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Assay for the determination of low dosage form of formoterol dry syrup by capillary electrophoresis with head-column field-amplified sample stacking

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Abstract

The development of a capillary zone electrophoresis method with head-column field-amplified sample stacking injection for the determination of formoterol (FMTR) in a low dosage dry syrup form was described. To obtain the highest sensitivity, the sample solution was prepared by high content of organic solvent with the presence of a small amount of H^+ (60–100 μM) and the capillary inlet end was dipped in water before electroinjection. This method was fully validated in terms of repeatability (RSDs for migration time, peak area of FMTR and peak area ratio between FMTR and I.S. at 1 μg/ml of FMTR was 0.76, 1.10 and 0.55% respectively), reproducibility (RSDs from different capillaries, analytes, days and instruments were 1.52%, 1.04%, 1.16% and 1.93% respectively), linearity ($y = 0.827x - 0.085$, $r = 0.9993$ ($n = 6$) over the range of 0.25–2.0 μg/ml), limits of quantitation, ruggedness and robustness. The method was applied to the determination of the drug in commercial dry syrup preparation (recovery was 100.9%, RSD = 1.5%, $n = 5$) and proved to be fast and reliable for the quantitation analysis of FMTR in the pharmaceutical form.

Keywords: Formoterol; validation; CZE; Head-column field-amplified sample stacking

1. Introduction

Formoterol (FMTR, Fig. 1a) is a β-receptor stimulating bronechodilator with excellent bronchoselectivity, anti-allergic effect and pulmonary edema-inhibitory effect [1]. There was only 40 μg of formoterol fumarate contained in one gram of the formoterol dry syrup and others were inactive ingredients such as sugar, sodium benzoate etc. It is troublesome to assay it by HPLC directly because the dosage of FMTR was low and the HPLC column may be contaminated by sugar or other ingredients contained in the dry syrup. Although an HPLC method had been developed in our laboratory that these inactive ingredients may be removed by a cation-exchanged solid-phase

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extraction procedure, poor results of the reproducibility of extraction were observed by use of different batches of resins or resins from different manufacturers used. Therefore, the extractive recovery validation must be performed every time if the different resin was used. This is an over-elaborate procedure and there was no report found about the assay of FMTR in a preparation except for some papers about the determination in plasma, urine [2–5] or enantiomeric separation [6].

There is growing interest in the use of capillary electrophoresis (CE) for the analysis of bulk drugs and pharmaceutical preparations [7,8]. High separation efficiency, selectivity, large separation capacity, flexibility and relatively low operational cost are the attractions of the technique. Since the capillary was hardly contaminated by sugar or other inactive ingredients that often occurred in HPLC column, the CE technique was more suitable to determining drug. However, the quantitative aspects of CE, particularly in pharmaceutical applications need to be explored more fully owing to the wide spread of parameters that can influence the analytical results. To determine a very low dosage drug, the solute was generally concentrated by solvent–solvent extraction or by solid-phase extraction. These were time-consuming procedures and large amounts of organic solvents were used. On the other hand, errors would be increased during tedious extraction procedures.

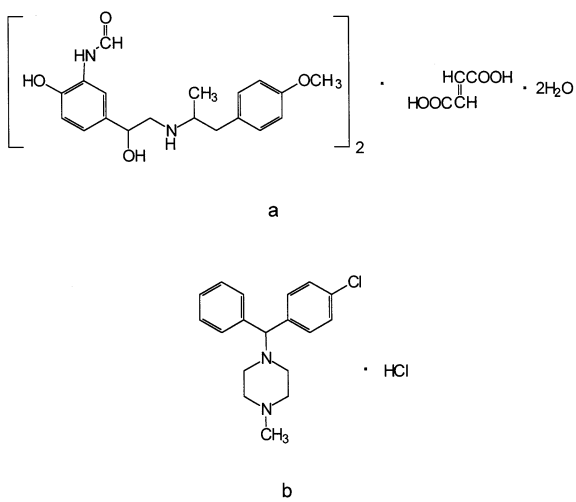

Fig. 1. Structure of formoterol fumarate (a) and internal standard (b).

