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Validated micellar electrokinetic capillary chromatography method for quality control of the drug substances hydrochlorothiazide and chlorothiazide

B.R. Thomas*, X.G. Fang, X. Chen, R.J. Tyrrell, S. Ghodbane

Analytical Methods and Support, Merck & Co., Inc., WP38-3, West Point, PA 19486, USA

Abstract

A stability-indicating, quality control analysis method was developed and validated for the diuretic drug substances hydrochlorothiazide (HCTZ) and chlorothiazide (CTZ). Micellar electrokinetic capillary chromatography employing the anionic detergent sodium dodecyl sulfate at 30 mM in 20 mM sodium borate buffer pH 9.5 was utilized to separate and quantify the active drug substance HCTZ from CTZ and the common impurity, 4-amino-6-chloro-1,3-benzenedisulfonamide (DSA). A 100 μm I.D. uncoated fused-silica capillary was necessary to provide the sensitivity, *i.e.* 1 μg/ml, for quantification of the DSA impurity. In this study, the linearity, precision, selectivity, accuracy, reproducibility and limit of quantitation for the method were investigated for HCTZ, CTZ and DSA. As the first validation of a drug substance method by capillary electrophoresis in this laboratory, unusual care was taken to insure reliability and ruggedness with multiple instruments, capillaries and analysts. Precision and reproducibility in the range of 1% R.S.D. was achieved by controlling subtle injection factors. These included minimizing the time in which the capillary ends were not immersed in buffer or sample during the injection process and minimizing the number of assays for each anode or inlet buffer vial. Stacking induced by differences in ionic strength between sample and capillary buffer was reduced by using a sample buffer concentration similar to that of the capillary buffer. Although stacking accomplished by using lower sample buffer concentrations increased sensitivity, reproducibility was decreased. Achievement of the 1% R.S.D. precision level means that many quality control assays for drugs with good absorbance characteristics can be validated with HPLC reproducibility and CE efficiency. These micellar electrokinetic capillary chromatography methods conform to the USA and European Pharmacopoeial validation guidelines.

1. Introduction

Validation of capillary zone electrophoresis (CZE) [1] or micellar electrokinetic capillary chromatography (MEKC) [2] methods for the quality control analysis of small molecule pharmaceuticals has been limited because of problems with sensitivity and reproducibility [3]. An instructive investigation of CZE quantitative analysis addressed many of the experimental parameters that affect precision and reproducibility including the relationship of sample concentration to the relative standard deviation, temperature control of capillaries and sample vials, high-capacity autosamplers, evaporation control and maximum absorption wavelengths [4]. However, MEKC and the types of analyses often found in pharmaceutical applications were not specifically investigated. Pharmaceutical assays often involve MEKC and generally require

* Corresponding author.

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precision on the order of 1–2% R.S.D. over a large number of assays and over several days utilizing multiple independent sample and capillary buffer preparations, instruments and analysts.

Although quality control (QC) pharmaceutical methods have been described [3,5,6], CE or MEKC methods that rival the reproducibility of HPLC methods and satisfy the United States and European Pharmacopoeial guidelines for validation [7,8] have not appeared in the literature. The most extensive body of work in the area of quantitative pharmaceutical analysis using CZE is that of Altria and co-workers [9–14]. These authors have focussed on parameters of importance in QC validation of CZE pharmaceutical methods including sensitivity, speed of analysis, linearity, within-day (intraday) and day-to-day (interday) reproducibility, determination of trace impurities in the presence of high drug concentrations, and comparison to HPLC methods. Reproducibility in other QC related validation studies, although valuable, was limited by the instrumentation employed [15,16].

Previously in this laboratory, an attempt to complete a validation procedure was handicapped by lack of sensitivity for the degradates of the active drug substance and variability induced by the presence of excipients in the finished pharmaceutical product [3]. That method has been modified to provide greater sensitivity, reproducibility and precision, then applied to the assay of drug substances and their impurities. Control of factors more subtle than those previously reported [4] allowed the successful validation of a MEKC method for the two drug substances, hydrochlorothiazide (HCTZ) and chlorothiazide (CTZ).

