# Improved Performance in Capillary Electrophoresis using Internal Standards

**Kevin D. Altria,** GlaxoSmithKline R&D, Ware, Hertfordshire, UK.

**The use of internal standards significantly improves the quantitative performance of capillary electrophoresis methods in terms of precision, linearity and recovery data. This instalment of "CE Currents" explains why and provides advice on internal standard selection.**

## Introduction

Injection precision performance is probably the most criticized aspect of commercial capillary electrophoresis (CE) instrumentation. Today's analysts are generally experienced high performance liquid chromatography (HPLC) users accustomed to obtaining injection precisions in the region of 1% RSD. This is possible on commercial HPLC equipment because of well-controlled total or partial loop-fill injection with volumes in the order of 5–50 µL. However, in CE, injection volumes are typically 5–50 nL and loop injectors for these tiny volumes are not available for current CE instruments. Injections in CE are normally achieved by inserting the capillary into a sample solution vial and then pressurizing the vial to force sample solution into the capillary. The volume injected is directly related to the pressure difference and the time that the pressure is applied.

Equation 1 shows that several other factors affect the volume injected during a CE injection. In addition, subtle factors, such as siphoning effects and the surface tension of the sample solution, affect the injection volume and can lead to variable injection volumes and peak areas. Note also that the area of a CE peak is related to its migration time.[1] Therefore, poor peak area precision will be obtained if variable migration times occur throughout a sequence, and precautions such as capillary rinsing and elimination of buffer depletion effects[2] are employed in routine applications. Consequently, many aspects must be considered when optimizing the quantitative performance of CE. The primary solution to improved precision is an internal standard and these will be the focus of this instalment of "CE Currents." Use of an internal standard also improves linearity and recovery data.

$$\text{Injection volume} = \frac{\Delta P\, d^4}{128\, \eta} \frac{\Pi t}{L} \qquad [1]$$

where ΔP is the pressure difference (mbar), $d$ is the capillary diameter (µm), L is the capillary length (cm), $\eta$ is the viscosity (of sample solution and buffer) and $t$ is the time (s).

Table 1 gives injection volumes for 1 s injections using 65 cm capillaries of varying bore, $\eta = 1$ and various ΔP values. These volumes generally correspond to sample plug lengths of less than 1 mm in the capillary.

Internal standards were widely used when HPLC and gas chromatography were in their infancies and when manual sample injections were routinely employed. Internal standards are still occasionally employed in HPLC as they add extra reliability to an assay method by reducing volumetric errors during sample dilutions. Use of an internal standard is simple and represents a very minor increase in workload or expense. It simply involves dissolving an appropriate substance into the solvent used to prepare both the sample and calibration solutions.

It has been shown that CE can produce acceptable precision for 5–10 replicate injections of a simple standard solution.[3] However, unless an internal standard is employed the required injection precision level is difficult to maintain during long routine injection sequences and/or analysis of samples in complex matrices. Several reports have shown improvements obtained using internal standards in CE.[4–7] Table 2 shows several operating effects that can lead to variable peak areas in CE and indicates whether the use of internal standards can reduce or eliminate the particular problem.

**Table 1:** Injection volumes on different diameter capillaries.

| ΔP | 50 µm | 75 µm | 100 µm |
|---|---|---|---|
| 50 mbar | 1.0 nL | 5.2 nL | 16.4 nL |
| 75 mbar | 1.5 nL | 7.8 nL | 24.6 nL |
| 100 mbar | 2.0 nL | 10.4 nL | 32.8 nL |

**The use of an internal standard…eliminates all injection volume-related sources of error and leads to dramatically improved precision.**