Although the lowest detectable mass of CE was very low, the lowest detectable concentration in CE with UV–VIS absorption detection is in the 1–10 μM range because of the short optical path length within the detection. This concentration sensitivity is 1–2 orders of magnitude worse than that encountered in HPLC [9]. Sample stacking technique was a remedy for this drawback [10,11]. Sample stacking is a distinctive on-column concentration method for CE and the head-column field-amplified sample stacking (HCFASS) can provide a sensitivity enhancement over 1000-fold [12]. The principle of head-column field-amplified sample stacking is based on Ohm's law. After replenishing the capillary with running buffer, a short zone of low conductivity (e.g. water plug) at the inlet side was introduced before making an electrokinetic sample injection from a sample solution of low conductivity. During electroinjection, the charged solutes migrate rapidly through the water zone. When the charged solutes reach the interface of the water zone and the running buffer, their electromigrational transport is decreased because the electric field within the water plug is much higher than that within the buffer. Consequently, many of the charged solute molecules are effectively concentrated before their electrophoretic separation.

In the present work, the potential utility of CE was studied as an efficient alternative method for the determination of FMTR in dry syrup dosage formulation by capillary zone electrophoresis (CZE). In order to achieve a robust, validated assay for FMTR, close attention was paid to the capillary conditioning, the optimization of ion strength, sample stacking effects and sample introduction.

2. Experimental

2.1. Instrumentation

Instruments from two CE manufactures, a TSP SpectroPhoresis 1000 system with FOCUS fast

scanning UV–VIS detector (Thermo Separation Products, San Jose, CA, USA) and an IBM 350-450DX2 PC running with PC1000 version 3.0 software for the method development, and a P/ACE system 5500 with DAD detector and control with Gold system software (Beckman, Fullerton, CA, USA) for ruggedness and robustness study, were used in this study. For TSP instrument, the electrophoretic separation was performed on a fused-silica capillary of 40 cm × 50 μm I. D. (32.5 cm of effect length, Polymicro Technologies, Phoenix, AZ, USA). New capillary was conditioned with 1M NaOH for 10 min at 60°C, followed with 0.1 M NaOH for 10 min at 60°C and water for 10 min at 20°C prior to use. For Beckman instrument, the fused silica capillary was 57 × 75 μm I.D. (50 cm of effect length). New capillary was conditioned with 1M NaOH for 10 min at 50°C, followed with 0.1 M NaOH for 10 min at 50°C and water for 10 min at 20°C prior to use. After each run, the capillary was rinsed for 2 min with running buffer. A constant voltage of 20 kV was applied throughout the run and the average current was about 80 μA. In both instruments, UV absorbance detection wavelength was set at 200 nm and the capillary temperature was controlled at 20°C. The running buffer was 80 mM phosphate buffer (pH3.0). All buffers were filtered through a 0.25-μm filter.

2.2. Chemicals

All reagents: 1-propanol (Beijing Chemical Reagents Company), phosphoric acid, dipotassium hydrogen phosphate (both from Beijing Hongxing Chemical Factory) were of analytical grade. Water was redistilled before use.

Formoterol fumarate (racemic compound), its dry syrup and the pre-mixed placebo were from Yamanouchi Pharmaceutical Co. Ltd. Chlorcyclizine hydrochloride used as I.S. (Fig. 1b) was from our division.

2.3. Standard and sample solutions

2.3.1. Standard stock solution and I.S. solution

The standard stock solution was prepared to 10 μg/ml of formaterol fumatate with 1-propanol. Other standard solutions used for the determining of linearity, recovery, limit of quantitation (LOQ) and repeatability etc. were diluted from this stock solution. The internal standard solution (I.S.) was prepared to 47 μg/ml of chlorcyclizine hydrochloride with water.

2.3.2. Preparation of sample solution and the procedure of FASS

A suitable amount of dry syrup was ground to fine powder with mortar and pestle. 6 g of the powder was accurately weighed and transferred to a flask, 25 ml of 1-propanol was added accurately and the mixture was sonicated for 5 min. A portion of the solution was transferred to centrifuge tube and centrifuged for 3 min at 15 000 rpm (13 000 × g). 1.0 ml of the supernatant solution was pipetted in a 10 ml volumetric flask, 0.2 ml of I.S solution and 6.0 ml of 1-propanol were added and then make up to the volume with 200 μM phosphoric acid solution. The final sample solution contained about 1 μg/ml FMTR, 0.95 μg/ml of I.S., 70% 1-propanol and 60 μM phosphoric acid. After mixed up thoroughly, the solution was filtered through a 0.25-μm membrane filter. The control solution was prepared under the direction of Section 3.2.3.

