Essential Peak Area Normalisation for Quantitative Impurity Content Determination by Capillary Electrophoresis

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Key Words

Capillary Electrophoresis
Quantitative determinations
Peak area normalisation

Summary

A number of papers have been published [1–5] which mention that normalisation of CE peak areas (ie division of peak areas by migration time) is necessary to ensure correct quantitation. However, there is a general unawareness of the impact of not performing this simple data manipulation upon impurity results when expressed as %area/area. An exercise has been conducted to exemplify the need for this normalisation using the separation of selected pharmaceuticals as illustrative examples. The impact of normalisation on %area/area results was demonstrated using the pharmaceutical ranitidine, and a synthetic precursor, as test solutes. The UV absorbance of each compound was determined and found to be equivalent. A solution of ranitidine hydrochloride was then spiked with a weighed amount of precursor. The %area/area CE results, when normalised, matched the known weighed radio. Use of uncorrected peak area data in this instance would have resulted in a severe underestimation of impurity levels. A quantitative analysis of drug related impurities was conducted at three levels of operating voltage whilst keeping all other operating parameters constant. This produced electropherograms having identical peak profiles but each peak having a different retention time and peak area. However, when normalised, the results were identical at each level of operating voltage confirming the validity and necessity of normalisation. A chiral separation of a racemic pharmaceutical was conducted. The uncorrected peak area data indicated the test sample was not racemic whilst the corrected data correctly confirmed that the compound was racemic. The significance and impact upon reported purity data of not normalising peak area data has been clearly demonstrated in this paper.

Introduction

Capillary Electrophoresis (CE) is finding considerable application in a wide and diverse range of analytical areas. The application range reported includes biomolecules, inorganic anions, metal ions and small synthetic organic molecules such as pharmaceuticals. Of particular importance is the use of CE to determine the levels of impurities present in test samples. In many analytical areas impurity data generated by CE are used to support analytical results obtained from alternative techniques such as HPLC:

Although the detector output is similar in both HPLC and CE (ie detector signal changes are monitored as a function of time) data handling is somewhat different in CE. In order to directly compare impurity levels between HPLC and CE a normalisation of CE peak areas is required. This operation is relatively simple but must be performed to allow direct comparisons of impurity levels to be conducted.

Solutes are separated in CE by virtue of differences in their mobilities (charge to mass ratios). More mobile species migrate more rapidly along the capillary, and therefore pass through the detector before less mobile species. This results in differential migration rates of solutes through the detector (2). Since the area of a peak is proportional to both the solute concentration (peak height) and peak residence time in the detector (peak width) direct comparisons of peak areas of components resolved within a CE separation are not possible without peak area normalisation.

Ackermans et al. [1] have mathematically examined the need to correct peak areas to migration times when employing conductivity and indirect UV detection in CE. The same requirement holds for use in UV absorbance detection which is the most frequently employed CE detection mode used in commercial instruments.

In HPLC all solutes are swept through the detector at a constant flow rate and accordingly have equal detector residence times. Therefore it is possible to directly quote HPLC results in terms of %area/area. However, in CE %area/area results cannot be directly calculated from the observed peak areas as these must be first

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corrected to compensate for their different residence times in the detector. This normalisation simply consists of the division of the each measured peak area in the separation by its corresponding retention time.

Peak area normalisation is not essential for quantitative main peak assays as calibration standards are employed which have identical migration times to the sample. Several reports have been published [6–8] concerning the reproducibility of peak areas on automated CE instruments with typical values being in the range of 1–2 %RSD. However, if a severe drift in migration times occurs during the course of a number of separations then the peak areas observed will also alter, leading to a calculated loss of precision.

By employing an internal standard, the effect of migration time drift can be reduced since the peak areas of both the sample and internal standard would change simultaneously. RDS's of below 1% have been obtained [9] using internal standards in CE.

CE and the associated capillary electroseparation technique of Micellar Electrokinetic Capillary Electrophoresis (MECC) have been applied to the area of pharmaceutical analysis [10–13]. A particular area of importance is the determination of drug impurities where CE can be used in combination with HPLC to provide complimentary data. The concordance of impurity levels as determined by two independent separation methods strongly endorses the validity of the results from either method and could form a suitable part of method validation.

