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Capillary electrochromatography as an alternative separation technique to high-performance liquid chromatography and capillary zone electrophoresis for the determination of drug related impurities in Lilly compound LY300164

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Abstract

Capillary electrochromatography (CEC) has been used to separate pharmaceuticals from their related impurities; however, this has not been fully explored to date within the pharmaceutical industry. Generally capillary electrophoresis is used in either free-flow mode or in combination with micellar electrokinetic mode to complement the results obtained from the traditional method of high-performance liquid chromatography (HPLC). This paper explores the various separation modes now at hand in pharmaceutical laboratories using a developmental Lilly compound LY300164 and its process impurities. Possible benefits and concerns for each of the separation modes are discussed using the same sample and impurities to generate the results. Regulatory bodies prefer that purity assays for pharmaceuticals be complemented with another technique. This is to guarantee that no other hypothetical impurities which could potentially be present are seen in another technique. Traditionally, HPLC has been complemented with the use of thin-layer chromatography. This paper suggests that CEC can be used as a alternative purity assay for pharmaceuticals.

Keywords: Pharmaceutical analysis; LY300164

1. Introduction

The separation of bulk drug substances from their related impurities has generally been performed by high-performance liquid chromatography (HPLC) with thin-layer chromatography (TLC) generally considered as a complimentary technique [1]. Capillary zone electrophoresis (CZE) offers differences in the selectivity of various analytes due to their electrophoretic mobilities, and hence is a complimentary and orthogonal technique to HPLC. Many papers and application notes now show CZE methods for the separation of pharmaceuticals from their related impurities [2–5]. Good agreement between CZE and HPLC data provides an assurance that all impurities have been accounted for due to the different modes of operation of the two techniques. Capillary electrochromatography (CEC) is a relatively new separation technique, and is currently being investigated for similar analyses. Recently two publications [6,7] have used CEC to analyse neutral pharmaceutical products and related impurities. At

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present no batch comparison data comparing traditional separation techniques (HPLC and CZE) with CEC have been reported for evaluation of drug-related impurities.

Over the past few years interest has grown rapidly in capillary electroseparation techniques, as they show high efficiency and resolution compared to liquid chromatography, providing rapid results and minimal sample and solvent requirements [8]. The advantages of micellar electrokinetic capillary chromatography (MECC) for separating uncharged analytes has been demonstrated by Terabe et al. [9] using micelles as a pseudostationary phase. MECC is used in the pharmaceutical industry; however, the micelles present in the background electrolyte can present difficulties when coupling this technique to mass spectrometry. CEC, unlike MECC, has a wide variety of stationary phases available (generally those used in HPLC), which makes this technique potentially more versatile.

CEC was first demonstrated by Pretorius et al. [10] in 1974, as a hybrid separation technique combining the stationary phase of LC with the electrically driven mobile phase transport of capillary electrophoresis. Additional differences in packed column flow dynamics result in significantly less band broadening for electrochromatography peaks in comparison with conventional LC separations. In CEC the electrodriven system offers the potential to pack capillaries with smaller particles than can be used in LC (less than 3 μm) yielding columns with more than 200 000 plates. The conditions for packing CEC columns have been well documented. Several methods of packing capillaries have been established, from conventional pressure packing techniques employing slurry packing, to supercritical fluid procedures, and electrokinetic packing [11–14]. Several reviews on CEC have described instrumental requirements for basic operation and will not be outlined in detail in this paper [15–21]. CEC has shown great promise as an alternative separation method to present conventional separation techniques such as HPLC or CZE.

CEC has since been utilized to analyze neutral compounds that are not easily separable by MECC (e.g., polycyclic aromatic hydrocarbons) [22,23]. For neutral analytes, selectivity is often but not always identical in HPLC and CEC [24]. For charged analytes, however, CEC and HPLC will differ as ions migrate at different rates in response to the applied electric field. There are several separations reported using CEC, the majority of which are neutral compounds with well-defined chromatographic characteristics (e.g., steroids) [25,26] and a few are pharmaceuticals [25,27,28] although information on reproducibility of migration or peak area is limited.

