The Use of a Short-end Injection Procedure to Achieve Improved Performance in Capillary Electrophoresis

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Key words

Capillary electrophoresis
Short-end injections

Summary

Minimum capillary lengths on commercial instruments are fixed and cannot be decreased further. To effectively reduce the capillary length used for separation the sample can be injected from the end of the capillary nearest the detector. This procedure is known as a 'short-end' injection and can reduces analysis times by at least two-thirds compared to conventional injections. The time reduction benefits are shown in rapid separations of basic drugs, drug-related impurities and chiral compounds. Short-end injections, in combination with both increased electrolyte strength and reduced voltage are an effective approach to reducing the detrimental impact of high sample solution ionic strength. They can also lead to improved resolution by increasing stacking effects and reducing peak tailing. Peak area and migration time precision obtained are shown to be equivalent to those obtained for conventional injection procedures. It is concluded that short-end injections should be considered for routine operation as they are a useful means of reducing analysis time, increasing sensitivity, decreasing buffer depletion effects. They also allow use of higher electrolyte strengths which can improve resolution and reduce peak tailing, and can overcome significant problems which occur when analysing samples containing high salt contents.

Introduction

In CE, as with all emerging techniques, the options available to optimize performance are still not fully characterised. Performance gains in CE can be achieved by use of the shortest capillary lengths possible. However, reduction in capillary length is limited with current commercial instruments by the distance between the point of injection and the position of the detector along the capillary. The minimum length of capillary on a typical CE instrument may be 25–50 cm depending upon the particular instrument. The migration time of a peak is related to the V cm^{-1} (volts/total length [l]) and the distance (l_d) to the detector (Figure 1) from the point of injection. To obtain rapid analysis a high voltage should be applied across as short a length of capillary as possible but, due to reasons of electrical insulation, the maximum voltage that can be applied on standard CE instruments is usually about 30 kV.

Previously the use of short capillary lengths (l) has been explored [1, 2] to reduce analysis times in CE, for example 34 cm long capillaries have been employed in combination with 25 kV to perform MEKC analysis of paracetamol in biosamples [1] with an analysis time of 1 min. Rapid separations of drug impurities have also been obtained by applying high voltages across 27 cm capillaries [2]. However resolution is diminished [1, 2] when using short separation lengths

Injection is conventionally performed at the capillary end furthest away from the detector. On certain CE instruments it is possible to introduce the sample at the capillary end nearest to the detector which minimises the distance the solute has to travel prior to detection. This procedure is known as a short-end injection. It is necessary to reverse the voltage polarity following a short-end injection as the solutes need to move backwards towards the detector. The use of short-end injections procedures is well recognised in CE, particularly during method development. However, the extent of the potential benefits of this procedure is not well appreciated and these benefits are clearly summarised and reviewed in this paper.

It is generally advantageous to perform separations using electrolytes with the highest possible concentration, as this can have benefits in terms of sensitivity, resolution and prolonged routine performance. Sensitivity and resolution increases are obtained due to increased "sample stacking" [3], which concentrates the

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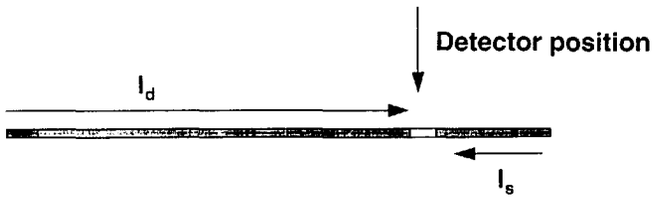
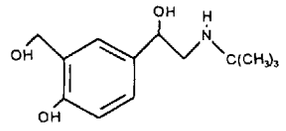
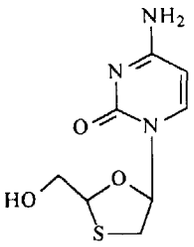


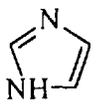
Figure 1
Configuration of the capillary lengths.