**Internal standard procedure:** The simplest approach is to use a diluent containing the internal standard to prepare both the sample and calibration solutions. Alternatively, an accurate and precise volume of the internal standard solution may be added to both the sample and calibration sample solution such that they both contain an identical concentration. This second approach is sometimes employed if several dilution stages are required in sample preparation. When the sample and calibrations solutions are analysed, the peaks for both the internal standard and the analyte are integrated. The area of the analyte peak is divided by the area of the internal standard peak to produce a *peak area ratio* (PAR) value. If a higher injection volume inadvertently occurs then the areas of both the internal standard and solute peaks will increase to the same extent and the PAR will remain constant. Conversely, if the injection volume is smaller both peaks will be correspondingly smaller and the PAR value will remain constant. The use of an internal standard, therefore, eliminates all injection volume-related sources of error and leads to dramatically improved precision. Injection volume-related imprecision can occur from both sample solution variability and operational considerations.

Table 3 shows the calculation of precision, accuracy and linearity data for the analysis of sample solutions using either peak areas or PARs.[6]

**Table 2:** Parameters affecting imprecision and whether they are solved by use of internal standard.

| Problem | Solved by Internal Standards? |
|---|---|
| Viscosity variation | Yes |
| Low sample concentration | No |
| Short injection time | Yes |
| Variable migration times | Yes (especially if peaks migrate close together) |
| Matrix effects | Sometimes |
| Spontaneous injection | Yes |
| Adsorption/peak tailing | Sometimes |
| Surface tension differences | Yes |
| Height differences | Yes |
| Baseline noise | No |
| Temperature variations | Yes |
| Capillary tip damage | Sometimes |
| Volume expansion losses | Yes |
| Inappropriate injection procedure | Yes |
| Pressure variations | Yes |
| Evaporation | Yes |
| Carryover | Yes |

**Table 3:** Accuracy, precision and linearity for tromethamine analysis.

| | No Internal Standard | With Internal Standard |
|---|---|---|
| Precision (n = 6) | 2.53% RSD | 0.35% RSD |
| | 5.77% RSD | 0.87% RSD |
| Recovery (n = 2) | 1.21% RSD | 0.50% RSD |
| | −6.48% | −0.01% |
| Linearity correlation | 0.98633 | 0.99997 |
| | 0.99684 | 0.99973 |

* Reproduced with permission from reference 6.

## Factors Affecting Injection Volumes

**Viscosity:** The sample solution viscosity is affected both by the temperature and the presence of viscosity-altering additives such as cellulose. Therefore, it is essential to maintain a constant temperature for both sample/calibration solutions and the electrolyte in the capillary and vials. It is also necessary to match the viscosity of the sample solution to that of the calibration solution. For example, a tablet containing cellulose was analysed by CE using an aqueous standard.[7] The tablet solution was more viscous than the standard prepared in water and, therefore, lower injection volumes were obtained for the sample solution compared with the calibration solution. This viscosity difference produced an assay result of only 90% of the expected value. Use of an internal standard gave the correct result because the PAR value of an injection is unaffected by the viscosity of the solutions. An alternative approach to the problem was to add a placebo tablet to the calibration solution to match the viscosity of the standard to that of the sample.[7]

**Evaporation problems:** Many of the sample vials used in CE are not sealed to allow the fragile capillary to enter the vial. Evaporation can occur, and the sample and calibration concentration will increase throughout the sequence leading to poor long-term precision. However, the PAR is unaffected by evaporation as both the analyte and internal standard become equally more concentrated. Alternatively, evaporation can result in migration time drifts during an injection sequence. Because the peak area is related to migration time this can lead to deterioration in precision. This problem can be addressed by placing a film of mineral oil on the surface of the buffer in the vial.[8] This film prevents evaporation and has been shown to significantly improve migration time precision.[8] Table 4 shows a comparison of different evaporation rates for various solvents in uncapped 4 mL vials. Methanol evaporated fastest followed by acetonitrile, isopropanol and lastly water.

After 24 hours approximately 44% of the methanol, 25% of the acetonitrile and 12% of the iospropanol had evaporated from uncapped vials. The evaporation was approximately four times slower for all solvents in capped vials.