2. Experimental

2.1. Sample and standard preparation

Water, acetonitrile and methanol were HPLC grade (Fisher Scientific, Fair Lawn, NJ, USA). Sodium dodecyl sulfate (SDS) was 99% pure (Sigma, St. Louis, MO, USA). Samples and standards for the drug substances were obtained internally at Merck. A series of phenolic substances tested as internal standards were obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were ACS reagent grade (Fisher Scientific). A 0.1 *M* sodium borate pH 9.5 stock solution used for anode and cathode buffers was made by adjusting a 0.1 *M* sodium borate solution to pH 9.5 with 0.1 *M* sodium hydroxide. This was diluted five-fold to make the 20 m*M* sodium borate solution used as the cathode buffer to make the anode or capillary buffer with 30 m*M* SDS. The capillary buffer was degassed under vacuum and passed through 0.45-μm filter before using. A 0.2 *M* monosodium phosphate stock solution was diluted to 10 m*M* and adjusted to pH 3.0 for HCTZ samples [17] or pH 2.0 for CTZ samples [18]. 4-Amino-6-chloro-1,3-benzenedisulfonamide (DSA) was dissolved at either pH depending on the drug substance validated. HCTZ samples (1 mg/ml or the method concentration) were first dissolved in methanol at 10% of the final volume, sonicated for 5 min in a bath at room temperature, then brought to the final volume with phosphate buffer. CTZ samples at 0.5 mg/ml or the 100% method concentration level were first mixed with 10% of the final volume of phosphate buffer and 10% of the final volume of acetonitrile and sonicated for 10 min, then brought to the final volume with phosphate buffer. All samples were passed through a 0.45-μm filter before use. The sample preparation was essentially the same as that used for HPLC QC analyses.

CE was performed on a Spectra-Physics (San Jose, CA, USA) SpectraPhoresis 1000 system (SP) using version 1.04 or 1.05 software. A manually threaded 70 cm (63 cm detection length) × 100 μm I.D. Chrompack (Raritan, NJ, USA) or a 70 cm (63 cm detection length) × 100 μm I.D. Polymicro Technologies (Phoenix, AZ, USA) internally uncoated fused-silica capillary was used in a Spectra-Physics cassette. Some ruggedness testing of the method was performed with a Beckman Instruments (Palo Alto, CA, USA) P/ACE 2100 with a 67 cm (60 cm detection length) × 100 μm I.D. capillary. Separating conditions were 20 kV applied voltage at 15°C or

17.5 kV at 25°C. Injections were hydrodynamic for 2.5 s following a 2-min capillary buffer fill time. Detection wavelength was 225 nm for most assays, but 214 nm for the P/ACE. One assay was performed from one 1.8-ml anode buffer vial or two assays for each P/ACE buffer vial to prevent buffer depletion and significant volume changes. The cathode reservoir was flushed after five assays with the SP unit or after six assays with the P/ACE unit the cathode vial was changed. At the start and end of each day, a 5-min wash with 0.1 *M* sodium hydroxide then a 10-min wash with water prepared the capillary for use or storage. Three to five blank runs were made after washing the capillary with capillary buffer for 10 min to equilibrate the system for quantitative assays.

2.2. Validation

The validation procedures employed were those used to validate HPLC methods for QC applications based on the US Pharmacopoeial (USP) [7] and European Pharmacopoeial (EC) [8] guidelines. Precision, accuracy, limits of detection and quantitation, range, linearity and method stability or ruggedness were determined. For DSA, these factors were determined by spiking the DSA impurity into HCTZ or CTZ from production samples (retention lots). On each of two days, duplicate spiked placebo samples were assayed at each of the five levels, between 50 and 150% bracketing the 100% concentration level. The assays were performed by two operators on two different instruments. Impurities were assayed similarly at 0.1, 0.5, 0.75, 1.0, 1.25 and 1.5% of the 1% of drug concentration level in the presence of the drug substance at its method concentration level (1 mg/ml for HCTZ, 0.5 mg/ml for CTZ). Accuracy was determined by comparing peak areas of sample solutions to a standard over the 50–150% concentration level range.

Limit of detection was determined by finding the concentration of the DSA impurity at which a peak area had signal-to-noise (S/N) ratio of 3. Limit of quantitation was the minimum concentration for the DSA impurity that could be reproducibly (<10% R.S.D.) integrated.

2.3. Ruggedness

Assays of five raw materials lots (retention samples) were conducted by two analysts on two different CE instruments over several days. Additionally, ruggedness testing was extended to assays on an instrument from a different manufacturer (P/ACE) under conditions that were as similar as possible to the method used on the Spectra-Physics instrument. The primary difference between instruments was the use of 214 nm as the detection wavelength with the P/ACE unit because it utilized a filter type UV detector with wavelengths of 200, 214, 254 and 280 nm installed. Although there were substantial differences in the design of the instruments, all other parameters were held constant. From each lot, duplicate samples were prepared and two injections were made for each sample. Peak areas were compared to standard peak areas to determine accuracy and precision between instruments and analysts.