This paper describes CEC as a alternative to LC and CZE for the analysis of a developmental Lilly compound and related impurities employing a commercially available CEC C_{18} column. The paper also provides an insight into CEC method development and validation.

1.1. LY300164 structure and physicochemical properties

LY300164 is an early phase pharmaceutical compound. The structures of LY300164 and some process impurities are shown in Fig. 1.

LY300164 is a hydrophobic basic compound with $\log P$ determined as 2.13 [29]. The $\log P$ value is supported by solubility studies which show LY300164 to be almost insoluble in aqueous conditions above pH 4.5, whilst being soluble in a range of organic solvents [30]. The pK_a value for the amine group on LY300164 has been determined as 2.83, and the distribution of species graph indicates that above values of pH 5 LY300164 is only present as the neutral species [29]. LY300164 exhibits a strong UV absorbance at 240 nm [31] and all subsequent chromatographic/electrophoretic methods used 240 nm as the detection wavelength. This enabled us to compare separate methods but with similar detection characteristics. All methods used percent peak area normalisation for quantification purposes.

The pK_a values for the process impurities I, II and III were not determined solely on the basis that in regard to their structures it is unlikely that any of the three compounds will accept a proton [32]. Hence it is safe to assume that these three process impurities will all be neutrals within the pH ranges of 2.5–8.0. Preliminary investigations into solubility of these three impurities show that they also are very hydrophobic compounds, and we would expect these to be more hydrophobic in nature than LY300164 due to

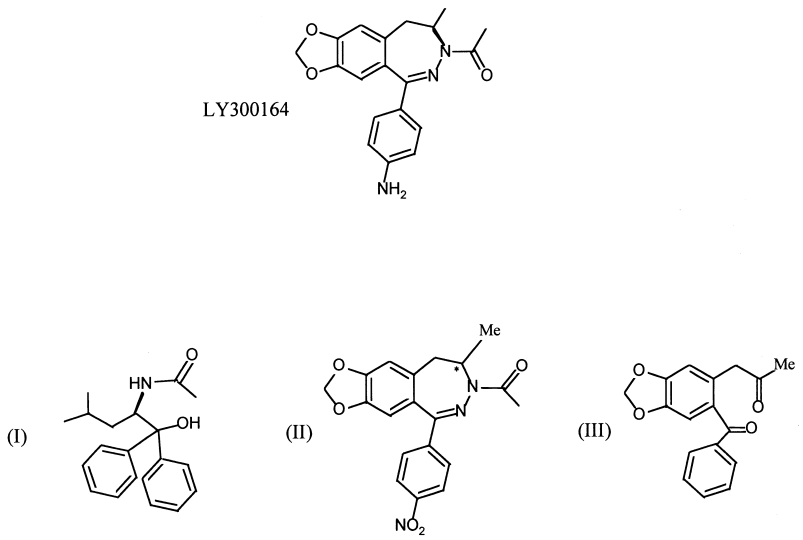

Fig. 1. Structures of LY300164 and process impurities.

the fact that there are no charge contributions at any pH. Unfortunately log *P* results for the process impurities were unobtainable due to the insufficient quantities of these impurities available at the time. Another process impurity present in final bulk LY300164 material is a very closely related analogue to LY300164, note that this structure is proprietary information and therefore cannot be disclosed. This impurity will be referred to as impurity '**x**' throughout the rest of this article and, due to its similarity in structure to LY300164, looks likely to accept a proton at low pH.

2. Experimental

2.1. Materials

LY300164 and process impurities were synthesized at Eli Lilly and Company (Indianapolis, IN, USA). The analytical reagents were obtained from various sources due to analyses being carried out at two different sites.

For CZE and HPLC analyses, sodium dihydrogenphosphate, acetonitrile and dimethylformamide (DMF) were purchased from Fisons (Loughborough, UK) with orthophosphoric acid, glacial acetic acid, 1.0 *M* sodium hydroxide and sodium dodecyl sulphate (SDS) being purchased from BDH (Poole, UK).

For CEC analyses, the buffer components disodium hydrogenorthophosphate (analytical reagent 99%) and ammonium acetate (Analar grade 98%) were obtained from BDH and acetonitrile (far UV grade) was purchased from Rathburn Chemicals.