Before electroinjection the inlet end of capillary was dipped into a vial containing water, designating the purge time as zero. Depending on the performance of the instrument, dipping time intervals were about 25–30 s. Then the inlet end was moved to the sample vial to perform electroinjection. The heights of water vial and of sample vial were remained the same as that of the buffer in cathode chamber (or cathode vial). The sample solution was introduced with electrokinetic mode at 10 kV for 20s with the anode on the injection side and the current was about 10 μA.

3. Results and discussion

3.1. Method development

Accurate and precise quantitative results in CE depend on many parameters. These parameters include instrumental factors and operational vari-

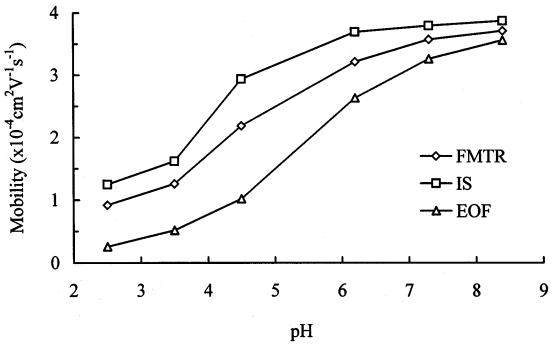

Fig. 2. The effect of pH on the separation of FMTR, I.S. and excipients (combined with electro-osmotic flow, EOF). Column: 40 cm × 50 μm I.D. (32.5 cm effect length) fused-silica capillary. Buffer: 80 mM phosphate (pH was adjusted from 2.5 to 8.0 with phosphoric acid). Other conditions are same as in text.

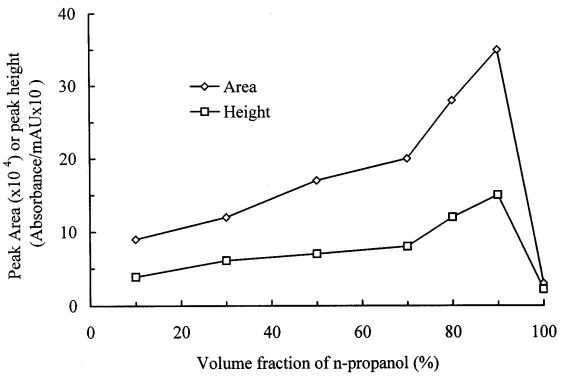

Fig. 3. Effect of 1-propanol concentration in sample solution on peak area and peak height. Buffer: 80 mM phosphate (pH 3.0). Other conditions are same as in Fig. 2.

ables. In order to achieve robust and reliable methods with CE for subsequent validation and application to the analysis of pharmaceutical on a regular basis, the analysts have to consider these parameters and their effects on the analytical responses and results. In terms of the separation, preliminary experiments in this work shown that it was well separated from the pH range of 2.5–6.0 (Fig. 2). As the buffer pH was increased from pH 2.5–6.0, migration times of two peaks were decreased and the electro-osmotic flow (EOF) was increased. When pH at 7.0, the resolutions of FMTR peak with I.S. and FMTR peak with the EOF peak were worsened. Although a shorter migration time with better separation was obtained at higher pH range (up to pH 6.0), low pH buffer could offer higher stacking efficiency because the EOF was small and the diffusing effect of solvents during electroinjection.

The concentration of electrolyte in the running buffer was not significantly influencing the resolutions of all peaks, but was an influential factor to stacking efficiency. The higher the conductivity of the buffer, or the lower the conductivity of the sample solution, the higher stacking efficiency is obtained [10]. Nevertheless, higher conductivity of buffer solution results in higher Joule heating at the inner of the capillary.

The stacking efficiency was also effected by the sample matrix. Because this preparation contains ionic inactive ingredients, such as sodium benzoate, the conductivity of sample solution was higher than that of standard solution if directly prepared with a solvent. A suitable solvent should be chosen to dissolve the main component with the lower dissolubility to other inactive ingredients. In comparison, 1-propanol was better than other solvents tested, such as water, methanol, ethanol and acetonitrile. On the other hand, the volatility of 1-propanol was less than other common organic solvents.

As presented in Fig. 3, the volume fraction of 1-propanol in the sample solution was found to drastically influence the stacking efficiency. This effect is based on the changes in conductivity [10]. As the volume fraction of 1-propanol increasing from 10 to 90%, both peak height and peak area were increasing. The highest sensitivity was obtained with the volume fraction of 90% of 1-propanol. However, when 1-propanol exceeded 90%, peak height and area was drastically decreased. The percentage of 1-propanol employed in this work was 70% considering sensitivity, precision and accuracy (discussed below).