Few examples of the quantitative analysis of drug related impurities have been reported. Swartz has reported [13] %area/area results for salicylamide related impurities by CE using areas which had not been corrected. Retrospective correction of the peak reduces the impurity level from 1.7 % to 1.5 % and also reduces the area% of the principal impurity from 0.53 % to 0.42 %. This disparity would be of great importance when attempting to cross-correlate impurity levels as determined by CE with another technique such as HPLC.

The application of CE to the chiral resolution of pharmaceuticals has been demonstrated by several workers [14–18]. However, there has been little emphasis placed upon the quantitative aspects of chiral CE. Free Solution CE has been employed [17] to quantify low levels (0.2 %w/w) of the inactive isomer present as an impurity in the active enantiomer picumeterol, (structure given in Figure 1). Nishi et al. [18] have reported the use of MECC, using chirally selective bile salt micelles, for the optical purity determination of trimetoquinol with a detection limit of 1 % of the inactive enantiomer being obtained.

A racemic compound consists of a mixture of two stereoisomers present at equal concentrations. Each isomer has identical UV activity therefore the peak area ratio (α) of the two peaks arising from the separation of a racemate should be 1.00. This would be the case when a racemate is baseline resolved by HPLC. Clearly peak area normalisation is essential in chiral CE as the results are quoted in %area/area. If the data are not corrected then an overestimation of the latter migrating enantiomer will occur in terms of area count.

Experimental

20 mM sodium citrate (pH 2.5) was obtained from Applied Biosystems Ltd. (San Jose, CA, US) and inorganic chemicals from Aldrich Ltd. (Poole, Dorset, UK). Water was obtained from a Millipore Q system (Watford, Herts., UK). UV absorbance measurements were performed on a Hewlett Packard 8452 A spectrophotometer (Bracknell, Berks., UK) using a 1 cm cell.

The work was performed on a P/ACE 2000 CE instrument (Beckman, Palo Alto, CA, US) which was connected to a Hewlett Packard data collection system. The fused silica capillaries used in this study were purchased from Beckman.

The separation conditions are given below, the method consists of five automated steps:

Capillary Electrophoresis Separation Method

Step I Rinse cycle 1 : 0.5 M NaOH 2 minutes
Step II Rinse cycle 2: run buffer 4 minutes
Step III Set detector
Step IV Hydrodynamic sampling
Step V Separate

Operating temperature: 25 °C
Capillary dimensions: 75 µm × 57 cm fused silica

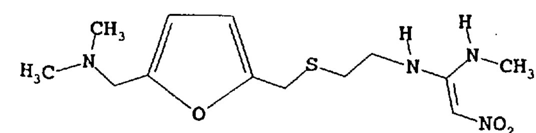
Results and Discussion

Various experiments were conducted to experimentally illustrate the quantitative effect of peak area normalisation on area/area results.

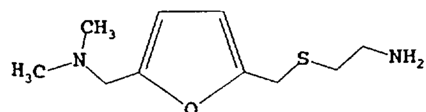
Calculation Impurity Levels as %Area/Area

The impact of peak area dependence upon a quantitative impurity determination was demonstrated employing ranitidine hydrochloride and a synthetic precursor as model compounds (structures given in Figure 1). The UV absorbance of solutions of ranitidine and the precursor (1 mg/100 ml solutions in the carrier electrolyte) was found to be equivalent (0.506 and 0.511 absorbance units respectively) using a spectrophotometer set at the CE detection wavelength (230 nm).

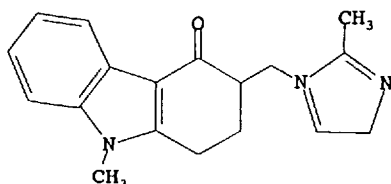
A solution of ranitidine hydrochloride (0.6 mg/ml in water) was spiked with the precursor at 9.3 % of the total combined sample weight. Analysis of the spiked sample by CE produced the separation given in Figure 2. The peak area data (mean of 2 injections) are given in Table I.



