\text{concentration of the concentrated band}}.$$

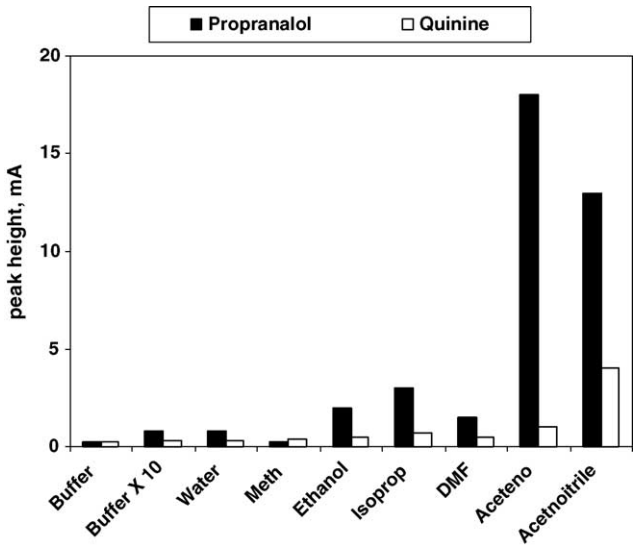

Fig. 1. Effect of different solvents on sample stacking of propranolol and quinine by electroinjection, 3 s at 3 kV: electrophoresis buffer, electrophoresis buffer diluted 10 times, water, methanol, ethanol, isopropanol, dimethylformamide, acetone and acetonitrile, respectively.

Table 1
Stacking efficiency for quinidine by electroinjection, 10 s, 3 kV, 214 nm

Acetic acid, pH 4 (mol/l)	Anhydrous		Sulfate	
	Acetonitrile	Water	Acetonitrile	Water
0	17	2	48	5
0.35	30	6	45	9
0.70	24	5	41	9

The sulfate form is a water-soluble compound while the anhydrous is hardly water-soluble. The stacking efficiency was better for the water-soluble (sulfate) form of the drug in both water and in the acetonitrile (Table 1). The anhydrous gave in plain water two small peaks probably representing the ionization of the two N atoms. The stacking improved in water by keeping the compound ionized (below the pk_a) (Table 1). Thus both the solubility and the ionization play a role in the stacking as expected. But in all cases, the acetonitrile gave better stacking than water or in the acetate buffer reflecting the higher field strength in this solvent. The *N* for sulfate form in water and acetonitrile was 43,000 and 74,000, respectively. In simple words, organic solvents stacking in practice is more efficient and less stringent than that of the aqueous buffers.

Based on sample introduction by electromigration, the four cationic compounds in Fig. 2, can be separated well regardless if they were dissolved in water or acetonitrile. All the peaks are much taller (better stacking) in the presence of acetonitrile compared to that for water (Fig. 2A and C, note the four times difference in the absorbance scale). Overall, there is about 15 times on the average increase in peak height due to the presence of acetonitrile in the sample compared to that for water. However, the degree of stacking for each compound (as relative peak heights) is different for water from that for acetonitrile (Fig. 2A versus Fig. 2C) similar to what has been noted in Fig. 1. For example, in water, amitriptyline has the same peak height as that for quinine; while in acetonitrile the amitriptyline peak has more than twice the peak height of quinine. Again, this may be a reflection of the difference in solubility or in ionization of these compounds in the two types of diluents.

The addition of acids such as phosphoric acid, 75 mmol/l decreased all the peaks in the electromigration (Fig. 2B and D) but more in the case of samples dissolved in water. For example, the quinine peak decreased by ~67% in water and by only 50% in the acetonitrile. The peaks remained much taller in the presence of acetonitrile (note the 4× difference in the scale) compared to that in water. Addition of salts (8 mmol/l NaCl) decreased also the peak height similar to that for acids with more effect on samples dissolved in water. For example, the quinine dissolved in water decreased by 88% and for that dissolved in acetonitrile by 78%. Thus, acetonitrile is a different and better alternative as diluent for samples containing acids or salts.