3. Results

3.1. Methods development

The drug substances HCTZ, lisinopril (LIS) and CTZ were used in various combinations in several pharmaceutical finished products, *e.g.* Hydrodiuril (HCTZ), Diuril (CTZ), Prinivil (LIS) and Prinzide (LIS and HCTZ). A MEKC method was developed that separated these bulk drug substances and their impurities (DSA and Lisinopril DKP) simultaneously (Fig. 1). The objective of the study was to develop a single method that with few modifications could be used to assay all these drugs as they would normally occur in a QC situation. In developing the assay, a general strategy was adopted in which the high resolution obtainable with CE was partially sacrificed to acquire the sensitivity and speed necessary for QC methods.

A relatively simple and commonly used capil-

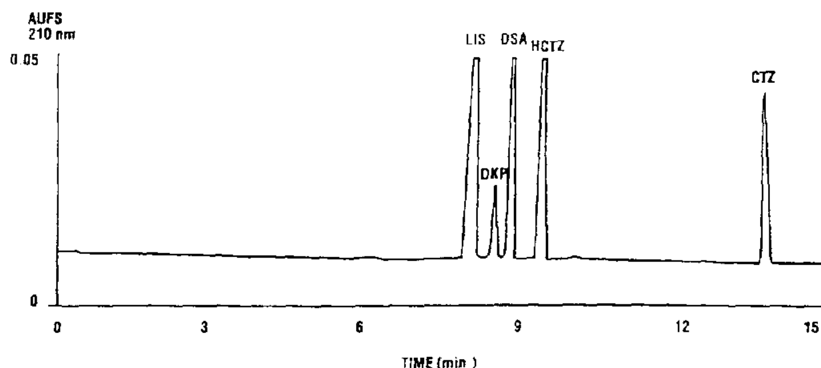

Fig. 1. Method selectivity. LIS = Lisinopril; DKP = lisinopril impurity; DSA = impurity; HCTZ = hydrochlorothiazide; CTZ = chlorothiazide. Absorbance 210 nm. Separations conditions were 20 m*M* sodium borate pH 9.5 and 20 m*M* SDS as capillary buffer, 20 kV applied voltage, and 70 cm (63 cm detection length) × 100 μm I.D.

lary buffer was chosen for the separation which included 20 m*M* sodium borate and 30 m*M* SDS at a pH of 9.5. Sodium borate has been commonly used in CE separations and has an appropriate buffering range (pK_a 9.2). The ionic strength of the buffer was relatively low to reduce joule heating and to accelerate the assay run time. SDS was used to create micelles that yielded the selectivity necessary to separate all the drug substances and impurities. The SDS concentration was minimized to maintain low ionic strength, lower the current and joule heating while providing the required selectivity. The pH chosen allowed weak anions ($RCOO^-$) to be deprotonated thereby increasing their negative charge and weak cations (RNH_2) to be deprotonated thereby decreasing their positive charge. Thus, migration times of the cationic and anionic compounds were increased to allow better separation and reduce the wall interactions of cations.

To obtain the sensitivity required for impurity quantification, a wide-bore capillary, 100 μm I.D., was used in preference to the more commonly available 75 and 50 μm I.D. capillaries. The use of this diameter resulted in reliable quantitation of DSA at 1 μg/ml. The minimum detection and quantitation limits were an order of magnitude better than with 75 μm capillaries. Large diameter capillaries can be used on any instrument reducing the need for esoteric optics or capillary configurations to achieve adequate sensitivity. The larger diameter capillary generated more joule heat and was more difficult to cool than smaller capillaries which resulted in selection of a subambient temperature for the capillary oven with 20 kV applied voltage or 25°C with 17.5 kV applied voltage. When subambient temperatures were used, condensation of atmospheric water vapor sometimes resulted in stray currents that disrupted separations.

Instrumental conditions were set to provide the highest possible applied voltage without generating excessive heat, because resolution in CE depends upon the field strength which is the voltage applied over the capillary length (V/cm) [1]. The injection time of 2.5 s was found to be optimal for peak shape and peak area reproducibility compared to injections of 1, 5, 7.5, or 10 s at the concentration of CTZ, HCTZ and DSA chosen. The detection 225 nm wavelength was a compromise for CTZ, HCTZ and DSA under the pH and buffer conditions used. CTZ and HCTZ peak areas were large enough for good reproducibility and the DSA peak areas were large enough for quantitation.