For all analyses, the purified water was obtained from a laboratory system which produces HPLC-grade quality water.

2.2. Instrumentation set-up and methods

2.2.1. High-performance liquid chromatography

HPLC was performed using an automated integral instrument from Thermoseparations (Stone, UK). The analytical column used was a Zorbax RX C_8, 25 cm×4.6 mm I.D., obtained from Hichrom (Reading, UK). Analog data were collected directly from the absorbance detector at 240 nm for LY300164 and process impurities.

The method employed was a fully validated early phase development method which is used to support the percent purity assessment of development batches of LY300164 bulk drug substance [33]. The method is an isocratic system using a mobile phase of water (adjusted to pH 3.0 with acetic acid)–acetonitrile (60:40) mobile phase. A flow-rate of 1

ml min^{-1} and an injection volume of 10 μl was utilised for all analyses. The column was maintained at ambient temperature. The sampling rate was 10 Hz on a laboratory-built centralized chromatography system based on a Hewlett-Packard model 1000 minicomputer (Bracknell, UK). The minicomputer was used for all the calculations. A system suitability test using three replicate injections was performed prior to running samples. Samples of concentration 0.1 mg ml^{-1} were accurately prepared in mobile phase diluent prior to injection.

2.2.2. Capillary zone electrophoresis and micellar electrokinetic chromatography

CZE was performed using an automated SP1000 instrument from Thermoseparations. The capillary cartridge was fitted with a 70 cm×50 μm fused-silica capillary obtained from Composite Metal Services (Worcester, UK), with an effective length of 63 cm. Analog data was collected directly from the absorbance detector at 240 nm for LY300164 and process impurities. The sampling rate was 100 Hz on a laboratory-built centralized chromatography system based on a Hewlett-Packard model 1000 minicomputer (Bracknell, UK). The minicomputer was used for all the calculations. A separation voltage of 30 kV (476 V cm^{-1}) was employed for all CZE separations. The column temperature was maintained at 35°C by the application of a forced air blown oven. Initially 0.1 mg ml^{-1} solutions of bulk drug LY300164 substance and process impurities were prepared in 10 m*M* phosphate buffer, pH 2.5, but it was noticeable that the signal-to-noise ratios for some of the samples needed to be increased to enhance detection. However, as the sample concentration was increased, limited solubility of LY300164 and process impurities was noticeable, hence the use of a acetonitrile–10 m*M* phosphate buffer, pH 2.5 (20:80, v/v), diluent for 1 mg ml^{-1} samples. The running buffer of 100 m*M* phosphate was adjusted to pH 2.5 using 10% orthophosphoric acid. A low pH running buffer was chosen, as LY300164 is partially ionised at low pH values and the validated HPLC method uses a low pH system. The samples were introduced into the capillary at the anodic end by the electrokinetic injection (30 kV for 2 s) and the voltage was then ramped up to the required operating voltage over 0.3 min.

Micellar electrokinetic chromatography was attempted using a buffer containing 50 m*M* phosphate adjusted to pH 3.5 using 10% orthophosphoric acid and 20 m*M* SDS reversing the polarity. An experiment using positive polarity was also performed at pH 9.4 using 50 m*M* sodium tetraborate and 50 m*M* SDS.

2.2.3. Capillary electrochromatography

CEC was performed on a Hewlett-Packard HP^{3D} CE unit (Waldbronn, Germany). CEC separations were carried out using a commercially available C_{18} capillary column (Hypersil, C_{18} ODS2 total length 33 cm, packed length 25 cm×50 μm I.D.) A separation voltage of 30 kV (909 V cm^{-1}) was employed for all CEC separations. The column temperature was maintained at 25°C. The data was collected directly from the diode array detector employing a sample wavelength of 240 nm and a reference wavelength of 380 nm for LY300164 and process impurities. The sampling rate was 20 Hz, employing a Hewlett-Packard data chromatography system.