The effects of preparing the sample in acetonitrile versus water are compared also using the pressure injection (Fig. 3). When the compounds are prepared in water and injected by

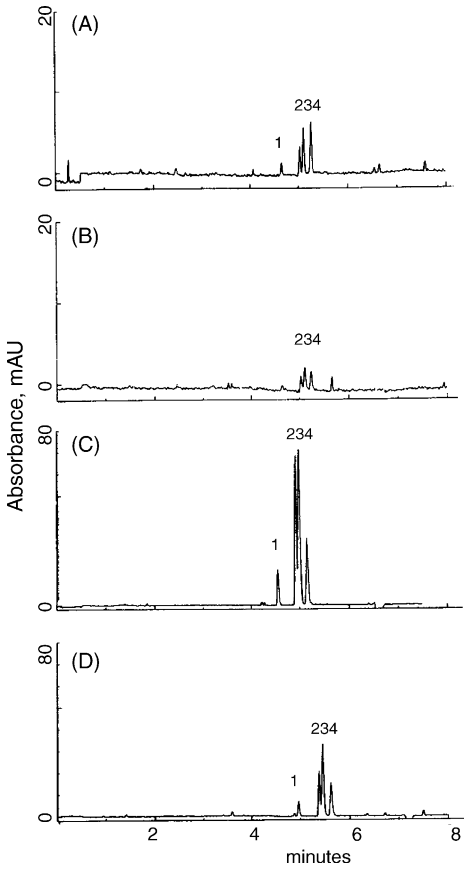

Fig. 2. Effect of acid and acetonitrile in the sample on the separation in the electroinjection mode, 3 s at 2 kV: sample dissolved in (A) water in absence of acid; (B) water and 75 mmol phosphoric acid; (C) acetonitrile in absence of acid; and (D) acetonitrile and 75 mmol/l phosphoric acid. Stock compounds at 20 mg/l: 1-tyramine, 2-amitriptyline, 3-propranalol, 4-quinine sulfate dissolved in 1 ml methanol + 5 ml water. This was diluted 20 folds either in water, or acetonitrile.

pressure, a reasonable separation is observed (Fig. 3A). However, if the sample is diluted in acetonitrile rather than water (or in the separation buffer) and injected by pressure, the separation is improved as the propranolol and amitriptyline peaks are better resolved (Fig. 3C compared to Fig. 3A). The difference becomes more noticeable when the sample contains an acid such as phosphoric acid, 75 mmol/l (Fig. 3B compared to Fig. 3D). In the pressure injection, the acid ruins the separation when the sample is diluted in water (Fig. 3B); while the acetonitrile counteracts this effect and improves slightly the resolution and the peak height (stacking) (Fig. 3D). However, for the acetonitrile, the peaks are much taller by the electroinjection compared to that by pressure injection (Fig. 2C versus Fig. 3C); provided excess of salts and acids are absent.

The effect of acids in the pressure injection is different from that observed in the electroinjection. A low concentration of acid in the sample <100 mmol/l, improved the peak height when the sample is introduced by pressure injection and contained acetonitrile (Figs. 3 and 4). However, in the electromigration, most of the concentrations of acid decreased the peak height but it was dependant on the