The stacking efficiency was influenced by the concentration of phosphoric acid in sample solution as well. With electrokinetic sample introduction, the amount of solute injected is proportional to the effective electrophoretic mobility [10,11], low conductivity and low pH appeared to provide the highest stacking of positively chargeable so-

lutes [12]. For a given conductivity, the solution with the lowest pH gives the highest sensitivity. As the concentration of phosphoric acid increased from 0 to 60 μM, both peak height and peak area were increased. When the concentration of phosphoric acid increased from 60 μM to 100, although the peak area was increased, however the peak height was decreasing and the peak was broadened. As the concentration of phosphoric acid increased to 200 μM, both peak area and peak height were decreased, so the concentration of phosphoric acid was chosen at 60 μM (Fig. 4).

Dipping the capillary inlet end and electrode in a vial containing water was found to be necessary to prevent contamination of the sample solution with running buffer of high conductivity. Without this procedure, smaller and irreproducible peaks were observed. The length of the water plug within the capillary should remarkably influence the sensitivity. It was reported that the sensitivity decreased as the length of water plug introduced by hydrodynamically was increased [12], however, the dipping time was uncontrollable by the used software with the TSP instrument in this study. The dipping time and the length of water were estimated as 25–30 s and 4 mm respectively.

In all of above conditions, the excipient peak was fused in the EOF peak. Typical electrophoregram was presented in Fig. 5.

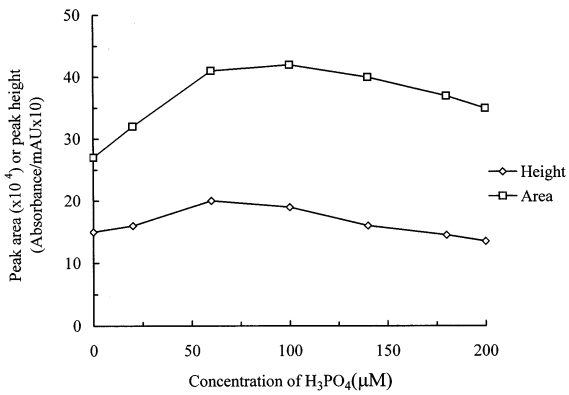

Fig. 4. Influence of the concentration of phosphoric acid on peak area and peak height of formoterol. Conditions are same as in Fig. 3.

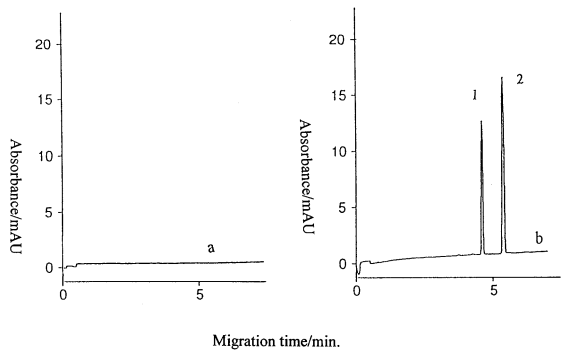

Fig. 5. Electrophoregram of the extract of (a) a placebo; (b) a formoterol dry syrup sample. Peaks: 1, chlorcyclizine (I.S.); 2, formoterol. Conditions are same as in the text.

3.2. Validation of the method

The CZE method for quantitation of FMTR in the dry syrup dosage form was validated in terms of linearity, limit of quantitation (LOQ), repeatability, reproducibility, ruggedness and robustness.

3.2.1. Linearity

The linearity for FMTR was assessed over the range of 0.25–2.0 μg/ml. Varying volumes of standard solution were spiked to 10 ml volumetric flasks to give concentrations of 0.25, 0.5, 1.0, 1.5, 1.8, 2.0 μg/ml of FMTR then 0.2 ml of I.S solution was added to each flask. After several volume of 1-propanol was appended to each volumetric flask to make the total volume of 1-propanol to 7.0 ml, The flasks were made up to the volume with 200 μM phosphoric acid solution. Duplicate injections were made at each concentration. The linearity of the standard curve was confirmed by plotting the ratio of the FMTR and I.S. peak areas versus the concentration of FMTR. A straight line obtained in the 0.25–2.0 μg/ml range was $y = 0.827x - 0.085$, $r = 0.9993$ ($n = 6$), where y is the ratio of FMTR and I.S peak areas and x is the concentration of FMTR (in μg/ml).