CTZ and HCTZ were stable in solution at pH 2 and 3, respectively for at least 24 h as determined by testing and as noted in the literature [17,18]. The phosphate sample buffer was chosen for its pH buffering range and its concentration selected to minimize stacking or focusing that caused peak area variability. Stacking induced by differences in ionic strength between sample and

capillary buffers provided increased sensitivity, but decreased peak area reproducibility.

The method concentration level of 1 mg/ml for the HCTZ was chosen to fit with practical constraints. The 1 mg/ml yielded good peak areas for quantitation of the HCTZ at 50% through the 150% levels, yet HCTZ was completely soluble in the sample buffer over that range. The DSA concentration was 10 μg/ml and was easily quantified. The method concentration for CTZ was determined in a similar manner to be 0.5 mg/ml. This level was less than that of the HCTZ because CTZ was less soluble than HCTZ in the phosphate buffer even with acetonitrile added to increase its solubility. The DSA concentration was 1% of the CTZ level or 5 μg/ml. Because of the differences in the drug substance solubilities, DSA was validated at 10 μg/ml in the presence of HCTZ and 5 μg/ml in the presence of CTZ. These concentrations were 1% of the drug substance level which was the established maximum specification for this impurity.

3.2. *Subtle factors in improving R.S.D.*

The control of subtle factors allowed the improvement in precision from the 2–4% R.S.D. achieved by controlling major and minor parameters to the required 1% R.S.D. In studying the literature and in this laboratory, there appeared to be a limit of 2–4% R.S.D. beyond which methods could not be improved. From many efforts to improve the precision of the methods, it was found that some relatively simple and perhaps overlooked techniques enhanced precision and reproducibility to the required 1% R.S.D. for QC methods. Frequent change of anode (inlet end) buffer vials yielded significant improvement in R.S.D. from approximately 2–4% to 1–2%. This was achieved by allotting one anode (inlet) buffer vial for each assay with the Spectra-Physics instrument and two assays per vial for the P/ACE due to limited autosampler space. Several effects may explain the necessity for changing buffer vials after each assay including buffer depletion, sample contamination of the buffer vials, thermal (thermoclines) or concentration layer formation and volume reduction in the buffer vials with each sample assayed. The effects appeared to be identical with the small 1.8-ml Spectra-Physics and the larger P/ACE vials.

It was also observed that reducing the time that the capillary was not immersed by buffer during the injection process improved the 1–2% R.S.D. to better than 1%. During the injection process when the capillary end(s) were exposed to the atmosphere, many events affecting reproducibility may have occurred including siphoning, evaporation, formation of droplets and movement of the sample or capillary buffer to an area on the outside surface of the capillary. The buffer vials used as anode (inlet) end reservoirs during the separations and used to fill the capillary before the assay were placed in the autosamplers in the positions directly adjacent to the samples or standards to be injected. This minimized the time that the inlet end of the capillary was exposed during movement of buffer vial to sample vial and back to buffer vial and thus may have limited siphoning effects. Controlling minor parameters in this way was necessary to meet the precision limits (1–2% R.S.D.) imposed by QC specifications and allowed CE methods to approach HPLC precision and reproducibility. It may be possible for manufacturers to increase the overall precision of their instruments by controlling these factors in their future designs.

3.3. *Internal standard*

The utility of an internal standard for quantitation was investigated using a series of phenols including 3,5-dimethylphenol, 2,3,5-trimethylphenol and 4-*tert.*-butylphenol. However, peak areas for these phenols were less reproducible than the drug substance peak areas. The phenols appeared to have different solubility than the drugs in the capillary buffer and 4-*tert.*-butylphenol had significant wall interaction. Therefore, validation assays were performed using an external standard method because integration was more accurate and sample preparation simpler than with these internal standards. Finished pharmaceutical product assays for the

drug substances and degradates in the presence of excipients may require internal standards.

3.4. Sensitivity

The 75 μm I.D. capillaries commonly used in CE studies allowed only a 1 μg/ml minimum detection limit (LOD) and 10 μg/ml quantitation limit (LOQ). Sensitivity was increased by increasing capillary internal diameter with an acceptable loss in resolution. A 100 μm I.D. capillary yielded an order or magnitude increase in sensitivity allowing reproducible integration at 1 μg/ml and minimum detection limit of 0.1 μg/ml for the impurities. The increased column diameter allowed a greater loading capacity and a longer light path for the detector. Maximum resolution was traded for sensitivity by use of the larger bore capillary. This sensitivity increase was the key to obtain reproducibility in the validation studies. Larger peak areas yielded a lower percentage error that made this validation possible with R.S.D. less than 1% for drug substances and less than 10% for impurities when other factors were controlled.