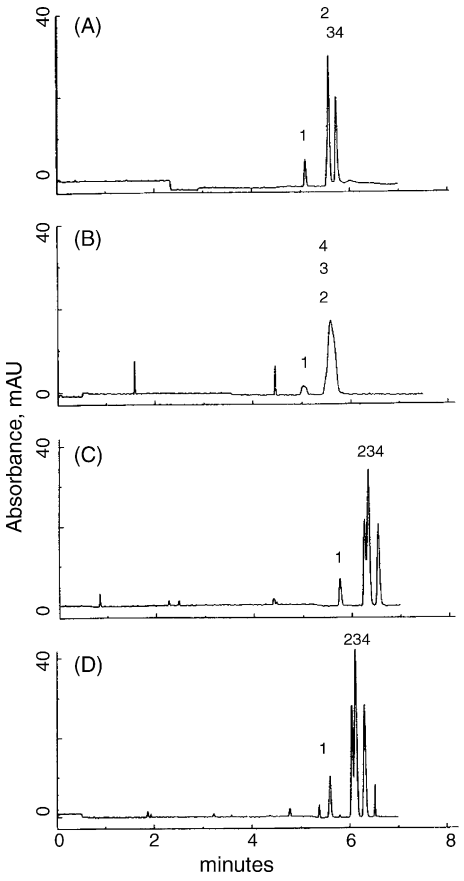

Fig. 3. Effect of acid and acetonitrile in the sample on the separation by pressure injection, 10 s: sample dissolved in (A) water in absence of acid; (B) water and 75 mmol/l phosphoric acid; (C) acetonitrile in absence of acid; and (D) acetonitrile and 75 mmol/l phosphoric acid; compounds as in Fig. 2.

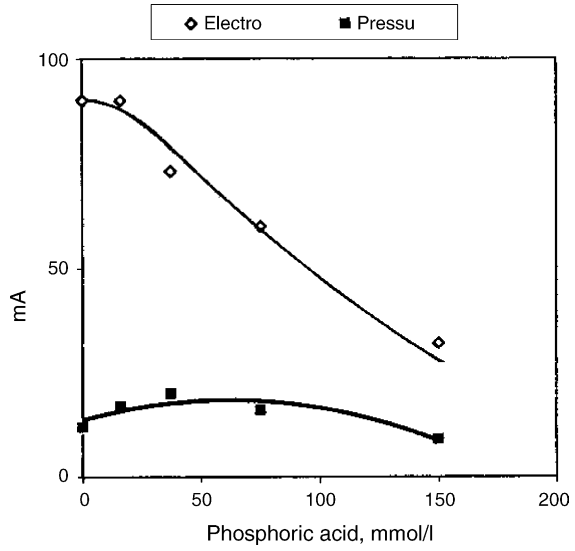

Fig. 4. The effect of different concentration of phosphoric acid in the sample on peak height for propranolol in both pressure and electroinjection.

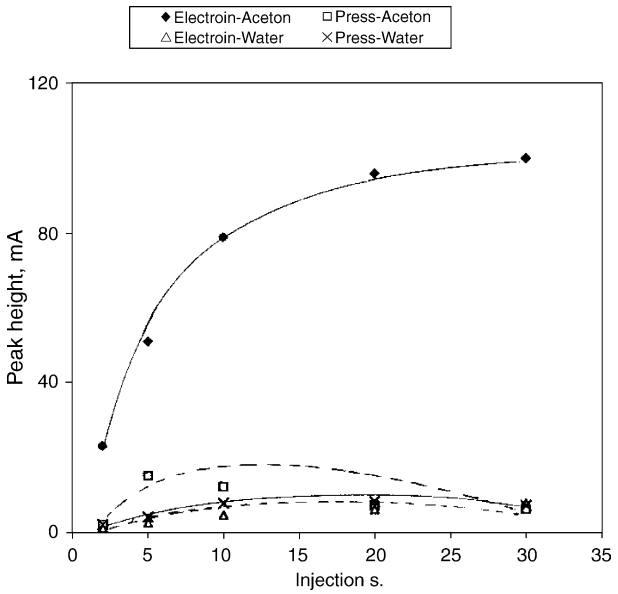

Fig. 5. Effect of different sample volumes in seconds by pressure and by electroinjection on the peak height of propranolol. The sample was dissolved in 90% acetonitrile or water in presence of 50 mmol/l phosphoric acid.

compound too. The decrease is related to the concentration of the acid (Fig. 4). Thus, the influence of small ions on the separation is affected by the type of injection. Although the acid in conjunction with acetonitrile, in general, decreases the peak height by electro-injection; this can be compensated, to some extent, by increasing the time or voltage of the injection. Other acids such as trichloroacetic acid, acetic acid and hydrochloric acid gave similar results to the phosphoric acid.