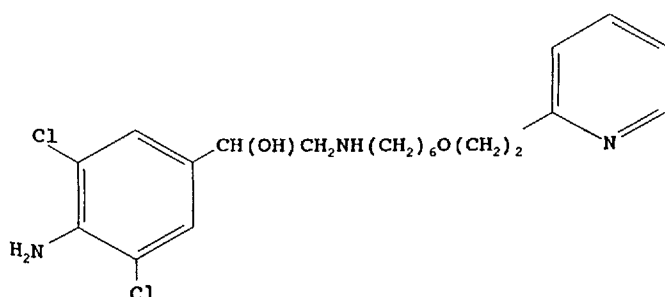
I Ranitidine



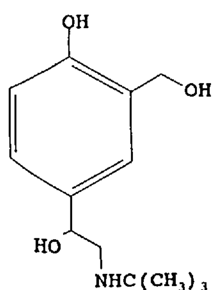
II Ranitidine precursor



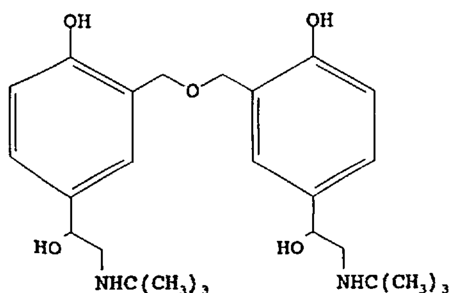
III Ondanestron



IV Picumeterol



V Salbutamol



VI Salbutamol dimeric impurity

Figure 1
Chemical structure of pharmaceuticals

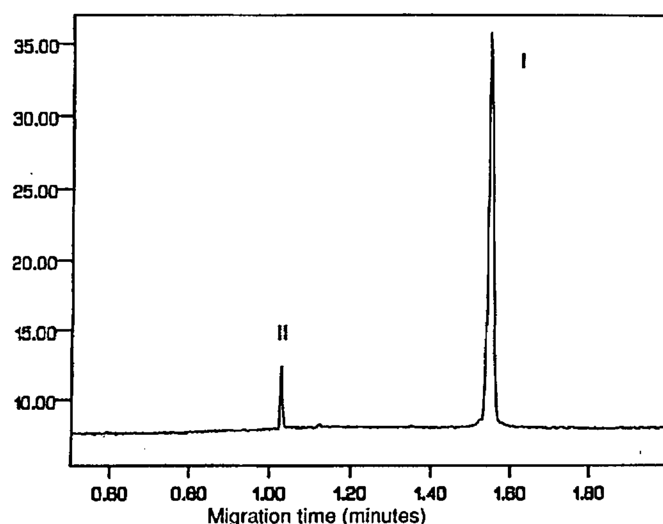


Figure 2
Separation of rantidine and precursor
Separation conditions: as above except 27 cm × 50 μm capillary, 20 mM Sodium citrate pH 2 · 5

Table I
Peak area data for CE analysis of spiked ranitidine sample

	Precursor	Ranitidine
Area	29092	436157
Migration time (minutes)	1.02	1.54
% total area	6.3	93.7
normalised peak area	28398	283013
% total normalised peak area	9.1	90.9

Since the UV absorbance activity of the two solutes are equivalent it would be expected that the precursor would give a % total area of 9.3 %, which is similar to the result obtained (9.1 %) using the normalised data. However, use of uncorrected peak areas would give a result of 6.3 % total peak area which represents a serious underestimation of the true value.

The determination of the related impurities of a Glaxo Group Research (GGR) development compound termed GGR1 was selected as an additional example of the use of corrected peak areas. This compound contains certain of the structural elements of ondansetron [19] the structure of which is given in Figure 1. A 1.0 mg/ml aqueous solution of the test sample was analysed in duplicate at three levels of operating voltage (9, 14 and 18 kV). Figures 3–5 are representative separations. This exercise was conducted to simulate an exaggerated drift in migration times.

Table II show that the migration time of the main peak is extended with lower applied voltage. The relative migration time (RMT) of the principal impurity (Imp 1) remains constant at the 3 voltage levels. In addition the resolution between Imp 1 and GGR1 remained constant at the different voltage levels. This is expected as the selectivity remains constant although the analysis time varies with applied voltage [13].