3.5. Micellar window

Under the standard conditions of 20 kV and 15°C, the micellar migration marker, Sudan III, migrated at 17.7 min while the electroosmotic flow (EOF) marker, acetone, migrated at 6.5 min ($t_{micelle}/t_0 = 2.7$). This was a relatively narrow micellar window, yet it yielded adequate selectivity for the resolution of at least three drug substances and their impurities. The micellar $k' = (t_m - t_0)/t_0(1 - t_m/t_{micelle})$ (t_m = migration time) [2] was 4.8 for DSA, 2.6 for LIS, 4.1 for lisinopril DKP, 7.0 for HCTZ and 35.5 for CTZ. The k' values were similar at 17.5 kV and 25°C for the drugs with a micelle migration time for Sudan III of 16.3 min and a migration time for acetone of 6.2 min ($t_{micelle}/t_0 = 2.6$). The separation of all these drug compounds simultaneously indicated that methods may be developed for pharmaceutical finished products containing multiple drugs.

4. CE method validation assays

4.1. HCTZ and DSA method validation

HCTZ standard solutions were prepared in duplicate at the following concentrations: 0.5, 0.75, 1.00, 1.25 and 1.50 mg/ml (equivalent to 50, 75, 100, 125 and 150% of the target concentration, respectively). The samples were dissolved in 10 m*M* sodium phosphate pH 3.0. The resulting linearity data are shown in Table 1. The correlation coefficients, R^2, were greater than 0.998 which indicated good linearity bracketing the method concentration. On a different day, HCTZ solutions were prepared in duplicate at the concentrations listed above. R^2 was found to be 0.995. The solutions were analyzed *versus* the reference standard at 0.5, 0.75, 1.00, 1.25 and 1.50 mg/ml. The average assay values, which are listed in Table 2, were 100.4, 99.9, 100.1, 100.6 and 99.5% for the 0.5, 0.75, 1.0, 1.25 and 1.5 mg/ml levels, respectively. Moreover, the R.S.D.s were better than 1%. The data in Table 2 demonstrated that the method was precise and accurate for HCTZ.

To show selectivity, DSA, CTZ and other impurities were separated from HCTZ by the CE assay method as demonstrated in the electropherogram in Fig. 2. Resolution for DSA and

Table 1
HCTZ method linearity

Level (%)	First mass[a]	Second mass
50	2 414 309	2 423 459
75	3 291 564	3 249 930
100	4 059 126	4 111 284
125	4 807 602	4 829 569
150	5 502 642	5 534 600
R^2	0.998	0.998

[a] Masses are replicate weighings of samples and the data are peak areas from the assay of those weighings.

Table 2
Method accuracy for HCTZ

Assay	50%	75%	100%	125%	150%
1	101.6	100.2	99.0	101.6	99.9
2	100.1	99.7	99.9	100.4	99.5
3	99.5	99.8	100.5	99.8	99.0
4	100.6	99.8	101.0	100.8	99.6
Mean	100.4	99.9	100.1	100.6	99.5
S.D.	0.9	0.2	0.8	0.7	0.4
R.S.D. (%)	0.9	0.2	0.8	0.7	0.4

Mean ($n = 4$) accuracy for the bulk drug substance compared to a reference standard. The 100% concentration level was 1 mg HCTZ/ml. Assays 1 and 2 were performed on day 1, while assays 3 and 4 were performed on day 2 with a different instrument and by a different analyst.

HCTZ was 1.73. The unknown peaks were not identified, but may be intermediates in the synthesis of HCTZ [17].

4.2. DSA assay in the presence of HCTZ

The DSA solutions were prepared in duplicate by spiking the reference standard DSA into 1.0 mg/ml HCTZ at 1, 5, 7.5, 10, 12.5 and 15 μg/ml (equivalent to 10, 50, 75, 100, 125 and 150% of the DSA target 100% level, respectively). The correlation coefficients were 0.999 for the two different days which indicated good linearity over the 50–150% assay range. The R.S.D.s in Table 3 were well within an internal 10% R.S.D. limit for precision in handling impurity data. To demonstrate method performance with the commonly assayed retention samples, five samples from two HCTZ lots A and B were prepared. The solutions were analyzed *versus* the reference standard DSA. The average assay values were 0.12%, R.S.D. = 0.03% for HCTZ lot A and 0.18%, R.S.D. = 1.23% for HCTZ lot B. These values were within the 10% R.S.D. limit for accuracy of the sample DSA compared to a standard.