Regardless of the presence or absence of the acid in the sample, the peak height increased non-linearly and to a certain limit with increase in sample injection time (Fig. 5) in both types of the injections. However, the increase was much better in the case of acetonitrile by the electroinjection. The difference between the acetonitrile and water is more obvious as the concentration of acid in the sample increases. There was about 5-, 10- and 20-fold difference in peak height at 0, 100, and 150 mmol/l phosphoric acid, respectively. The decrease of peak height in the electromigration by the acids probably can be explained by the hydrogen ions migrating rapidly to the capillary inlet ahead of the analyte ions and competing with the transfer of analytes in addition to their effect on the ionization. On the other hand, the acetonitrile gives higher field strength (field amplified injection) compared to the aqueous solutions so the peak height remains higher in the presence of acetonitrile compared to that for water or dilute buffers. In addition to that, during the electromigration, the buffer co-anions migrate to the anode, which is immersed in the sample. These co-ions can surround the sample cations and hinder their movement especially at the inlet of the capillary. However, when acetonitrile is used to dissolve the sample, the co-ions probably migrate more rapidly in the acetonitrile compared to water (due to the higher field

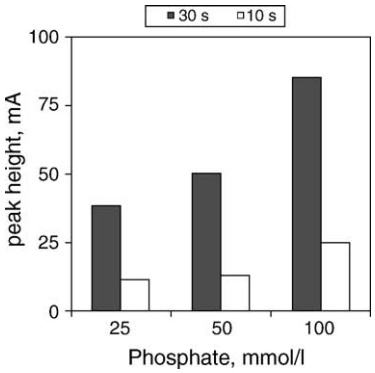

Fig. 6. Effect of the concentration of the separation buffer, phosphate, on peak height of propranolol by electroinjection at 10 and 30 s.

strength) moving further away, far from the outlet of the capillary where the cations are stacking.

As the concentration of the separation buffer is increased the peak height is increased too in the electromigration (Fig. 6). This is a reflection of the difference of the field strength between the sample and the buffer. Separation buffers, with high concentration, enhance peak height, improve the resolution and allow a better toleration of salts and acids in the sample.

The stacking of basic compounds is different from that of anionic compounds. By pressure injection, acetonitrile as a diluent in the sample gives good stacking (ES ∼ 5–30 times) for the anionic compounds [4,5]. However, the stacking is more difficult for the cationic compounds [6]. On the other hand, the effect of acetonitrile in the electroinjection can be very dramatic ∼2–20 times compared to pressure injection, provided again the salts and acids are absent or very low in concentration. The stacking by field-amplified injection depends on the transfer of the compound from the sample zone to the capillary inlet tip. This in turn depends on the field strength, ionization, salts, and injection time among other factors. Thus many factors have to be optimized for the field-amplified injection. The stacking efficiency was much better for organic solvents in all cases compared to that for aqueous buffers (Table 1).

The mechanism behind stacking of basic compounds from aqueous buffers in the electromigration and pressure injection is similar and depends on the high-field strength. On the other hand, stacking from water-miscible organic solvents is different in the electromigration from that by pressure injection. In the electromigration, simple high-field strength is responsible for the stacking while in the pressure injection often transient pseudo-isotachophoresis step causes the stacking. The small amounts of salts in the sample aids the stacking by acting as leading ions while the organic solvent acts as pseudo-terminator [5]. Thus the electroinjection in organic solvents is the preferred method when the salts and acids are very limited. On the other hand, pressure injection in organic solvents is preferred when the sample has excess of salts or acids.