3.2.2. Limits of quantitation (LOQ)

For the TSP instrument, The LOQ based on a signal-to-noise ratio of 10 was found to be 0.12 μg/ml. For the Beckman instrument, the LOQ

was 35 ng/ml. The sensitivity from this method compared with that obtained from hydrodynamic injection mode indicated that a <260-fold of magnitude of concentration sensitivity was improved. The sample solution used for non-FASS injection was diluted from the standard stock solution by the running buffer.

3.2.3. Recovery assessment

The recovery is evaluated by comparing the peak area or peak–area ratio of the solute with I.S of the test solution with that of the control solution. Generally, the control solution was constructed with a simple solvent or the solvent mixture. It was not acceptable for this study to use this control solution because its background did not have the same conductivity as that of the test solution. For HCFASS injection of CE analysis, different matrix of sample solution results in different peak area of a solute. The higher the conductivity, the less the peak area. So this is one of the important factors to obtain a 'true' recovery. Some ingredients, especially sodium benzoate may increase the conductivity of sample solution. Although most of the excipients were removed by the use of 1-propanol as a solvent, because some ionic components, especially sodium benzoate, were slightly dissolved, the conductivity of test solution was still larger than that of the control solution. The measured conductivity of test solution prepared by 1-propanol (2.0×10^{-5} Ω^{-1}/cm) was smaller than that prepared by water (1.0×10^{-4} Ω^{-1}/cm). This was because that the dielectric constant of water is larger than that of 1-propanol, and the fraction of sodium benzoate dissolved in water was larger than that in 1-propanal. In order to maintain the same background of the control solution as that of sample matrix, the conductivity of control solution was adjusted similar to that of test solution with sodium benzoate solution.

The recovery of FMTR from drug syrup placebo was evaluated by comparing the peak area ratio of the solute and I.S in the recovery test solution and in the control solution. The test solution was prepared by spiking a set of 1.0 ml of standard stock solution to 10 ml volumetric flask contained 25 mg of placebo, adding 0.2 ml of I.S solution and 6 ml of 1-propanol, then made up to volume with 200 μM phosphoric acid solution, shaken and filtered through 0.25 μm membrane filter. The control solution was prepared by spiking 1.0 ml of standard stock solution to a 10 ml volumetric flask, adding 0.2 ml of I.S solution, about 4 drops of 1% sodium benzoate solution (the conductivity of final solution could be adjusted to about 2.0×10^{-5} Ω^{-1}/cm), 6 ml of 1-propanol and then made up to volume with 200 μM phosphoric acid solution. The recovery was 100.8%, RSD = 1.5% ($n = 5$).

3.2.4. Repeatability

Ten consecution injections of a test solution of FMTR which prepared as same as the control solution mentioned in Section 3.2.3 (at a concentration of 1 μg/ml) were performed. The relative standard deviations (RSDs) of migration times, peak areas and the ratio of peak area between FMTR and I.S were shown in Table 1.

To obtain good precision for peak area and migration time, great care was observed with many aspects of sample introduction, capillary preparation and peak integration. This includes temperature control, capillary conditioning, washing at each run, buffer composition, the vial height of buffer, sample and water for dipping, sample matrix and capillary-end geometry etc. Optimization of the injection time and injection voltage should also be considered. The water vial for the capillary dipped before the electroinjection must be renewed for each run. This step prevented the contamination of the sample solution with running buffer of high conductivity. Another aspect was that one sample vial could not performing more than five injections. For example, ten replicate injections from the same vial on the TSP instrument gave an RSD of 1.8% for peak area ratio and 6.4% for FMTR peak area. However, ten replicate injections from two vials (five injections per vial) gave the RSD of 0.55% for peak area ratio and 1.1% for FMTR peak area. All precision data obtained in the various repeatability tests gave acceptable RSD values of $<1\%$ for peak area ratio.

Table 1
Repeatability results of formoterol and I.S. with ten replicate injection from two vials of the same solution. The concentration of FMTR was 1.0 μg/ml

No. of Injections	t_m (FMTR)	Area (FMTR)	t_m (I.S.)	Area (I.S.)	PAR[a]
1/vial-1	5.56	101328	4.72	74577	1.359
2/vial-1	5.58	99128	4.75	71868	1.379
3/vial-1	5.60	100596	4.73	73334	1.372
4/vial-1	5.48	99872	4.60	73106	1.366
5/vial-1	5.52	98642	4.70	72107	1.368
1/vial-2	5.52	101005	4.68	73128	1.381
2/vial-2	5.61	102136	4.75	74661	1.368
3/vial-2	5.54	99541	4.71	72964	1.364
4/vial-2	5.49	99863	4.61	72800	1.372
5/vial-2	5.53	98685	4.69	71349	1.383
Average	5.54	100080	4.69	72989	1.371
RSD	0.73%	1.10%	1.06%	1.39%	0.55%

[a] PAR, peak area ratio of FMTR and I.S.