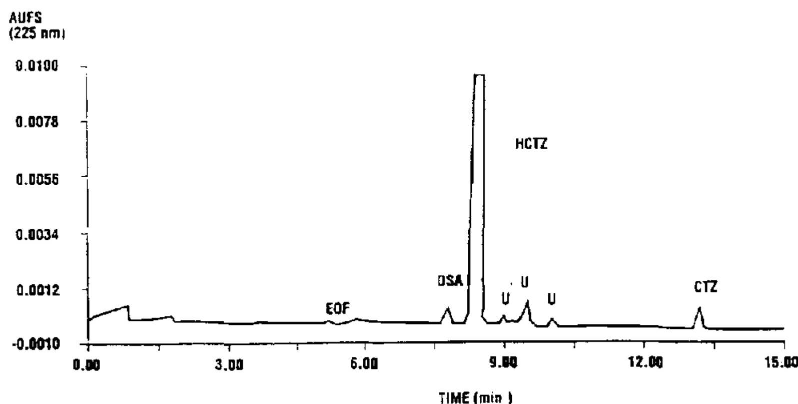

Fig. 2. HCTZ and DSA assay electropherogram. EOF = electroosmotic flow marker, methanol; DSA = impurity; HCTZ = hydrochlorothiazide; U = unknown; CTZ = chlorothiazide. Absorbance 225 nm. Separations conditions were 20 m*M* sodium borate pH 9.5 with 30 m*M* SDS as capillary buffer, 20 kV applied voltage, and 70 cm (63 cm detection length) × 100 μm I.D. capillary.

Table 3
Assay precision for DSA in HCTZ samples

Assay	10%	50%	75%	100%	125%	150%
1	7237	50 503	69 519	87 185	106 808	125 813
2	7350	49 602	68 342	86 797	107 322	126 237
3	7249	50 081	68 554	89 708	105 967	126 351
4	7227	49 939	67 604	88 628	105 288	125 583
Mean	7266	50 031	68 505	88 080	106 346	125 996
S.D.	57	373	789	1341	900	360
R.S.D. (%)	0.8	0.7	1.0	1.3	0.7	0.3

Mean ($n = 4$) peak areas for assays of DSA spiked into samples containing HCTZ at its method concentration (1 mg/ml). The 100% level of DSA was 10 μg/ml or 1% of the HCTZ concentration. Assays 1 and 2 were performed on day 1, while assays 3 and 4 were performed on day 2 with a different instrument and by a different analyst.

The limit of detection of 100 ng/ml with an S/N ratio of 3. The limit of quantitation was 1.0 μg/ml. These values were achievable by use of the 100 μm I.D. capillary. In system-suitability determinations, the R.S.D. of five injections was 2.24% at the 100% level. This precision was within a 10% R.S.D. internal limit.

4.3. *Ruggedness testing*

Table 4 illustrates duplicate assays on five HCTZ lots performed by two analysts on three instruments on two days. Precise and reproducible data were obtained from each analyst and instrument. The Beckman and Spectra-Physics instruments had very different basic designs, especially in regard to the injection methods, yet separation selectivity and precision were similar which indicated a basic ruggedness for CE methods among instruments from different manufacturers. The comparison with the Beckman P/ACE was for illustration purposes only. The method was validated only on the Spectra-Phoresis instrument.

Table 4
HCTZ method ruggedness

Lot	Analyst 1[a]		Analyst 2[a]		Analyst 1[b]	
	HCTZ (%)	DSA (%)	HCTZ (%)	DSA (%)	HCTZ (%)	DSA (%)
Day 1						
1	99.3	0.24	99.5	0.23	99.8	0.27
2	99.5	0.16	100.2	0.16	99.9	0.19
3	99.9	0.19	100.2	0.19	99.7	0.22
4	99.3	0.14	100.1	0.14	100.5	0.18
5	99.9	0.08	100.2	0.08	99.9	0.12
Day 2						
1	100.5	0.25	100.0	0.24		
2	100.0	0.16	99.6	0.16		
3	101.0	0.20	99.4	0.18		
4	101.3	0.13	100.3	0.13		
5	100.1	0.08	100.1	0.07		

[a] Spectra-Physics 1000 CE System.
[b] Beckman P/ACE 2100.
Mean ($n = 4$) assay accuracy of HCTZ sample lots compared to a reference standard.