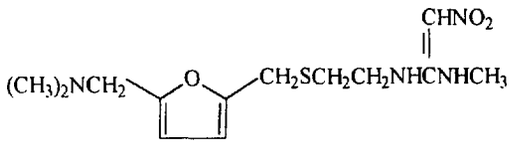
Salbutamol



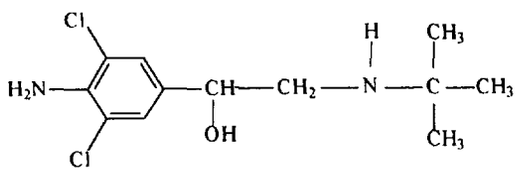
Lamivudine



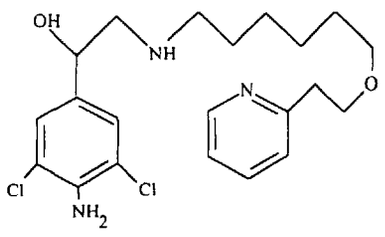
Imidazole



Ranitidine



Clenbuterol



Picumeterol

Figure 2
Chemical structures.

sample prior to separation resulting in improved peak height and improved resolution. Stacking increases [3] with use of higher electrolyte concentrations. However, the maximum permissible electrolyte concentration is limited by the Joule heating and current generated within the capillary. Operation with currents above 100 μA can lead to noisy and perturbed baselines due to refractive index changes of the electrolyte. This therefore limits the electrolyte concentrations that can be used for a given capillary bore and length and typical electrolyte concentrations are 20–100 mM. Reducing the capillary diameter permits increases in electrolyte concentrations but with losses in sensitivity.

Band broadening due to molecular diffusion of the solute is related to the diffusion coefficient (D) of the solute, the electrolyte viscosity and the analysis time. Large biomolecules such as proteins and DNA have low diffusion coefficients and are generally multiply charged with appreciable mobilities which therefore ensures that diffusion-related band broadening is minimal. However, diffusion-related band broadening are appreciable for small organic molecules, such as pharmaceuticals and agrochemicals. Increasing the electrolyte viscosity by addition of polymers such as cellulose [4] reduces diffusion but also reduce the electro-osmotic flow [5] causing alterations in selectivity. Rapid analysis reduces diffusion and can therefore give taller peaks and improved sensitivity.

The analytical benefits of using short-end injections has been assessed using a range of separations. The chemical structures of the compounds separated are given in Figure 2. Factors measured include analysis time, precision, sensitivity, tailing reduction, and reductions in sample and matrix interference.

A test mixture of 4 basic compounds (imidazole, aminobenzoic acid, aspartame and salbutamol) were separated using pH 2.5 phosphate buffer (50 mM or 250 mM NaH_2PO_4 adjusted with conc. H_3PO_4). These conditions are slight modifications of those previously validated [6] for a range of basic drugs. A degraded solution of ranitidine HCl was analysed using a 100 mM triethanolamine-phosphoric electrolyte (pH 2.5). Ranitidine impurities have previously been separated using a low pH phosphate buffer [7]. However, a triethanolamine-phosphoric acid combination has been demonstrated [8] to be a useful alternative to phosphate buffer, and in this instance gave improved performance.

Chiral separation of two racemic basic drugs, clenbuterol and picumeterol, was performed using pH 2.5 phosphate buffer (50 mM or 250 mM NaH_2PO_4 pH adjusted with conc. H_3PO_4) containing 30 mM dimethyl-β-cyclodextrin. Modified β-cyclodextrin has previously been used to resolve both clenbuterol [9] and picumeterol [10]

Electrolysis of the buffer (known as buffer depletion) occurs [11] with prolonged application of voltage across the system causing pH and ionic strength differences in the buffer vials at the ends of the capillary. This effect

can lead to a loss of selectivity and drifts in migration time. Therefore, it may be necessary to programme use of fresh vials of electrolyte throughout a prolonged injection sequence to maintain acceptable analytical performance [12] or to replace the contents of the separation vials. A reduction in analysis times will minimise depletion effects.