**Surface tension effects:** The volume injected is also related to the surface tension of the sample solution, which can be altered by the presence of surfactants.

**Sample dissolving solvent viscosity:** Organic solvents have different viscosities when compared with water. The use of variable mixtures of solvents will alter the viscosity of the sample solution. Therefore, an identical solvent composition should be used for both sample and calibration solutions to control injection volumes. The nature of the sample dissolving solvent also affects the separations achieved. As a result, it is important to optimize sample diluent during method development and essential to maintain the same diluent throughout the use of the method.

**Temperature control:** Thermostatting the capillary is often achieved by placing the capillary in a cartridge device and a temperature-regulated liquid or gas is flushed through the cartridge. Temperature control is essential to obtain consistent viscosities. The mobility of the compound is directly related to temperature, with a 2% change occurring per 1 °C change in temperature.

**Sample solution levels:** Siphoning can occur if the capillary is dipped into vials that contain different levels (heights) of solution. This siphoning flow is related to capillary diameter[3] and can be highly significant for 100 µm or greater bore capillaries. If the sample solution level is lower than the solution level at the other end of the capillary then a siphoning flow will counteract, to some extent, the injection pressure resulting in a lower injection volume. If siphoning-related errors were obtained then the vials would need to be filled to a certain height or with a fixed volume.[3] Siphoning-related errors would not be noticed if, for example, 10 replicate injections were performed from a single sample, but may be noticeable if a single injection was performed from each of 10 vials containing the same solution. The use of internal standards eliminates injection volume-related siphoning effects. However, it is good practice to ensure that the buffer vials used are all filled to an equivalent height as siphoning will occur during the separation resulting in variable migration times.

A useful procedure may be to use an autopipette to dispense a consistent volume of liquid into each vial used.[3]

**Voltage ramping/volume expansion:** Current is generated the instant separation voltage is applied. Generation of this current heats the electrolyte within the capillary causing a volume expansion. The expanded volume forces a volume of liquid from the capillary ends.[9] This can have a detrimental affect on precision, as it is an uncontrolled process. However, the PAR is unaffected by this process. The problem can be overcome by injecting a portion of electrolyte behind the sample injection prior to application of the separation voltage.[10]

**Injection vial positioning:** If an empty vial is positioned at the non-injection end drops of liquid can form at the non-injection end of the capillary during the injection process. If the drop(s) fall during the period that the capillary is dipped into the sample solution vial then it will alter the pressure across the capillary and produce a higher injection volume. This problem can be eliminated if a filled vial is always placed at the non-injection end of the capillary.[10]

**Injection process control:** If the injection pressure varies during the injection then a variable volume will be injected. Many instruments have a pressure monitor facility, which has a feedback mechanism to automatically compensate for this problem to some extent. The problem is greatest with short injection times. If an internal standard is not used then it is preferable to employ a longer injection time at a lower pressure to reduce this effect.[10] For example, a 10 s injection at 5 mBar would give better precision than a 1 s injection at 50 mBar.

**Sample carryover effects:** Small volumes of sample solution can be inadvertently transferred onto the tip of the capillary after the injection procedure is complete. This carryover is variable and can, therefore, influence the precision obtained throughout an injection sequence. The effect is eliminated by use of an internal standard, as the peak area for the internal standard will also be increased.

## Linearity Data Improvements using Internal Standards

The use of internal standards can also improve correlation coefficients obtained for detector linearity during method validation. Because the use of internal standards improves injection precision, this in turn reduces the scatter of points along the line giving a higher correlation coefficient. For example, both peak areas and PARs were plotted (Table 5) for a range of sample solutions injections[11] — the PARs gave considerably better correlation coefficients. Table 3 also confirms this improvement[6] plotting data using either peak areas or PARs — the slope of the line is the same but the correlation coefficient is better.