Prior to each days work the installed C_{18} column was equilibrated using the separation buffer by applying a suitable voltage (25 or 30 kV) for 30 min and a pressure of 8 bar at both inlet and outlet capillary ends until a stable current and baseline was observed. The samples were introduced into the capillary at the anodic end by the application of an electrokinetic injection (3 kV for 4 s or 3 kV for 8 s) or a combination of electrokinetic and pressure (5 bar×3 kV for 4 s), the voltage was then ramped up to the required operating voltage over 0.3 min.

LY300164 and process impurities were prepared in a 100% acetonitrile (far UV grade). All samples (20 mg ml^{-1}) were filtered using Sartorius Ministart SRP 15 0.2-μm syringe filters (hydrophobic solvent resistant) prior to injection and stored in a refrigerator at 4°C prior to injection.

CEC buffers were prepared as for typical CE buffers. The pH for phosphate buffers was adjusted by 10% phosphoric acid. The final CEC mobile phase employed acetonitrile–10 m*M* phosphate, pH 3.50 (60:40, v/v), this was filtered using 0.2-μm HPLC filters or Sartorius Ministart SRP 15 0.2-μm syringe filters. Samples and CEC mobile phases were sonicated prior to use using an ultrasonic bath for 10

min at room temperature in order to remove any air bubbles. Calibration standards were prepared from a stock solution of LY300164 (50 mg ml^{-1}) in 100% acetonitrile using the appropriate dilutions. The prepared standards were stored in a refrigerator at 4°C prior to use.

3. Results and discussion

3.1. HPLC of LY300164 and process impurities

Prior to analysis of samples the system required conditioning. Relative standard deviations of three peak areas from three replicate injections of a sample should be below 2% before analysis. The separation of LY300164 and some of the process impurities can be seen in Fig. 2.

LY300164 elutes first followed by impurities I, III and then II in elution order. Two development batches of LY300164 were analysed by HPLC.

The highest individual impurity in development batches of LY300164 has been characterized as impurity '**x**' [34]. The run time for the analysis is set at 30 min and a zoomed chromatogram is shown in Fig. 3. In both development batches the possible process impurities I, II and III were not seen to be present, however, quantities of impurity '**x**' were seen in both batches. Isolated amounts of impurity '**x**' were unavailable at the onset of this study, hence in order to prepare a quantity of sample for CZE and CEC analyses, a fraction collection experiment was necessary. In order to achieve enough of impurity '**x**' on-column, a 20-mg ml^{-1} sample of LY300164 in 100% acetonitrile was prepared and 100 μl of this injected using the standard chromatographic conditions as stated previously in Section 2.2.1. A 2-ml fraction of impurity '**x**' was collected in mobile phase at 900 s. This enabled analysis of this fraction

