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Development and validation of a combined potency assay and enantiomeric purity method for a chiral pharmaceutical compound using capillary electrophoresis

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Abstract

A single capillary electrophoresis method was developed for both quantitative assay and determination of enantiomeric impurity in a pharmaceutical compound. The method uses a borate buffer containing β-cyclodextrin, and makes use of an internal standard for improved peak area precision. The quantitative assay uses a calibration curve from 50–150% of the nominal concentration, while the level of undesired enantiomer is determined by a comparison to an external limit test standard. The effects of run buffer pH and concentration, cyclodextrin type and concentration, capillary length, and detector type (diode array or UV filter) were investigated. Validation of the method included parameters of selectivity, linearity, limit of quantitation, robustness, and migration time and quantitation precision. The precision for the main component assay is approximately 1% R.S.D. In addition, the method is capable of determining 0.5% of the minor enantiomer in the presence of the major enantiomer. The CE method and an existing HPLC method for the same compound were compared and found to give similar results.

Keywords: Enantiomer separation; Pharmaceutical analysis; Validation; Buffer composition; Potency assays; LY231514

1. Introduction

Many papers have been published on the applications of capillary electrophoresis (CE) to the analysis of chiral compounds [1–8]. Relatively few have discussed the validation of such methods for routine use, although such information is becoming more readily available as CE matures [1,3–6]. Very little work has been reported using a single CE method for both assay and enantiomeric purity determinations [3,7]. This paper describes the development and validation of such a combined method and compares the CE method to an existing HPLC method for the same compound.

The chiral compound under study is currently under development in Lilly Research Laboratories for anti-tumor activity. Quantitative assay and chiral purity are critical parameters and are routinely determined using HPLC. Capillary electrophoresis was investigated as an alternative technique to confirm results obtained by HPLC, to gain experience in CE method validation, and to examine potential advantages and disadvantages offered by CE as a routine method.

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2. Experimental

2.1. Chemicals

β-cyclodextrin (β-CD), dimethyl-β-cyclodextrin (DM-β-CD), and trimethyl-β-cyclodextrin (TM-β-CD) were obtained from Sigma (St. Louis, MO, USA). Hydroxypropyl-β-cyclodextrin (HP-β-CD) was obtained from Aldrich (Milwaukee, WI, USA). γ-Cyclodextrin (γ-CD) was obtained from Fluka (Ronkonkoma, NY, USA). Boric acid, 5 *M* sodium hydroxide solution, and dimethylformamide were obtained from EM Science (Gibbstown, NJ, USA). Mannitol was obtained from Roquette Americas (Gurney, IL, USA). The other compounds were internal to Lilly Research Laboratories.

2.2. Buffer and sample preparation

Borate buffer was prepared by adjusting the pH of boric acid (0.4 *M* except as noted) with 5 *M* NaOH. Unless otherwise noted, the pH of the borate buffer was 9.1. The concentration of the major enantiomer and internal standard were 0.5 mg/ml respectively, and sample solvent was 5% buffer in purified water. Run buffers were prepared by diluting weighed samples of β-CD with the borate buffer. Buffers were filtered through 0.45 μm syringe filters (Gelman Science Nylon Acrodisc syringe filter) prior to use.

2.3. Equipment and conditions

The work was performed on a P/ACE 5500 capillary electrophoresis instrument (Beckman Instruments, Fullerton, CA, USA). It was equipped with either a single wavelength filter or photodiode array absorbance detector, and a 50 μm I.D. fused-silica capillary (Beckman Instruments). The capillary length was 85 cm (78 cm to detector) unless noted otherwise. Sample solutions were introduced into the capillary using applied pressure for 2 s. The detector was at the cathode. Finalized method separation conditions consisted of an applied voltage of 30 kV, detection at 230 nm, operating temperature of 20°C, and a run buffer containing 2.5 m*M* β-CD in 0.4 *M* borate at pH 9.1. The capillary was rinsed with run buffer between runs, and run buffer vials were changed every ten injections.

3. Results and discussions

3.1. Method development

The major factors considered in this method development were the run buffer pH range and concentration, chiral selector and concentration, instrumental operating conditions (e.g., capillary length), and use of an internal standard.