**Table 4:** Comparison of evaporation rates for various solvents in capped and uncapped vials – evaporation from 4 mL vials over 24 hours at room temperature.

| Solvent | % Evaporated Uncapped Vials | % Evaporated Capped Vials |
|---|---|---|
| Water | 2.6 | 0.6 |
| Acetonitrile | 26.4 | 7.0 |
| Methanol | 43.6 | 8.6 |
| Isopropanol | 12.4 | 3.7 |

**Table 5:** Detector linearity studies for various acidic drugs using peak areas or peak area ratios.

| | Correlation coefficient over the range 50–150 ppm | |
|---|---|---|
| **Solute** | **Peak Areas** | **Peak Area Ratios** |
| Glibenclamide | 0.9168 | 0.9989 |
| GW1 | 0.9933 | 0.9998 |
| GW2 | 0.9715 | 0.9979 |
| Omeprazole | 0.9958 | 0.9978 |
| Levothyroxine | 0.9986 | 0.9999 |

* Reproduced with permission from reference 12.

Surprisingly, the use of internal standards also brings the intercept value closer to the origin when peak area is plotted against injection time. When the capillary is inserted into the sample solution a small portion of the solution is drawn into the capillary by capillary action. This 'spontaneous' injection cannot be avoided[12] and results in a positive intercept value being obtained in linearity studies. Use of an internal standard eliminates this problem as it is also 'spontaneously' injected and the PAR values are automatically adjusted.

## Accuracy (Recovery) Improvements using Internal Standards

The injection volume in CE (Equation 1) is inversely related to sample viscosity. This can cause variability of injection volumes (and peak areas) if the viscosity of the samples and standards differ. For example, many pharmaceutical formulations contain excipients such as lactose, cyclodextrins and cellulose. The presence of these excipients increases the sample solution viscosity compared with the standard, which is prepared in pure diluent. Therefore, injection of a sample solution will give a lower relative injection (and peak area) than the less viscous standard solution. This effect will generate a low recovery (accuracy) result for the sample (e.g., the 6.48% low recovery shown in Table 3). However, the use of an internal standard accounts for the viscosity-related low injection area and calculation using PARs (Table 3) gives an acceptable 100% recovery. Similar recovery improvements have been reported for *L*-thyroxine recoveries from tablets using internal standards.[7] An improvement was also shown by adding placebo tablets to the calibration solutions to match standard and sample viscosities.

## Choice of Internal Standard

Analysts are often reluctant to use internal standards in HPLC as selection of an appropriate standard can cause a significant increase in method development time. This increase results from the additional injections required to establish the elution position of each potential internal standard relative to the other peak(s) in the chromatogram. The migration position of peaks can be more easily predicted in free-solution CE as the migration time is related to the size and charge of the compound. Therefore, to have an internal standard that migrates before a basic analyte of interest at low pH requires one with a smaller molecular weight or higher number of positive charges. The separation of anions at high pH would require an internal standard with larger molecular weight or fewer charges than the analyte to produce a peak that migrates before the solute peak. In MECC it is more difficult to predict an internal standard but solubility data is useful, as a lower water-soluble compound will generally elute later than a more soluble one.

The nature and concentration of the exact substance selected depends on several factors. The main requirement is that the substance gives good peak shape and is resolved from the analytes of interest and any other peaks in the separation. Other requirements for an appropriate internal standard include that it is stable in solution, commercially available in a high-purity form, readily soluble in the diluent required, possesses acceptably high UV activity at the desired wavelength, is inexpensive and is non-toxic.

**Stability:** The internal standard should be sufficiently stable in the sample dissolving solvent to prevent the formation of degradation products, which would interfere with the integration results. It should also be chemically stable in the solid state to allow suitable storage.

**Internal standard solubility:** The internal standards should be freely soluble in the sample solvent. If low conductivity sample diluents are used then 'stacking' may result in a 10-fold increase in on-column concentration, which may cause problems of precipitation and/or peak tailing. This problem can also occur for the analyte and should be checked.