Table II
Current and migration time versus applied voltage

Voltage (V)	Current (μA)	Migration time (minutes)		Resolution (Imp1 & GGR1)
		GGR 1	Greatest impurity (Imp1)	
9000	72	35.0	29.7 (RMT = 0.85)	6.6
14000	120	20.5	17.5 (RMT = 0.85)	6.7
18000	162	14.9	12.7 (RMT = 0.85)	6.6

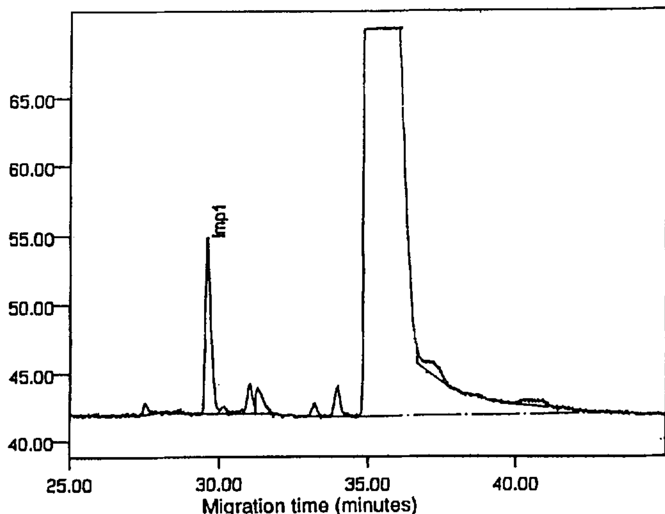


Figure 3
Separation achieved at + 9 kV
Separation conditions: 254 nm, 100 mM borate pH 2.1 with orthophosphoric acid, 50 μm x 57 cm capillary

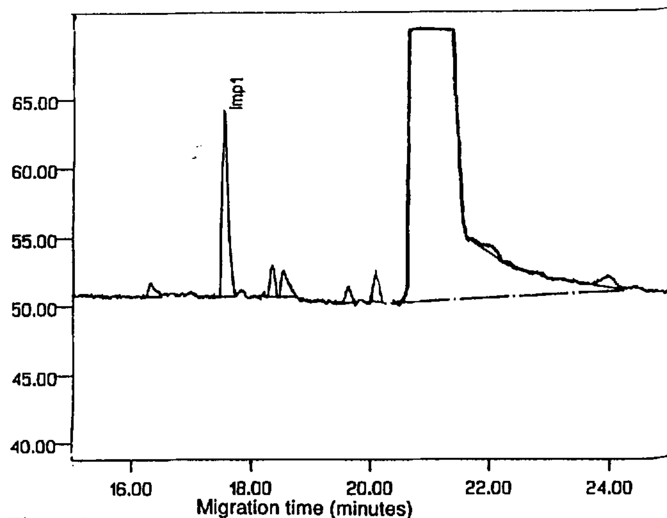


Figure 4
Separation achieved at + 14 kV
Separation conditions: as Figure 3 except voltage variation

The total peak area of the electropherograms increased at lower applied voltages (Table III). However, when each of the observed peak areas are normalised to their retention times then the normalised peak areas remain constant confirming the necessity and validity of the normalisation process.

Both the total impurity content, and principal impurity content (adjusted and unadjusted), are consistent at the 3 voltage levels (Table IV). In this example the %impurity increases with normalisation, however if the majority of impurities migrated after the main peak then the total would be reduced.

Table V shows that the peak heights for GGR1 remained effectively constant with applied voltage since the peak height is proportional to the on-column concentration. The slight decrease at lower voltages is due to increased solute diffusion as the solute spends a longer time migrating along the capillary. As expected, the peak width increases with decreased voltage (20) thereby increasing the peak area.

The need to normalise peak areas prior to reporting results as area% is therefore clearly necessary. However this is not widely appreciated as quantitative area% results are often reported without normalisation which gives an incorrect indication of both total and individual impurity levels.