The short-end of the capillary protrudes from the thermostatted cartridge holding the capillary and is therefore not temperature regulated. This may have untoward effects on repeatability therefore multiple injections were performed in this study to measure precision.

Short-end injections can also permit the use of higher electrolyte concentrations since a lower voltage can be employed to give an equivalent migration time to that obtained using long-end injections. For example, to obtain a peak at 5 min a voltage of 30 kV may be required for a 30 cm capillary whilst this voltage can be reduced to 10 kV with one of only 10 cm, this in turn, reduces the current to a third that at 30 kV. Therefore, if a three fold increase in electrolyte concentration were used in conjunction with the lower voltage then an acceptable level of current would be maintained. The ability to use the higher concentration of electrolyte could lead to improvements of resolution due to improved stacking. This would also be beneficial where samples with high ionic conductivity may cause reversed stacking effects leading to band spreading and loss of performance [13]. Samples containing high salt levels can produce separations with distorted peak shapes and extremely poor resolution [14]. These sample matrix effects can be addressed through use of increased electrolyte concentrations. Additionally analysis of proteins and peptides is difficult due to excessive peak tailing caused by solute adsorption onto the capillary wall. Previously, concentrated electrolytes have been used to minimise adsorption [15], and used inconjunction with short-end injection may result in a further reduction in tailing.

Experimental

Work was performed on a Hewlett Packard 3-D (Waldbronn, Germany) CE instrument. Capillaries were purchased from Composite Metal Services (Hallow, Worcs. UK). Conventional long-end injections were performed using positive pressure and positive polarity voltage. Short-end pressure injection was performed using a negative setting for the pressure injection (mbar) in combination with a negative voltage. Capillaries of total lengths 34 cm were employed with an l_d of 25.5 cm and a short-end length (l_s) of 8.5 cm. Reagents were obtained from Aldrich (Poole, Dorset, UK). Drug samples were obtained from within GlaxoWellcome. Dimethylated-β-cyclodextrin was from Chinion (Budapest, Hungary). Water was obtained from a Waters MilliQ System (Watford, Herts. UK).

Results and Discussion

Analysis Time Reduction

Figures 3a (conventional) and 3b (short-end) shows the expected reduction in analysis time for the separation of the 4 component test mixture of basic drugs when performing a short-end injection and maintaining the same voltage. Figure 3b shows the anticipated slight loss of resolution. The short-end procedure gave slightly improved precision for actual and relative migration times (Table I).

The same test mixture was also analysed using a 250 mM phosphate buffer and a short-end injection with a reduced voltage of –7.5 kV. The results (Figure 3c) clearly illustrates the improvement in resolution using the higher buffer concentration, compared to the 50 mM buffer (Figure 3a). However, excessive current would have prevented operation at the voltage used to produce Figures 3a and 3b.

A solution of degraded ranitidine HCl was injected both by the conventional and the short-end modes (separations given in Figures 4a and 4b respectively). The short-end injection gave peak area precision for ranitidine of 1.4 % RSD (n = 10) and consistent resolution of impurities was observed in the ten injections. Table II shows the ranges, and precision data, and also the consistency of % a/a for the impurity peak at 1.22 min in Figure 4b.

A low pH phosphate electrolyte containing 30 mM dimethyl-β-cyclodextrin was used for separation of the two racemic drugs, from each other and from their individual enantiomers. Figure 5a shows the conventional separation and Figure 5b confirms that the resolution is still obtained, but with a considerably reduced analysis time, using the short-end procedure.

Sensitivity

As diffusion processes are reduced by minimising analysis time then sharper peaks and improved sensitivity would be expected to result from short-end injections. This was confirmed for the basic compound test mix (Figures 3a and 3b) where peak heights (including that of the main peak) were found to be approximately 50 % greater than for the corresponding long-end injections.

Similar sensitivity improvements were observed in the ranitidine impurity separations (Figures 4a and 4b). The low level (< 0.05 % area/area) of the impurities was clearly detectable in the short-end injection (for example the peak at 0.83 min in Figure 4b) which are not evident in the long-end injection.