**Commercially available:** The material selected should be cheap and readily available in a high-purity form from commercial suppliers so that the method

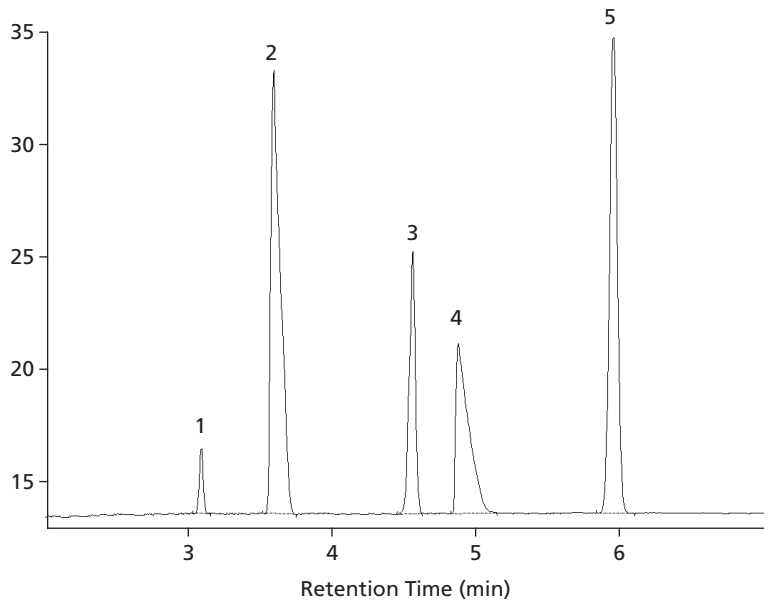
**Table 6(a):** Repeatability of peak area ratios when using different peaks as standard (n = 10).

| Peak area ratio repeatability, RSD values (%) | | | | | |
|---|---|---|---|---|---|
| **Peak used as standard** | **Imidazole** | **Dimethyl-biguanide** | **Diamino-benzoic acid** | **Quinacrine** | **Triamino-pyrimidine** |
| Imidazole | | 2.04 | 2.48 | 3.07 | 1.96 |
| Dimethylbiguanide | 2.04 | | **0.97** | 1.85 | **0.59** |
| Diaminobenzoic acid | 2.48 | **0.97** | | 1.35 | **0.81** |
| Quinacrine | 3.07 | 1.85 | 1.35 | | 1.50 |
| Triaminopyrimidine | 1.96 | **0.59** | **0.81** | 1.50 | |

**Table 6(b):** Repeatability of relative migration times using different peaks as the standard peak (n = 10).

| Peak used as standard | Relative migration time, RSD values (%) | | | |
|---|---|---|---|---|
| | **Imidazole** | **Dimethylbiguanide** | **Quinacrine** | **Triamino-pyrimidine** |
| Imidazole | | **0.18** | 0.94 | 1.17 |
| Dimethylbiguanide | **0.18** | | 0.81 | 0.98 |
| Diaminobenzoic acid | 0.78 | 0.65 | **0.17** | 0.38 |
| Quinacrine | 0.94 | 0.81 | | **0.24** |
| Triaminopyrimidine | 1.12 | 0.97 | **0.24** | |

**Figure 1:** Separation of five potential cationic internal standard compounds.




**Peaks:** 1 = imidazole, 2 = dimethylbiguanide, 3 = diaminobenzoic acid, 4 = quinacrine, 5 = triaminopyrimidine.

can be readily reproduced elsewhere.

**Toxicity:** The toxicity of the internal standard should be minimal to reduce any handling precautions that may be required.

The compound should have a good UV response at the detection wavelength so that a high signal can be obtained to reduce any integration-related variability generated with small peaks. Preferably, the internal standard should have a migration position near to the peak of interest so that if there is a drift in migration times throughout an injection sequence the migration times for both the solute and internal standard peaks will be similar.