Table 5
CTZ method linearity

Level (%)	First mass[a]	Second mass
Capillary 1		
50	1 849 954	1 865 108
75	2 807 003	2 806 748
100	3 686 523	3 676 258
125	4 632 717	4 590 615
150	5 483 839	5 522 213
R^2	0.999	0.999
Capillary 2		
50	2 175 439	2 180 318
75	3 260 843	3 297 954
100	4 293 019	4 337 297
125	5 313 571	5 374 834
150	6 449 737	6 412 312
R^2	0.999	0.999

[a] Masses are replicate weighings of samples and the data are peak areas from the assay of those weighings.

4.4. CTZ and DSA method validation

The reference standard CTZ solution and duplicate CTZ solutions were prepared at 0.25, 0.375, 0.50, 0.625 and 0.75 mg/ml (50%, 75%, 100%, 125% and 150% level) in acetonitrile–sodium phosphate buffer pH 2.0 (10:90). In a two-day validation, two different capillaries were used. The correlation coefficients were 0.999 (Table 5) well within the acceptable internal minimum of 0.99. The peak areas obtained with the two capillaries were different, but each set of data was linear, precise and reproducible over the range assayed. In a two-day validation, the CTZ lot A was analyzed *vs.* the reference standard CTZ. The accuracy ranged from 99.4 to 101.3% and the R.S.D.s were within the 1% limit (Table 6). The DSA, HCTZ and three unknown peaks, possibly including N-(2-amino-4-chloro-5-sulfamoylphenylsulfonyl) formamide and 5-chloro-2,4-disulfamoylformanilide [17], were separated from CTZ by the CE assay method (Fig. 3).

4.5. DSA assay in the presence of CTZ

The DSA solutions were prepared in duplicate by spiking the reference standard DSA into 0.5 mg/ml CTZ at 0.125, 0.5, 2.5, 3.75, 5.00, 6.25 and 7.5 μg/ml. In a two-day validation, the correlation coefficients were 0.999 (Table 7) which indicated linearity within the 0.99 limit. To determine precision of DSA assay, solutions were prepared in duplicate by spiking the reference standard DSA into 0.5 mg/ml CTZ. In the two-day validation, the R.S.D. was below the 10% internal limit indicating good precision (Table 7). The limit of detection was 100 ng/ml. The limit of quantitation was 0.5 μg/ml. The R.S.D. of five injections were 1.2% for day 1 and 0.4% for day 2 at the 100% level (5 μg/ml) for system suitability determinations.

Table 6
Method accuracy for CTZ

Assay	50%	75%	100%	125%	150%
1	100.5	101.4	100.5	100.5	100.7
2	101.3	101.4	100.3	99.6	101.4
3	99.9	99.3	98.9	98.2	101.9
4	100.1	100.4	99.9	99.3	101.3
Mean	100.4	100.6	99.9	99.4	101.3
S.D.	0.6	1.0	0.7	0.9	0.5
R.S.D. (%)	0.6	1.0	0.7	1.0	0.5

Mean ($n = 4$) accuracy for the bulk drug substance compared to a reference standard. The method concentration (100% level) was 0.5 mg CTZ/ml. Assays 1 and 2 were performed on day 1, while assays 3 and 4 were performed with a different instrument and by a different analyst.

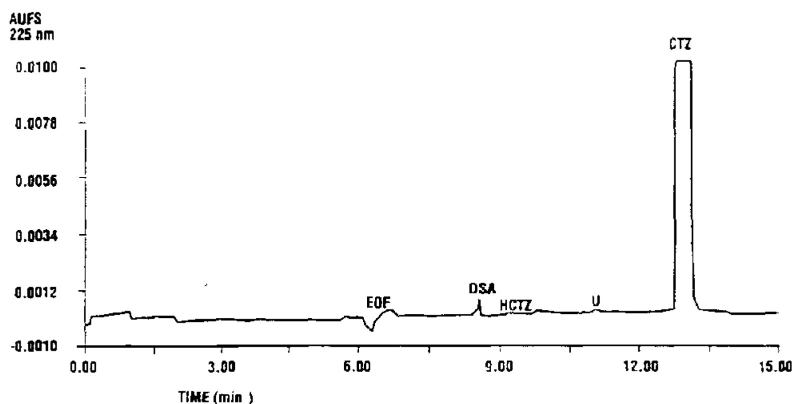

Fig. 3. CTZ and DSA assay electropherogram. EOF = electroosmotic flow marker, acetonitrile; DSA = impurity; HCTZ = hydrochlorothiazide; U = unknown; CTZ = chlorothiazide. Absorbance 225 nm. Separations conditions were 20 m*M* sodium borate pH 9.5 with 30 m*M* SDS as capillary buffer, 20 kV applied voltage, and 70 cm (63 cm detection length) × 100 μm I.D. capillary.