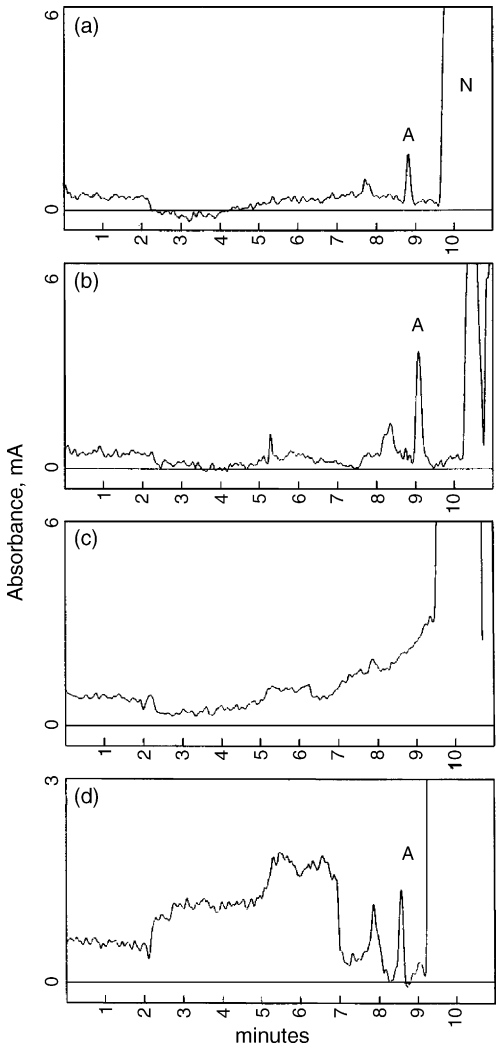

Fig. 7. Therapeutic monitoring of amiodarone (A); standard 5 mg/l diluted in water: (a) 100 μl of amiodarone standard and 200 μl acetonitrile with 70 s pressure injection; (b) the same previous sample but with 70 s electroinjection at 5 kV; (c) 100 μl of amiodarone standard and 200 μl water; and (d) 2.4 mg/l of amiodarone added to human serum acidified with 10 μl of phosphoric acid, 0.8 mol/l and deproteinized with 200 μl acetonitrile with electroinjection for 70 s at 5 kV. The separation buffer was phosphate, 170 mmol/l, pH 5.9, containing 30% isopropanol. The capillary was 32 cm × 50 um (I.D.), at 8 kV with 254 nm detection.

It is important to be able to extend these observations to the practical tests such as for the assay of drugs in biological fluids. As a preliminary experiment, amiodarone-HCl stock standard was diluted in water and added also to serum at final concentration of 2.4 mg/l. This value is within the therapeutic level (1–2.5 mg/l). Two volumes of acetonitrile to one volume of serum were used to remove the proteins and dissolve the drug. In spite of using a general filter of 254 nm, where the drug has about half of the UV absorption of 245 nm (the maximum absorbance of the drug), values <1 mg/l, i.e. below the therapeutic range, can be detected by this technique (Fig. 7d). Amiodarone standard gave better peak height by the electroinjection (Fig. 7b). When acetonitrile is absent from the sample, no peak can be detected by the electroinjection

(Fig. 7c). The RSD for five injections of the standard for peak height was 3.45% and for migration time was 0.97%. This example illustrates the practicality of the organic solvent for field-amplified injection especially for compounds not readily soluble in water. It provides enough sensitivity to enable therapeutic monitoring of many drugs in blood within the therapeutic range without the need for sample extraction or concentration. Of course, some drugs are present in serum at very low concentration beyond this technique.

4. Conclusion

Regardless of the type of injection method, preparing the sample in water-miscible organic solvents yields better stacking than that in aqueous buffers. This work illustrates that high sample concentration is obtained based on field-amplified injection from organic solvent. Many factors affect the electroinjection in CE—especially the presence of acids and salts. However, stacking by electroinjection in organic solvents is less stringent. Under the proper conditions, this stacking can yield better concentration and better separation than that by the hydrodynamic injection, provided the sample does not contain an excess of salts and acids. By selecting electroinjection in combination with acetonitrile a concentration factor of 50–70 folds can be obtained for basic compounds over the traditional method of pressure injection from aqueous solutions. Pressure injection from organic solvents can tolerate more salts and acids in the sample than the electroinjection. Although stacking from organic solvents under electroinjection can be affected more than the hydrodynamic injection, it is very simple to perform and deserves further investigations. From practical aspects, it is easy to prepare the sample in organic solvent and try both injection methods in order to choose the best one for a particular application. This data has practical implications in analysis of basic compounds such as drugs present in biological tissues. Based on the data presented here, dissolving the sample in the same separation buffer but at 10 times dilution or just in water is not always the best choice for improving the sensitivity in CE.

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