3.2.5. Reproducibility

In order to demonstrate the reproducibility of the method between capillaries, analytes, days and instruments, five granulate extracts were injected in the capillary in duplicate. The RSDs of the content of FMTR (calculated by internal standard method) from different capillaries (three capillaries from different manufacture), two analytes, 5 days (all were performed on TSP instrument) and two instruments (performed on TSP and Beckman instruments) were 1.52% ($n = 15$), 1.04% ($n = 10$), 1.16% ($n = 5$) and 1.93% ($n = 10$) respectively. Although the results shown that the RSD from different instruments was larger than that from other aspects, it was within an acceptable range ($< 2.0\%$).

3.2.6. Ruggedness and robustness

In this study, the separation conditions were shown to give reproducible performance on different capillaries, instruments, between analysts and between both laboratories and sites. Parameters examined by varied about 5–10% include temperature, pH, electrolyte concentration, rinse time, contents of 1-propanol and phosphoric acid in sample solution and sample loading. Separation selectivity and baseline resolution of FMTR and I.S. was maintained in each analysis showing slight variation. The content of 1-propanol which constructing sample solution was selected at 70% instead of the highest sensitive point of 90% because the values of RSDs for peak areas or for the ratio of peak areas at the concentration range of $70 \pm 5\%$ were smaller than those at the concentration range of $90 \pm 5\%$. In all cases, the migration times of the two peaks were maintained within 7 min. The RSDs for peak area were $< 2\%$ and for peak area ratio were $< 1.5\%$ in each analysis.

3.3. Determination of the main drug in dry syrup dosage and quantitative analysis

Five granulate extracts were compared with an equivalent amount of FMTR control solution which prepared under the direction of Section 3.2.3. The complete results were given in Table 2.

Quantitation calculations in this work were based on the internal standard method and the peak areas were not normalized to the migration times (t_m). Because the low concentration of test solution and many factors influence the precision of HCFASS, the significance of the peak areas varied among different solutions with the same concentration was observed. In electrokinetic injection mode, each analyte was drawed into the capillary at a rate proportional to its electromigration velocity, faster ions are overrepresented in the electrophoregram. Two problems arise in us-

Table 2
Results of quantitation of FMTR in dry syrup dosage form by use of two instruments

Batch No.	Instrument employed	Expected value (μg/g)	Observed value (μg/g)	RSD (%)
021Y	TSP	40.00	41.52	0.62
	Beckman	40.00	41.76	0.40
024Y	TSP	40.00	41.21	0.83
	Beckman	40.00	41.00	1.30
025Y	TSP	40.00	41.52	0.74
	Beckman	40.00	41.20	1.72

ing electrokinetic injection for quantitative analysis. First is the mobility bias problem, which can be corrected for if the ion mobilities are known or can be calculated. Second, run-to-run variations in the injection voltage, injection time and sample conductivity also generally exist [13]. These variations could be corrected for by using internal standard if the relative contributions of EOF velocity and electromotive migration velocities were constant. For internal standard method, a known amount of substance is added in the analytical procedure to enable correction for sample loss during the assay. Using internal standard can help to improve the precision of CE analysis from 2% to $<1\%$ [14–17]. In our laboratory, the precision of $<1\%$ RSD level was obtained with a TSP instrument and in another laboratory, the precision was $<1.5\%$.

4. Conclusion

The CZE method developed for the quantitation of FMTR is rapid, reliable and robust. It has been demonstrated, however, that complete method optimization is essential in order to obtain a fully validated procedure, which gives acceptable quantitative results, and that this should include consideration of instrumental and operational parameters. It has been shown that the fully developed and validated CZE method with the head-column field-amplified sample stacking injection can be applied to the assay of formoterol in a low dosage form. In this case, the robustness and stability of the migration times in the developed method and allows the assay to be carried out without the requirement of area/migration time normalization. Parameters were well controlled and an internal standard was used, precise quantitation results were obtained. It can be used as a routine method for the determination of this drug in the preparation.

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