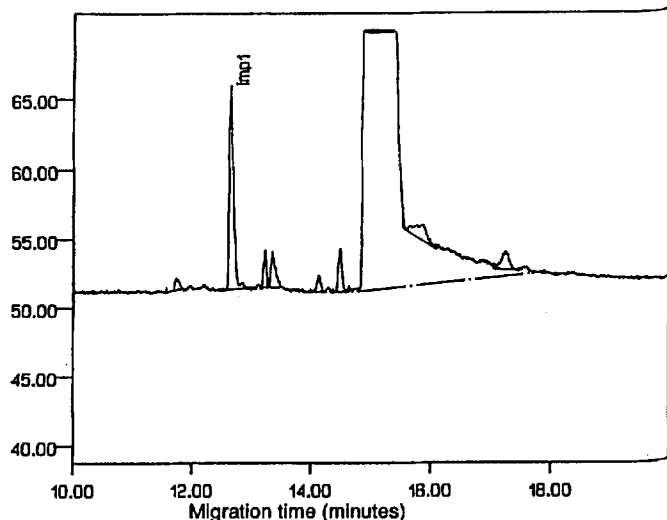


Figure 5
Separation achieved at + 18 kV
Separation conditions: as Figure 3 except voltage variation

Table III
Peak areas versus applied voltage

Voltage (V)	Total peak area	
	Unadjusted	Adjusted
9000	11958641	344289
14000	7212395	355165
18000	5235509	351438

Table IV
Impurity content versus applied voltage

Voltage (V)	Total impurities (%) Unadjusted	Total impurities (%) Adjusted	Greatest impurity (%) Unadjusted	Greatest impurity (%) Adjusted	No. imps
9000	5.5	6.1	2.9	3.4	9
14000	5.3	5.8	2.7	3.2	8
18000	5.5	6.1	2.7	3.2	8

Table V
Peak height and width versus applied voltage

Voltage	Peak Height		Peak width
(V)	GGR1	Imp 1	GGR1
9000	203051	25479	77.1
14000	211181	27871	45.1
18000	221619	29252	33.4

Chiral Analysis of Picumeterol

A racemate, of which picumeterol is the active enantiomer, was analysed 9 times using a chiral CE method. The chiral recognition agent employed was a derivatised cyclodextrin (see Figure 6 for a representative electropherogram). Table VI gives details of the performance of the replicate analyses in terms of peak area and migration time data. The replicate analysis gave a mean enantiomer ratio (α) value of 1.04 for uncorrected peak ratios (Enantiomer 2 / Enantiomer 1) whilst the expected α value for a racemate is 1.00. When the observed peak area where normalised prior to calculation of the α value an average figure of 1.00 was obtained. This figure of 1.00 is in accordance with an HPLC method [17] also used to verify the enantiomeric ratio of this compound.

Determination of Trace Impurity Levels

Relatively poor precision is obtained when quantifying trace levels of drug in solution which is in-part due to the errors involved in measuring the areas of small peaks. Utilising the variation of peak area with migration time can partially overcome this precision problem. The area of a peak can be increased by lowering the operating voltage as this extends the detector residence time of the peak. However this would be conducted at the expense of analysis time. Employing a step in the voltage level during a separation can produce the required peak area increase whilst minimising the increase in analysis time.

CE has been used [21] for the determination of selected dimeric impurities in the pharmaceutical salbutamol. The separation conditions involved the constant application of 30 kV. To increase the area for the peak obtained for low level impurity standard solutions a voltage gradient was employed. The initial portion of the separation was performed at a high voltage (30 kV) followed by application of 5 kV prior to the passage of the peak through the detector. Therefore, this voltage step resulted in the solute travelling through the detector at a rate six times slower than at the method held constant at 30 kV.