The structure of compound LY231514 is displayed in Fig. 1(a). It contains one chiral center and so can exist in either *S* (LY231514) or *R* form. The compound is slightly soluble in acid (0.2–0.5 mg/ml at pH 1.2), but freely soluble at pH 7 or higher (>100 mg/ml). Therefore, borate run buffer was tested from pH 8.5–9.5. This pH range was chosen to maintain good buffer capacity. Under basic conditions, the compound is negatively charged, which

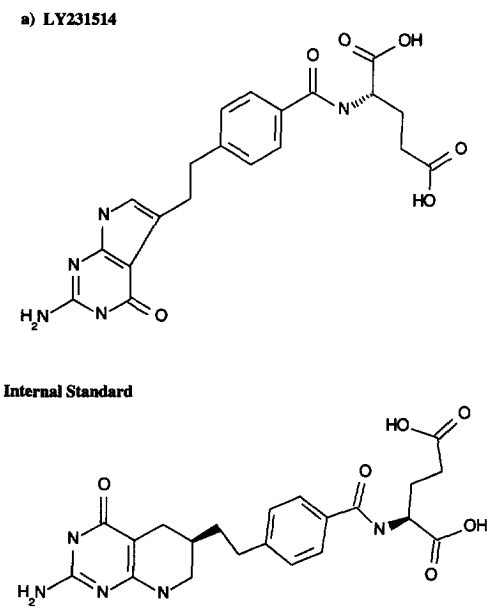

Fig. 1. Structures: (a) LY231514: N-[4-[2-(2-amino-4,7-dihydro-4-oxo-1H-pyrollo[2,3-*d*]pyrimidin-5-yl)ethyl]benzoyl]-L-glutamic acid. (b) Internal standard: N-[4-[2-(2-amino-3,4,5,6,7,8-hexahydro-4-oxopyrido[2,3-*d*]pyrimidin-6-yl)ethyl]benzoyl]-L-glutamic acid.

allows the use of neutral cyclodextrins as potential chiral selectors. Several cyclodextrins were studied: β-CD, HP-β-CD, DM-β-CD, TM-β-CD, and γ-CD. Using 1.5 m*M* β-CD in 0.4 *M* buffer (pH 9.1), near baseline resolution was obtained. Using the same buffer and cyclodextrin concentrations, HP-β-CD showed a partial separation, and DM-β-CD showed a hint of separation. No separation was obtained with the other two cyclodextrins. The results indicated that β-CD gave the best resolution for the enantiomers, and all further development work used β-CD as the chiral selector. With the use of β-CD and under the experimental conditions described in this paper, the order of migration of the enantiomers was *R*, *S* (i.e., minor before major enantiomer).

Variation of pH affects resolution and migration times. Fig. 2 shows the effect of increasing pH from 8.5–9.5. Better resolution and longer migration times were obtained with higher pH. To determine whether the increased migration time was due to decreased electroosmotic flow (EOF), the EOF marker dimethylformamide (1% in water) was injected over the same pH range. The migration time of DMF also increased consistently with pH: from 5.7 min at pH 8.5 to 6.6 min at pH 9.5. This confirmed that EOF was smaller at higher pH in this narrow range, contrary to our expectation. The result is likely caused by elevated ionic strength in the higher pH buffers, as supported by dramatic current increases from 60 μA at pH 8.5 to 120 μA at pH 9.5. It should be noted that pH was not increased beyond 9.5 due to the current increase.

A range of β-CD concentrations was examined from 10 m*M* to 0.7 m*M*. The results are shown on Fig. 3. The resolution increased as the β-CD concentration decreased from 10 m*M* to 3 m*M*, remained constant from 3 m*M* to 1 m*M*, then decreased at 0.7 m*M*. Note also that the peak was distorted and broadened significantly at 0.7 m*M* β-CD. Under these experimental conditions, the net migration rate of the compound was slower than the EOF since its electrophoretic mobility was in the opposite direction, due to its negative charge. The greater the extent of complexation with β-CD, the shorter the migration time, simply because the larger size of the complex lowered the electrophoretic mobility of the compound.

The concentration of borate buffer was evaluated in the range from 0.1 *M* to 0.4 *M*. High buffer concentrations reduce EOF, enhance sample stacking effects, and increase current. 0.4 *M* borate buffer was selected since it gave the best resolution (see Fig. 4) without excessive current.