Figure 1 shows the separation of a range of basic compounds using a low pH buffer. The compounds are selected to give a range of peak shapes and peak intensities. It can be seen, for example, that imidazole gives a good peak shape but only a small peak height (and area) because of its low UV absorbance. Quinacrine gives a good peak response but because of its highly basic nature shows a degree of peak tailing as it interacts with the capillary walls. The other three compounds give gaussian, non-tailing peaks with good UV responses suggesting that they would be appropriate internal standards. Table 6(a) shows that both imidazole and quinacrine give poor PAR precision data, confirming that they are inappropriate internal standards. Table 6(a) also confirms that dimethylbiguanide, diaminobenzoic acid and triamino-pyrimidine are suitable internal standards as the PARs between these compounds (bold figures in Table 6(a)) consistently show good precision (below 1% RSD).

During the course of each separation slight variability can occur in the level of current, solution viscosity and temperature. These changes lead to slightly different migration times for the peaks for each analysis. If the migration time of a peak is referenced against another peak then this variability is reduced. However, if the peaks are well separated in time then variability will still occur and the improvement will be less. Table 6(b) shows that the precision of measuring relative migration times is improved if the peaks migrate closely to each other. The closely migrating peaks give good precision (bold figures in Table 6(b)). The precision of the relative migration time for imidazole, for example, increases with the distance in time between the two peaks.

## Conclusions

Many subtle factors affect the volume introduced into the capillary during a pressure injection in CE. These factors mainly relate to variability in the pressure/timing of the injection and to changes in sample solution viscosity. Variability results in poor injection repeatability, as well as reduced detector linearity and recovery (accuracy) data. These problems can be efficiently eliminated by inclusion of an internal standard component in the diluent used to prepare the sample and standard solutions. The internal standard used should be chosen to give good peak shape and to migrate reasonably near the solute peak of interest.

## References


1. K.D. Altria, *Chromatographia*, **35**, 177–182 (1993).
2. M.A. Kelly, K.D. Altria and B.J. Clark, *J. Chromatogr. A*, **768**, 73–80 (1997).
3. B.R. Thomas et al., *J. Chromatogr.*, **657**, 383–394 (1994).
4. E.V. Dose and G.A. Guiochon, *Anal.Chem.*, **63**, 1154–1158 (1991).
5. H. Watzig and C. Dette, *J. Chromatogr.*, **636**, 31–38 (1993).
6. T. Wielgos, P. Turner and K. Havel, *J. Cap. Elec.*, **4**, 273–278 (1997).
7. K.D. Altria and J. Bestford, *J. Cap. Elec.*, **3**, 13–23 (1996).
8. K. Shimura, N. Uchiyama and K.-I. Kasai, *Electrophoresis*, **22**, 3471–3477 (2001).
9. J.H. Knox and K.A. McCormack, *Chromatographia*, **38**, 279–282 (1994).
10. K.D. Altria and H. Fabre, *Chromatographia*, **40**, 313–320 (1995).
11. K.D. Altria, S.M. Bryant and T. Hadgett, *J. Pharm. Biomed. Analysis*, **15**, 1091–1101 (1997).
12. H.A. Fishman et al., *Anal.Chem.*, **66**, 2318–2329 (1994).



"CE Currents" editor **Kevin D. Altria** is senior principal scientist in the pharmaceutical development group at GlaxoSmithKline R&D, Ware, Hertfordshire, UK, and is a member of the Editorial Advisory Board of *LC•GC Europe*. Direct correspondence about this column to "CE Currents," K.D. Altria, Pharmaceutical Development, GlaxoSmithKline, Park Road, Ware, Hertfordshire SG12 0DP, UK, fax +44 1920 882 295, e-mail: KDA8029@gsk.com, website: http://www.ceandcec.com/