Resolution and Peak Tailing

The use of higher ionic strength electrolytes was shown to be beneficial both in terms of increasing resolution but also in reducing tailing. For instance peak tailing was appreciably reduced for the chiral separations of

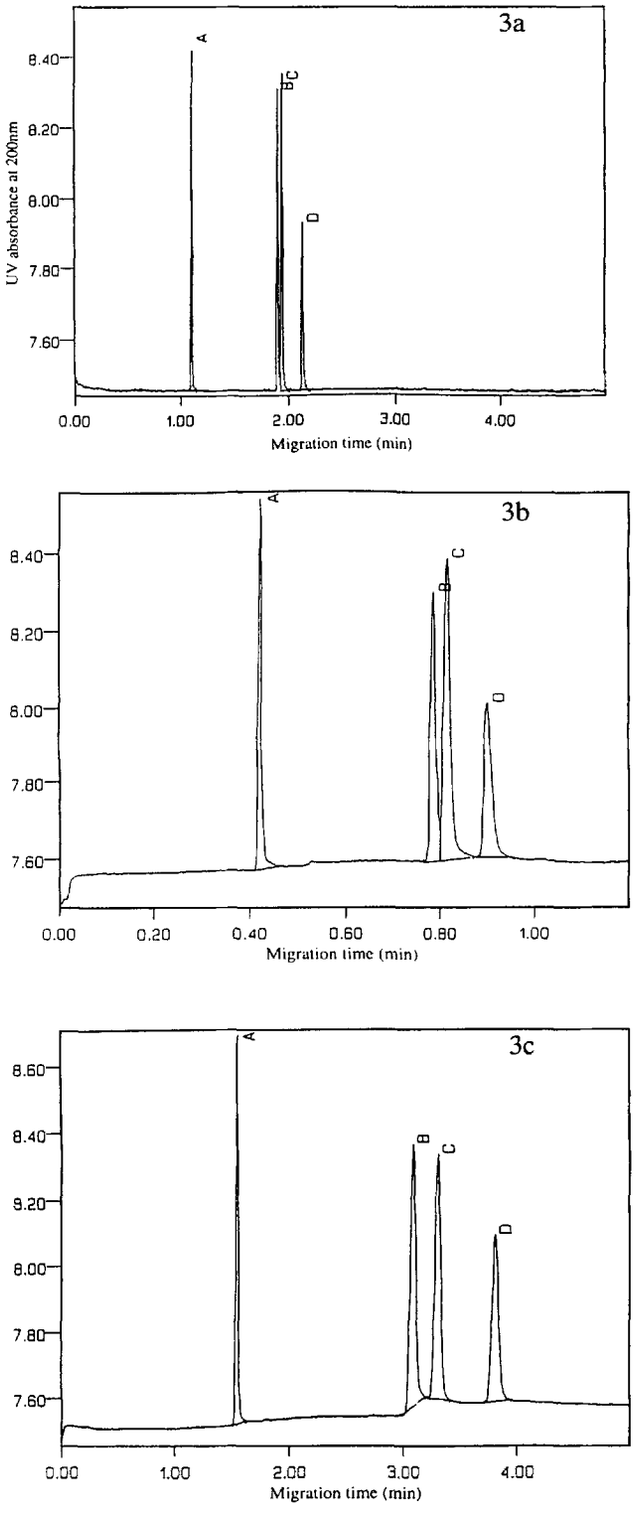


Figure 3

Separations of basic drug test mixture

a) Conventional-end injection of a basic drug test mixture containing **A** = imidazole, **B** = aminobenzoic acid, **C** = lamivudine, **D** = salbutamol. Separation conditions : 50 mM phosphate pH 2.5, +20 kV, 34 cm × 50 µm, detection at 200 nm, injection 5 s at 50 mbar.

b) Short-end injection of a basic drug test mixture. Separation conditions: 50 mM phosphate pH 2.5, –20 kV, 34 cm × 50 µm, detection at 200 nm, injection 5 s at –50 mbar.

c) Short-end injection with high concentration of electrolyte. Separation conditions 250 mM phosphate pH 2.5, –7.5 kV, 34 cm × 50 µm, detection at 200 nm, injection 5 s at –50 mbar.