After the initial optimization of the run buffer, the length of the capillary was optimized to achieve a baseline separation in a reasonable run time. Voltages applied to different length of capillaries were selected in order to obtain high field strength while

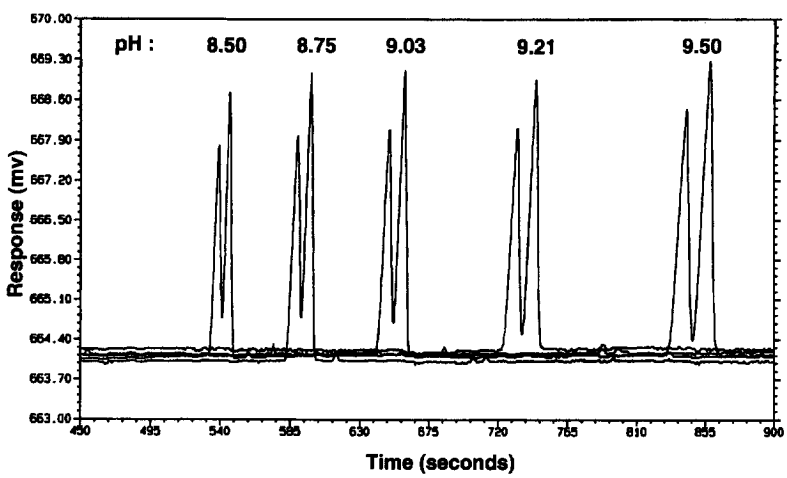

Fig. 2. The effect of pH on resolution and migration time. Conditions: 57 cm (50 cm to detector)×50 μm I.D. fused-silica capillary; 25 kV; wavelength: 280 nm; 0.4 *M* borate adjusted to pH 8.50, 8.75, 9.03, 9.21 and 9.50 prior to adding 1.55 m*M* β-CD. The sample solution contained 0.5 mg/ml of each of the enantiomers.

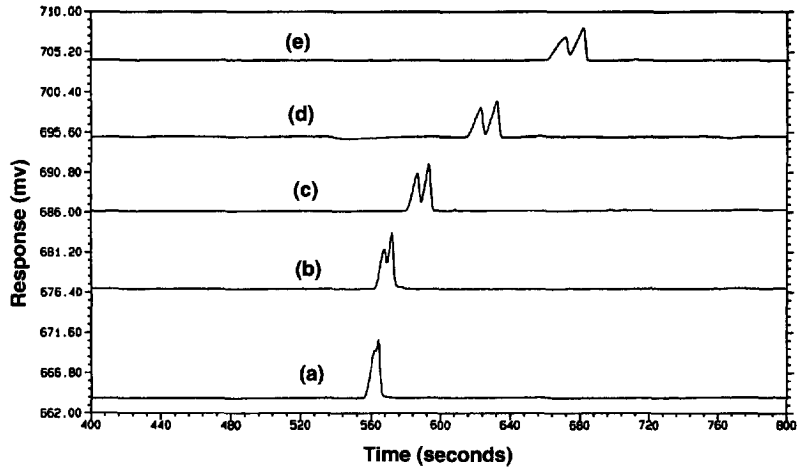

Fig. 3. The effect of β-CD concentration on the resolution and migration time. (a) 10 mM; (b) 5 mM; (c) 2.6 mM; (d) 1.3 mM; (e) 0.7 mM. Run buffers: 0.4 M borate, pH 9.0. The other conditions were the same as described in Fig. 2.

keeping current relatively low. Fig. 5 shows that the resolution was increased with capillary length up to 85 cm. When the capillary length increased from 85 cm to 95 cm, no further gain in resolution was realized.

Injection precision in CE is generally poorer than that for HPLC due to the difficulties involved in reproducibly injecting nanoliter volumes of sample into the capillary. Many quantitative reports have utilized internal standards to alleviate this problem and obtain acceptable precision data [2,4,5,7]. In this work, the precision of the method was examined with and without the internal standard. It was found that the R.S.D. of the LY231514 peak area was