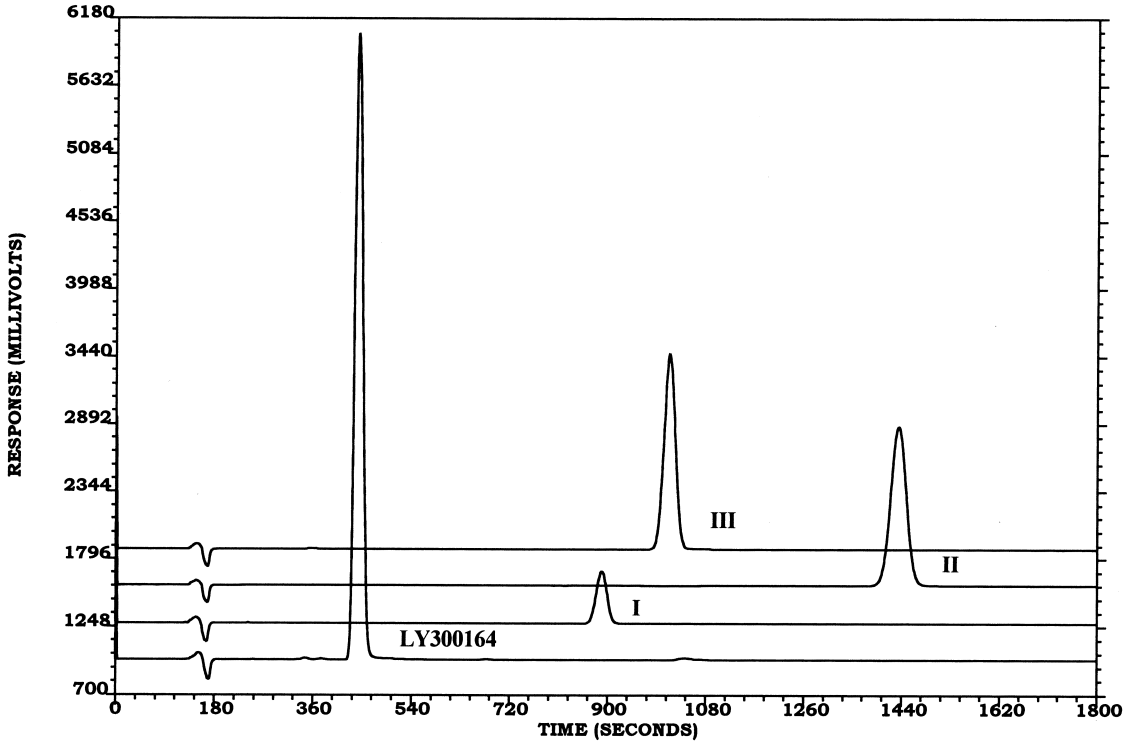

Fig. 2. HPLC separation of LY300164 and process impurities.

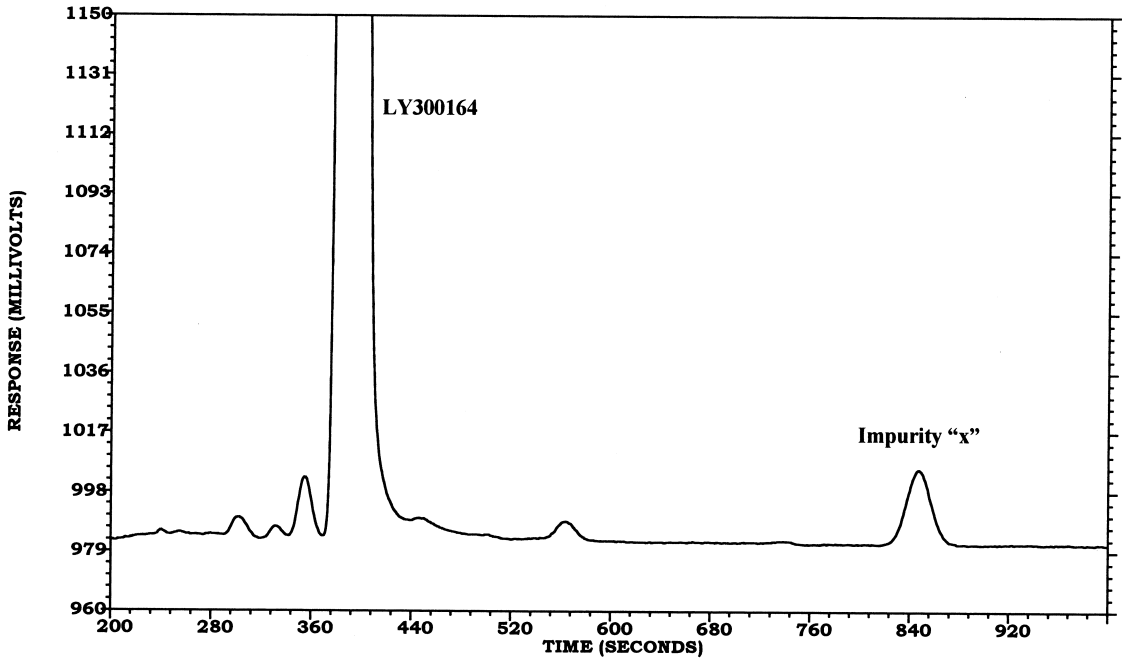

Fig. 3. Chromatogram of LY300164 batch.

by CE and CEC and also a percent impurity comparison of the three techniques in Section 4.

3.2. CZE and MECC of LY300164 and process impurities

CZE was considered to be a useful technique for LY300164 analysis due to its positive nature at low pH values. However, concerns over whether the three process impurities I, II and III would elute in a reasonable time were validated when the process impurities were run alongside LY300164.