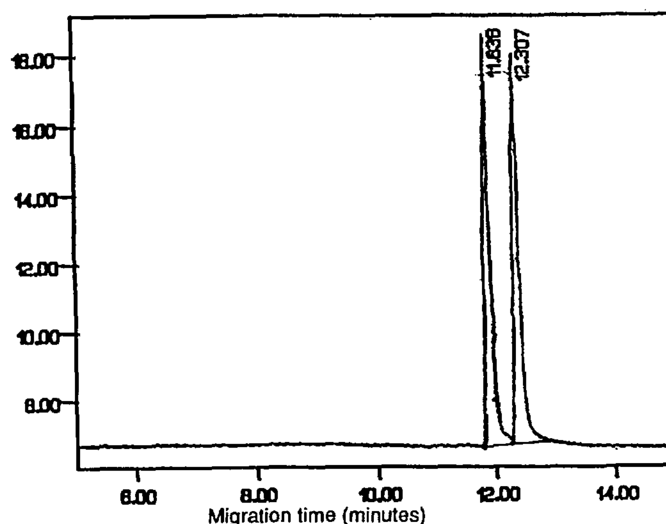


Figure 6
Chiral separation
Separation conditions: 214 nm, + 10 kV, 25 mM borate pH 2.3 with orthophosphoric acid with 30 mM dimethyl-beta-cyclodextrin

Table VI
Peak area and retention time data for 9 replicate chiral separations (n = 9)

	Enantiomer 1	Enantiomer 2	Peak area ratio
Mean peak area (observed)	1199781	1246293	1.04
Mean peak area (normalised)	103827	103841	1.00
Retention time (minutes)	Enantiomer 1 11.55 (1.5 % RSD)	Enantiomer 2 12.00 (1.5 % RSD)	RMT of enantiomer 1 0.962 (0.04 % RSD)

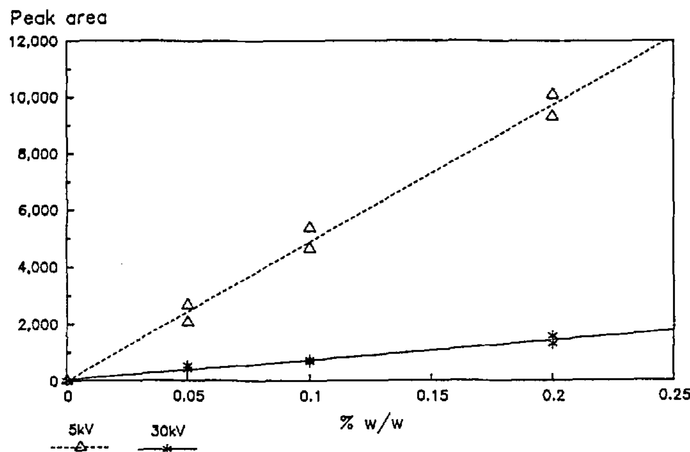


Figure 7

Plot of peak area versus concentration employing constant voltage or voltage step

Separation conditions: 200 nm, 20 mM sodium citrate pH 2.5, 75 μm x 57 cm capillary

The performance in terms of precision and peak area increase was assessed by analysing low concentration standards of a dimer impurity (structure given in Figure 1) of salbutamol using both the voltage step and constant voltage methods. Figure 7 shows the expected 6 fold increase in detector response using the adjusted voltage procedure. The precision of the measurements was improved as correlation coefficient for the voltage step method was 0.992 compared to 0.970 for the constant voltage. This improvement is due to both the reduction in quantitative errors when measuring the larger peak areas and to a longer time averaging of the signal.

The use of a voltage gradient in CE [22] has been reported as a means of altering separation selectively.

Petoney et al. [23] have showed similar sensitivity gains (as measured in counts/sec) for detection of radiolabelled compounds using a radioactivity detector when reducing the separation voltage from 20 kV to 2 kV.

Conclusions

This exercise confirms the normalisation of peak areas with migration times in CE is essential prior to reporting impurity level results as %area/area.

Unless area correction is adopted it makes the cross correlation of %area/area impurity results between CE and others separative methods such as HPLC impossible to accurately perform.

This normalisation is relatively simple in the case of a systematic variation in migration speed of the analytes during an analytical separation. However, the case becomes significantly complicated when employing voltage gradients [22] or pH gradients [24] where the peak velocities of the components of mixture vary markedly during the course of a separation.

It is suggested that is normalisation is an important consideration in handling CE generated data and that authors should indicate in future publications whether their data have been normalised.

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