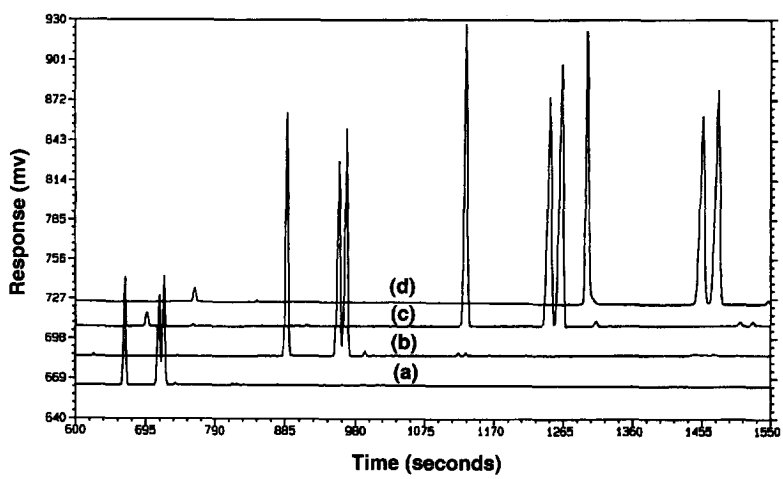

Fig. 4. The effect of buffer concentration on the resolution and migration time. (a) 0.1 M; (b) 0.2 M; (c) 0.3 M; (d) 0.4 M. Note that the internal standard was included and is the peak migrating prior to the two enantiomers. Also, the detector full scale output (AUFS) was 0.2 in (a) and 0.1 in (b)–(d). Other conditions: 85 cm (78 cm to detector)×50 μm I.D. fused-silica capillary; 30 kV; wavelength: 228 nm; 2.5 mM β-CD. The sample solution contained 0.5 mg/ml of each of the enantiomers and internal standard.

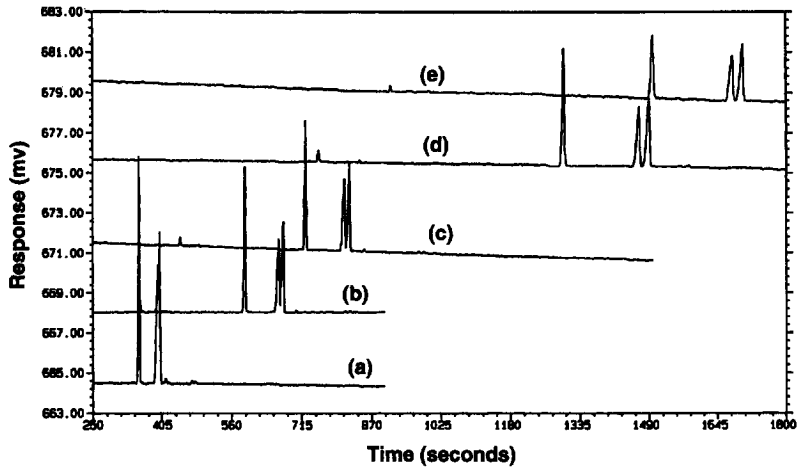

Fig. 5. The effect of capillary length on the resolution. The capillary length and voltage: (a) 35 cm, 15 kV; (b) 57 cm, 25 kV; (c) 67 cm, 27 kV; (d) 85 cm, 30 kV; (e) 95 cm, 30 kV. The length to the detector was 7 cm less than the total length listed. 0.4 *M* borate, pH 9.0. The other conditions were the same as described in Fig. 4.

typically in the range of 3–4%, while the R.S.D. of the peak area ratio (LY231514 peak area/int. std. area) was typically less than 1%. The structure of the internal standard is similar to LY231514 as displayed in Fig. 1(b).

One unexpected observation in this work was splitting of the internal standard peak (a pure enantiomer), which was initially observed when the concentration of β-CD was 1.6–1.7 m*M* (see Fig. 6). Further experiments showed that the peak splitting also occurred at other β-CD concentrations, but only after multiple injections using the same run buffer. This unusual splitting remains unexplained and is the subject of further investigation. However, the peak area appears unaffected even when splitting occurs. Using the final conditions described under the experimental section, in which the run buffer is replaced after every ten injections, the peak splitting