Neither of the three isolated process impurities could be seen within 20 min from injection. This confirmed the previous assumption that these three impurities will all be neutrals at a running buffer of pH 2.5 [32]. A 1% DMF solution in water was used as a neutral marker subsequent to these injections and eluted at 1641 s on this system.

Although this CZE method was far from ideal, when two development batches of LY300164 were screened using this technique the electropherograms were reproducible. Also, when the fraction collection of impurity '**x**' was analysed on this system it confirmed that impurity '**x**' elutes directly before LY300164 on CZE. This is consistent with the theory that impurity '**x**' is also positively charged in a low pH environment and will accept a proton in a similar manner to LY300164. A typical electropherogram for LY300164 by CE is seen in Fig. 4.

MECC experimentation gave variable results; however, limited resources reduced the experimentation time necessary to optimise the efficiency of this technique. The reverse polarity experiment was tried as SDS is negatively charged at low pH and it has been demonstrated that this technique works well for neutral components [35]. This would be ideal as the pH would be similar to the HPLC and CEC methods; however, unfortunately no results were obtained from this method and hence will not be discussed in further detail.

The MECC application using positive polarity at pH 9.4 was relatively successful in so far as a separation was achieved for impurities from LY300164. Due to poor peak efficiencies obtained this method was not investigated any further. The authors recognize that addition of an appropriate organic solvent would probably improve peak ef-

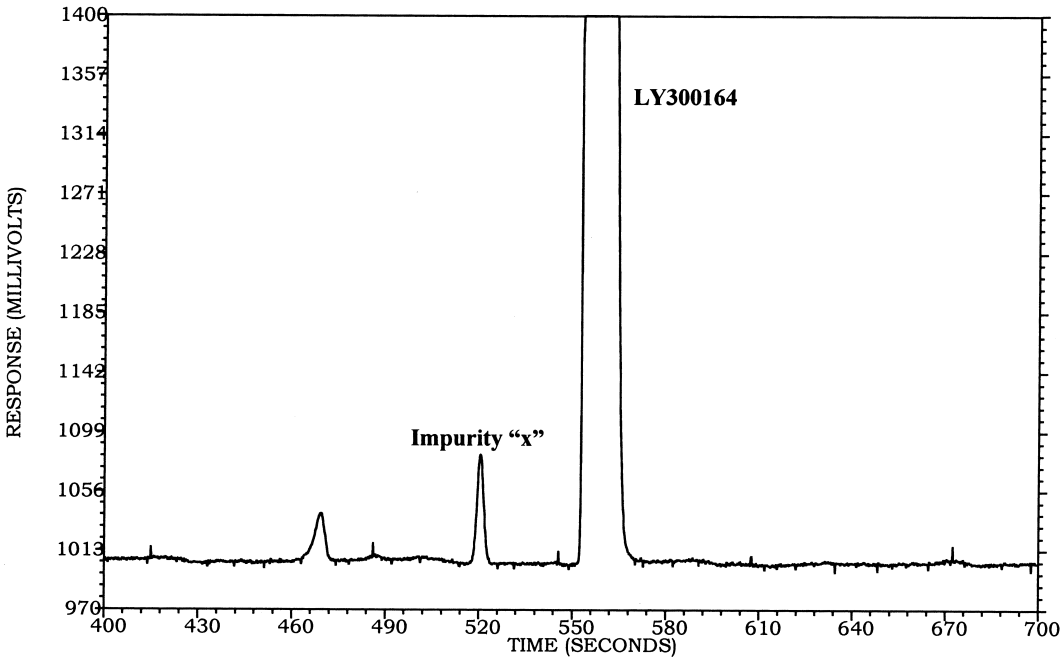

Fig. 4. Typical electropherogram of LY300164 batch.