Table I. % RSD of migration time and relative migration time for repeated injection of basic drug test mixture.

Separation conditions 1. Conventional injection of the basic test mix using 50 mM phosphate buffer at +20 kV; 2. Short-end injection of the basic test mix using 50 mM phosphate buffer at –20 kV; and 3. Short-end injection of the basic test mix using 250 mM phosphate buffer at –7.5 kV.

Precision Data %RSD (n = 10)							
	Peak A		Peak B		Peak C		Peak D
	Mt	*RMt	Mt	*RMt	Mt	*RMt	Mt
(1)	0.71	0.77	1.11	0.37	0.8	0.55	1.43
(2)	0.39	0.57	0.40	0.6	0.52	0.59	0.58
(3)	0.31	0.37	0.62	0.15	0.55	0.12	0.44

*(RMT= Migration time relative to migration time of peak D).

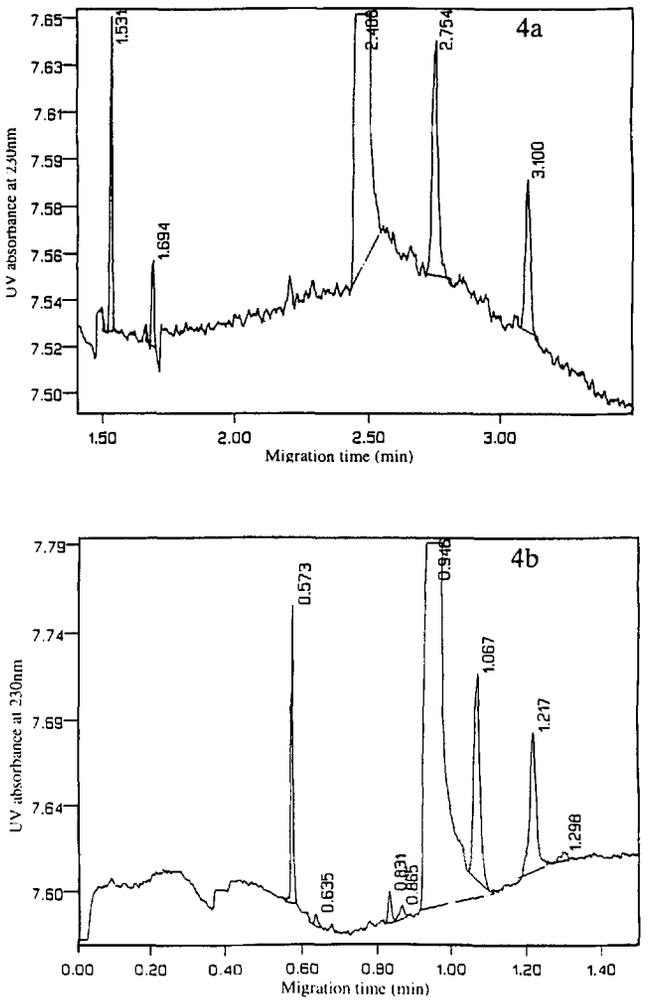


Figure 4

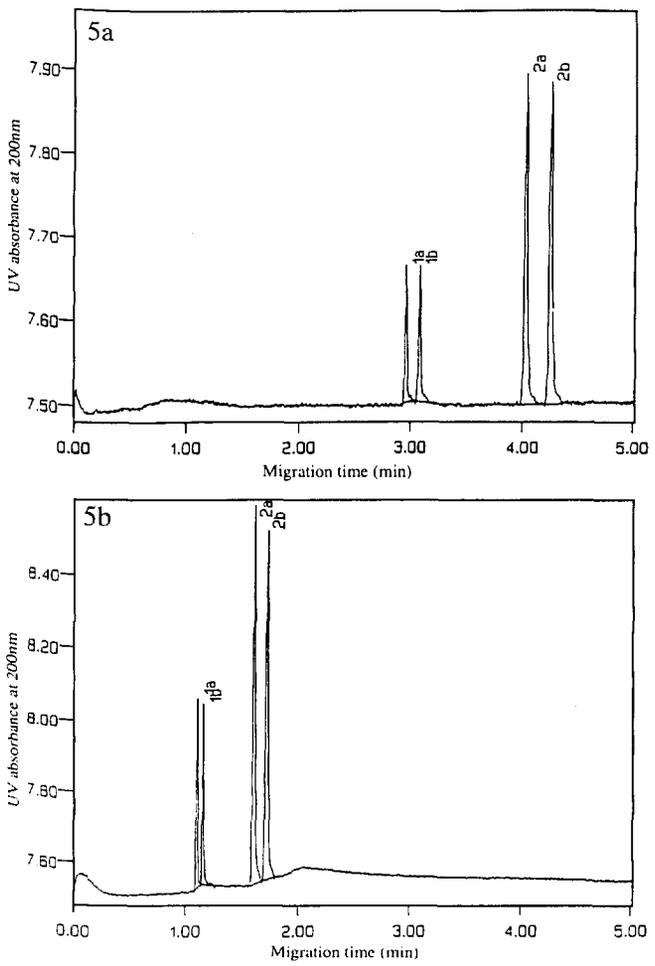
Separations of degraded ranitidine hydrochloride sample solution:

a) Normal-end injection of ranitidine. Separation conditions: 100 mM triethanolamine-phosphoric electrolyte pH 2.5, +20 kV, 34 cm × 50 µm, detection at 230 nm, injection 5 s at 50 mbar.

b) Short-end injection of ranitidine. Separation conditions: 100 mM triethanolamine-phosphoric electrolyte pH 2.5, –7.5 kV, 34 cm × 50 µm, detection at 230 nm, injection 5 s at –50 mbar.

Table II. Precision data for repeated short-end injections of a degraded ranitidine hydrochloride solution (n = 10).

Inj. no.	Ranitidine Mt (min)	Ranitidine % a/a	Ranitidine impurity % a/a	Main peak area
1	0.95	96.05	1.39	71339
2	0.93	96.19	1.43	70304
3	0.94	96.26	1.38	71962
4	0.94	96.12	1.43	71643
5	0.93	96.04	1.39	71724
6	0.93	96.29	1.36	73284
7	0.93	96.09	1.43	71802
8	0.94	96.07	1.38	70282
9	0.94	96.08	1.37	70184
10	0.94	96.11	1.39	70348
Mean	0.94	96.13	1.40	71287
%RSD	0.72 %	0.09 %	1.33 %	1.41 %