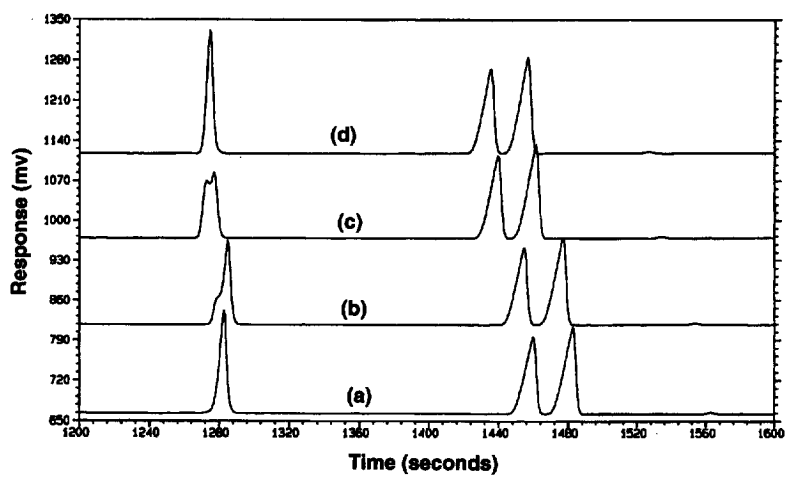

Fig. 6. Splitting of the internal standard peak. The concentration of β-CD: (a) 1.49 m*M*; (b) 1.62 m*M*; (c) 1.73 m*M*; (d) 1.82 m*M*. The other conditions were the same as described in Fig. 4.

has not been observed. It is also interesting to note that only the internal standard peak has been affected, not LY231514 or its enantiomer.

3.2. Method validation

In order for the method to be used in laboratories as a routine analysis procedure, performance criteria were examined for parameters including selectivity, linearity, precision, limit of quantitation, and robustness, as in HPLC validation. Two different types of samples were studied in the validation: LY231514·disodium drug substance and its drug product, which contains mannitol (50%). The lyophilized reference standard used in both assays also contains 50% mannitol as a bulking agent to aid in lyophilization of the drug substance.

3.3. Selectivity

Samples of all known impurities, degradation products, and matrix components were injected and separated from the internal standard and the enantiomers of LY231514 by this method.

3.4. Linearity

Linearity was evaluated using two sets of standard solutions: one prepared from reference standard (containing mannitol), and one from drug substance alone. Comparison between these two sets provides a means to determine if mannitol in the reference standard impacts the analysis accuracy. The reference standard set consisted of five standards in the concentration range of 20–200% of nominal concentration (0.5 mg/ml). The drug substance standard set consisted of nine standards in the concentration range of 0.4–200% of nominal concentration. The wider concentration range was investigated for the drug substance to get some information at a low concentration level, relevant to the enantiomeric purity test.

The similar slopes (Table 1) from both sets indicate no interference from mannitol. This is confirmed by the comparable results obtained from the quantitation analyses by CE and HPLC (see comparison with HPLC, below). The data from Table 1 also showed that the response is linear over the range that will be used to determine the potency.

3.5. Precision

The method demonstrates adequate precision as evaluated by using ten replicate sample preparations for drug substance, and seven replicate sample preparations for drug product. The relative standard deviation of the area ratio obtained from the LY231514 peak versus internal standard was 0.6% for drug substance and 1.4% for drug product.

3.6. Long term injection precision

Migration time precision was examined to determine the maximum number of injections that could be performed using a single pair of run buffer vials (inlet and outlet vials) without significant buffer depletion effects [1]. Although migration times decreased slightly with repeated runs, 18 injections using the same run buffer vials gave migration times with a R.S.D. of less than 0.6%. However, it was determined that no more than ten injections be run using a single pair of run buffer vials due to the peak splitting described above.

3.7. Limit of quantitation (LOQ)

The limit of quantitation of the *R*-enantiomer was determined at a signal-to-noise ratio of ten using a

Table 1
Method linearity

	Ref. std. vial	Drug substance
Linear slope (area counts/mg/ml)	2.365	2.334
y-Intercept (area counts)	1.7×10^{-4}	5.2×10^{-3}
Correlation coefficient:	1.0000	0.9999
Relative standard deviation (%)	0.35	1.7
Normalized intercept/slope	0.0001	0.0051

filter UV detector. The value of LOQ was estimated to be approximately 2.5 μg/ml, 0.5% of the LY231514 assay concentration. This corresponds to the limit of maximum desirable level of enantiomeric impurity in the drug. Therefore adequate sensitivity is critical. The detection of enantiomeric impurity could not be enhanced by increasing either injection volume or sample concentration due to band broadening which compromised resolution in both cases. For this reason, a system suitability test includes an evaluation of sensitivity by assessing the precision of the *R*-enantiomer peak at the 2.5 μg/ml level in the presence of 0.5 mg/ml LY231514. Fig. 7 shows a typical electropherogram for a sample containing 2.5 μg/ml *R*-enantiomer in 0.5 mg/ml LY231514.