4.6. Ruggedness testing

Extensive ruggedness testing was performed with two Spectra-Physics instruments. Two analysts, three capillaries, and many sample and standard preparations were involved over a period of 2 months. The method was intended for daily use in a QC laboratory and as the first of its kind unusual steps were taken to ensure reliability and transfer of the method from development to utilization. Table 8 indicated that the method was reproducible. In these CTZ ruggedness assays, it was determined that more accurate data were obtained when the peak areas were corrected by dividing the areas by migration times of the peaks. This was not necessary with HCTZ and may be required only with specific drugs or analysts. Corrected peak areas or peak areas divided by migration time are commonly used in CE, was not found to be

Table 7
Assay precision for DSA in CTZ samples

Level (%)	First mass[a]	Second mass
Capillary 1		
10	4 301	4 770
50	21 557	21 993
75	32 320	33 297
100	43 767	45 010
125	55 059	56 494
150	66 710	68 722
R^2	0.999	0.999
Capillary 2		
10	4 624	5 226
50	21 962	22 240
75	33 169	33 430
100	44 325	45 047
125	55 532	56 566
150	67 651	68 555
R^2	0.999	0.999

[a] Masses are replicate weighings of samples and the data are peak areas from the assay of those weighings.

Table 8
CTZ method ruggedness

Lot	Analyst 1		Analyst 2	
	CTZ (%)	R.S.D. (%)	CTZ (%)	R.S.D. (%)
1	100.0	0.8	100.5	1.0
2	99.9	0.6	100.2	1.0
3	99.7	0.8	100.4	1.0
4	100.0	1.1	100.7	1.1
5	99.9	0.6	100.9	1.0

Mean (n = 4) assay accuracy and precision of CTZ samples compared to a reference standard.

necessary in this laboratory when migration times were extremely consistent.

5. Discussion

Development of precise (1% R.S.D.), reproducible assays in CE requires control of large number of factors [3,4,9–14]. The factors that must be controlled in HPLC assays are now well known as HPLC technology has matured, but the many minor or subtle CE factors that yield the precision and reproducibility necessary in quantitative assays have not been clearly elucidated in practical terms. In order to obtain the precision necessary to validate QC methods this laboratory has investigated the control of these more subtle parameters.

Parameters of minor importance for MEKC methods development generally, but having major importance in reaching the 1% R.S.D. levels required for precise quantification included minimizing siphoning effects while the capillary ends were not immersed. It was observed on both Spectra-Physics and Beckman instruments that placing sample vials and the corresponding buffer vials immediately adjacent to each other significantly enhanced R.S.D. Minimizing the number of assays performed in each buffer vial also enhanced R.S.D. Two or less assays for each anode buffer vial in the Spectra-Physics instrument and two or less assays for each pair of buffer vials in the Beckman instrument consistently yielded the best R.S.D. values. Maintaining a consistent fluid level in each anode vial for the Spectra-Physics instrument and identical fluid levels in the Beckman instrument vial pair (anode and cathode or inlet and outlet) also resulted in better precision. Stacking or sample concentration during injection by using a ten-fold lower buffer concentration in samples provided significant increases in sensitivity in preliminary investigations, but also resulted in variability and poor peak area reproducibility. Stacking resulted in skewed peak shapes that suggested an overload of the capacity of the capillary buffer to solubilize the high concentration of sample molecules injected. The sample buffer concentration selected was 10 m*M* to approximate the capillary buffer concentration. The relatively low pH for the sample buffer was necessitated by the stability of the drugs which prevented use of stacking by differences in pH [19]. Consistent vial fill levels were obtained by filtering solutions into the vials through a syringe filter with the tip of the filter placed as far as possible into the vial. The vials were filled above the level of the filter tip, then the fluid above the tip level was withdrawn to a level even with a filter tip. Siphoning effects were aggravated by the large-bore capillary utilized in this study to provide adequate sensitivity. Dose and Guiochon [20] discussed the theoretical background and accurately predicted the effects of "inadvertent hydrodynamic flow". Although these effects were not large scale their control was essential for precise quantitation.

6. References

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