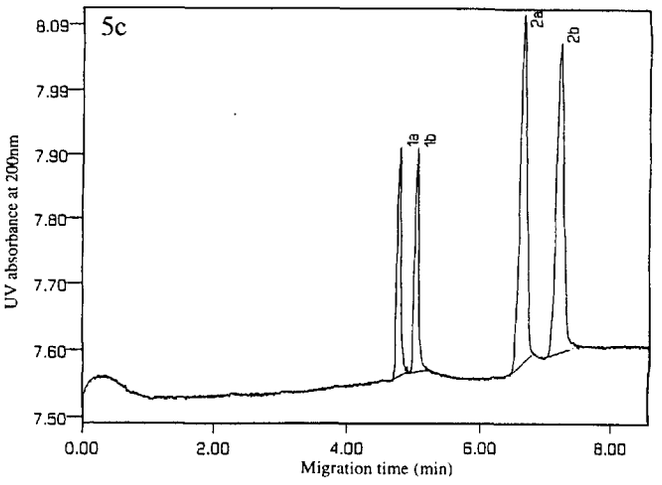


Figure 5

Chiral separations of picumeterol and clenbuterol:
a) Normal-end injection of picumeterol (1) and clenbuterol (2). Separation conditions: 50 mM phosphate pH 2.5 containing 25 mM dimethyl-β-cyclodextrin, +20 kV, 34 cm × 50 μm, detection at 200 nm, injection 5 s at 50 mbar. Peaks: **1a** and **1b**, **2a** and **2b** are picumeterol and clenbuterol enantiomers respectively.
b) Short-end injection. Separation conditions: 50 mM phosphate pH 2.5 containing 25 mM dimethyl-β-cyclodextrin, –20 kV, 34 cm × 50 μm, detection at 200 nm, injection 5 s at 50 mbar.
c) Short-end injection with high buffer concentration. Separation conditions: 250 mM phosphate pH 2.5 containing 25 mM dimethyl-β-cyclodextrin, –7.5 kV, 34 cm × 50 μm, detection at 200 nm, injection 5 s at –50 mbar.

picumeterol and clenbuterol using 50 mM and 250 mM buffer (Figure 5a and 5c). This higher electrolyte strength also generated improved symmetry factors (average 1.2 – calculated using USP definitions) compared to those obtained with 50 mM electrolyte (average symmetry factor of 1.4) This improvement is due to increased stacking and reduced tailing. These improvements would be of great benefit when quantifying low levels of undesired enantiomer present in a single enantiomeric drug.

Sample Matrix Effects

The presence of a high salt content in the sample solution can have an extremely deleterious effect on separation performance [15]. This is due to a "reverse stacking effect" resulting in localised heating of the sample zone, band broadening and significant deterioration in peak shape and resolution.

For example, 100 mM NaCl was added to the test mixture of basic compounds which had been previously separated (Figure 3a) using a 50mM phosphate buffer. The resultant anti-stacking effects caused by the high