Earlier work was performed using a photodiode array detector, which typically gave R.S.D. values in the range of 10–20% for the *R*-enantiomer at 2.5 μg/ml. A number of experiments were pursued to decrease baseline noise, including adjustments of bandwidth and sampling rates. The lack of success by these efforts to improve baseline noise indicated that the instrument limit was reached at 2.5 μg/ml using the photodiode array detector under these conditions. After replacing the photodiode array detector by a filter UV detector, the sensitivity was improved, thus making possible R.S.D. values of 5–10% at the 2.5 μg/ml level. The data confirmed the expectation that the UV filter detector provides greater sensitivity than the photodiode array.

3.8. Robustness

The robustness was checked by varying pH and the concentration of β-CD around the nominal run buffer conditions [0.4 *M* borate (pH 9.1) containing 2.5 m*M* β-CD]. In the pH range of 9.0–9.3, the migration time of LY231514 changed approximately 4% per 0.1 pH unit. In the concentration range of β-CD from 2.2–3.0 m*M*, the migration time changed approximately 0.3% per 0.1 m*M*. In both cases, the resolution between the two enantiomers remained almost constant. A capillary from a different batch was also used to generate validation data, and the results were very consistent between the two capillaries.

3.9. Comparison with HPLC

The CE method has been compared with an existing HPLC method by running the same sample solutions. The potency found for a sample of drug product was 99.7 mg/vial with 0.2% R.S.D. from HPLC, and 99.1 mg/vial with 1.4% R.S.D. from CE. The potency found for a sample of drug substance was 90.4% with 1.5% R.S.D. from HPLC, and 90.3% with 0.4% R.S.D. from CE. The precision of

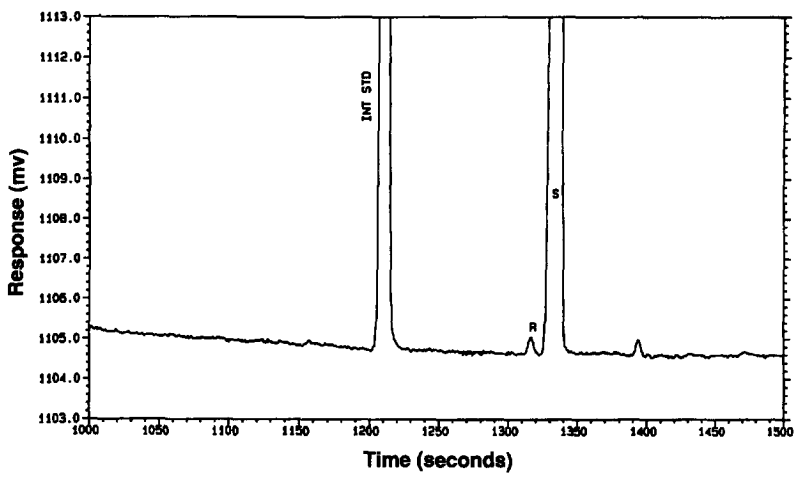

Fig. 7. A typical electropherogram containing 2.5 μg/ml *R* enantiomer in 0.5 mg/ml LY231514. The conditions were as stated in Section 2, using a filter UV detector.

the *S*-enantiomer at a 0.5% level was also compared. A R.S.D. of 17% was obtained from an HPLC run (ten injections). The CE method gave comparable R.S.D. values when the photodiode array detector was used, but the R.S.D. was typically 5–10% when the filter UV detector was used.

Some general advantages of CE over HPLC discussed elsewhere and applicable here include lower consumption of chemical reagents and analytes, less waste, lower cost of operation, and shorter analysis time. This method shortened the HPLC run time of 42 min to 30 min. However, the CE method requires an internal standard, which makes sample preparation more tedious. Also, the CE method requires one to change buffer vials every ten injections (see *Long Term Injection Precision*).

4. Conclusion

This work has demonstrated that capillary electrophoresis can be used for the determination of potency and enantiomeric purity in a single method. Method development and validation parameters have also been described. The performance of the method was evaluated and proved to be comparable to an existing HPLC method.

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