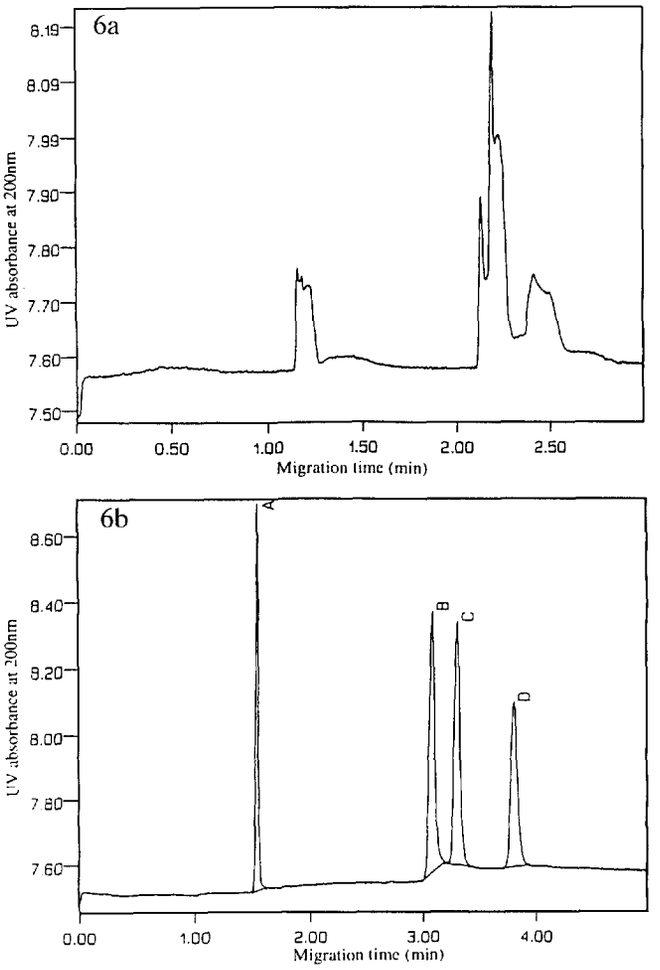


Figure 6

Separations of basic drug test mixture in 100 mM NaCl:
a) Conventional-end injection of a basic drug test mixture containing **A** = imidazole, **B** = aminobenzoic acid, **C** = lamivudine, **D** = salbutamol dissolved in 100 mM NaCl. Separation conditions: 50 mM phosphate pH 2.5, +20 kV, 34 cm × 50 µm, detection at 200 nm, injection 5 s at 50 mbar.
b) Short-end injection of a basic drug test mixture dissolved in 100 mM NaCl. Separation conditions: 250 mM phosphate pH 2.5, −7.5 kV, 34 cm × 50 µm, detection at 200 nm, injection 5 s at −50 mbar.

Table III. Benefits of short-end injections.

Feature	Advantage
Analysis time reductions	Increased sample throughput. Decreased buffer depletion problems.
Less diffusion	Increased sensitivity.
Lower voltage operation	Wider bore capillaries possible for improved sensitivity.
Higher electrolyte concentrations possible	Reduced peak tailing. Increased resolution possible. Reduced sample matrix effects.

salt content resulted in a very poor separation as shown in Figure 6a. A short-end injection of the 100 mM NaCl sample was then performed using 250 mM phosphate buffer (Figure 6b). Under these circumstances the ionic strength of the sample solution is lower than the buffer and stacking effects are therefore favourable. Operation with the higher electrolyte strength with a long-end injection would have been impossible due to excessive current (Joule heat) generation. This example of high ionic strength sample solutions represents conditions often encountered in biofluids where desalting is commonly required prior to assay and also in liquid formulations where isotonic saline is a frequent diluent.

Table III shows the benefits of routinely adopting short-end injections.

Conclusions

The use of short-end injections has been demonstrated for a range of applications and considerable benefits shown in comparison with conventional long-end injections. These benefits include significant reductions in analysis times and increased sensitivity. Short-end injections also allow higher electrolyte concentrations to be employed in conjunction with lower voltages, which has been shown to improve resolution, reduce peak tailing and reduce the impact of high salt content in samples. Short-end injections are possible on a number of commercially available instruments.

It is therefore concluded that short-end injections should be considered for routine operation as they offer considerable advantages with no apparent disadvantages.

References

[1] *D. Perrett,* G. Ross, J. Chromatogr. A, **700**, 179 (1995).
[2] *K. D. Altria, J.* Chromatogr. **636**, 125 (1993).
[3] *R. L. Chien, D. S. Burghi,* Anal. Chem. **64**, 489A (1992).
[4] *H. C. Birrell, M. D. Brightwell, P. Camilleri,* Chromatographia **39**, 325 (1994).
[5] *K. D. Altria, C. F. Simpson,* Chromatographia **24**, 527 (1987).
[6] *K. D. Altria, P. Frake, I. Gill, T. Hadgett, M. A. Kelly, D. R. Rudd, J.* Pharm. Biomed Anal. **13**, 951 (1995).
[7] *S. R. Rabel, J. F. Stobaugh,* Pharm. Res. **10**, 171 (1993).
[8] *K. D. Altria, J.* Chromatogr. **735**, 43 (1996).
[9] *K. D. Altria, D. M. Goodall, M. M. Rogan,* Chromatographia **34**, 19 (1992).
[10] *K. D. Altria, D. M. Goodall, M. M. Rogan,* Electrophoresis **15**, 824 (1994).
[11] *B. J. Clark, A. Shafaati,* Anal. Proceed. **30**, 481 (1993).
[12] *B. R. Thomas, X. G. Fang, X Chen, R. J. Tyrell, S. Ghodbane, J.* Chromatogr. **65**, 383 (1994).
[13] *L. Song, O. Quingyu, Y. Weile, X. Guifang,* J. Chromatogr. A **696**, 307 (1995).
[14] *M. A. Kelly, B. J. Clark, K. D. Altria,* J. Pharmacie Belgique **50**, 347 (1995).
[15] *H. H. Lauer, D. McManigill,* Anal. Chem., **58**, 166 (1986).

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