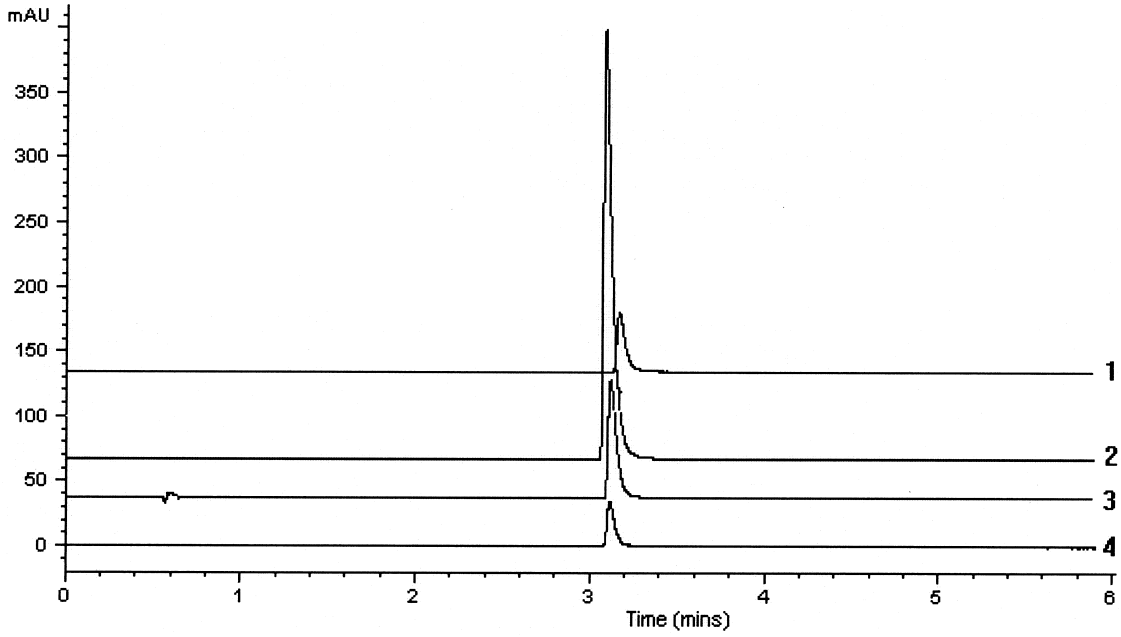

Fig. 5. LY300164 using different injection parameters, concentration and sample matrix. Employing (1) 3 kV for 4 s for a 2.0 mg ml^{-1} solution in acetonitrile–10 m*M* phosphate, pH 3.50 (60:40, v/v), (2) 3 kV for 4 s for a 20 mg ml^{-1} solution in 100% acetonitrile, (3) 3 kV for 8 s for a 2.0 mg ml^{-1} solution in 100% acetonitrile, (4) 3 kV for 4 s for a 2.0 mg ml^{-1} solution in 100% acetonitrile.

ficiencies; however, limited time was available for further method development work and also batch analysis.

3.3. CEC of LY300164 and process impurities

3.3.1. Investigation of CEC injection parameters

A preliminary investigation was performed on varying the injection conditions and sample matrix for different concentrations of LY300164. Electrokinetic injection was initially investigated and both voltage and injection time was varied as shown in Fig. 5. The primary aim of the study was to determine if detection of process impurities, peak area and peak shape was dependent upon sample matrix, concentration and injection parameters. It was found that when the sample matrix employed contained 100% acetonitrile or mobile phase no difference in peak area, peak shape or number of process impurities was observed. However, to determine all process impurities and obtain a similar sensitivity as LC, a large concentration was required and the best CEC conditions were determined to be 3 kV for 4 s for a 20 mg ml^{-1} solution in 100% acetonitrile as indicated in Fig. 5.

By utilizing the macro-programming facility of the Hewlett-Packard CE system a modified electrokinetic injection was employed which uses a combination of Hiflush and voltage to increase sample loading into the capillary. This was found to have a different impurity profile compared to electrokinetic injection indicating that the type of injection used is an important parameter for impurity determination. Further work is necessary to determine why different profiles were obtained (see Fig. 6 for typical CEC electrochromatogram).

3.3.2. CEC of LY300164 and process impurities

An investigation of LY300164 and its process impurities was performed employing CEC. Reproducibility for the migration was found to be adequate, however peak area gave a higher R.S.D. It was found that improved R.S.D. values for peak area were obtained when an internal standard was employed, as shown in Fig. 7. In a pharmaceutical development laboratory routinely performing HPLC

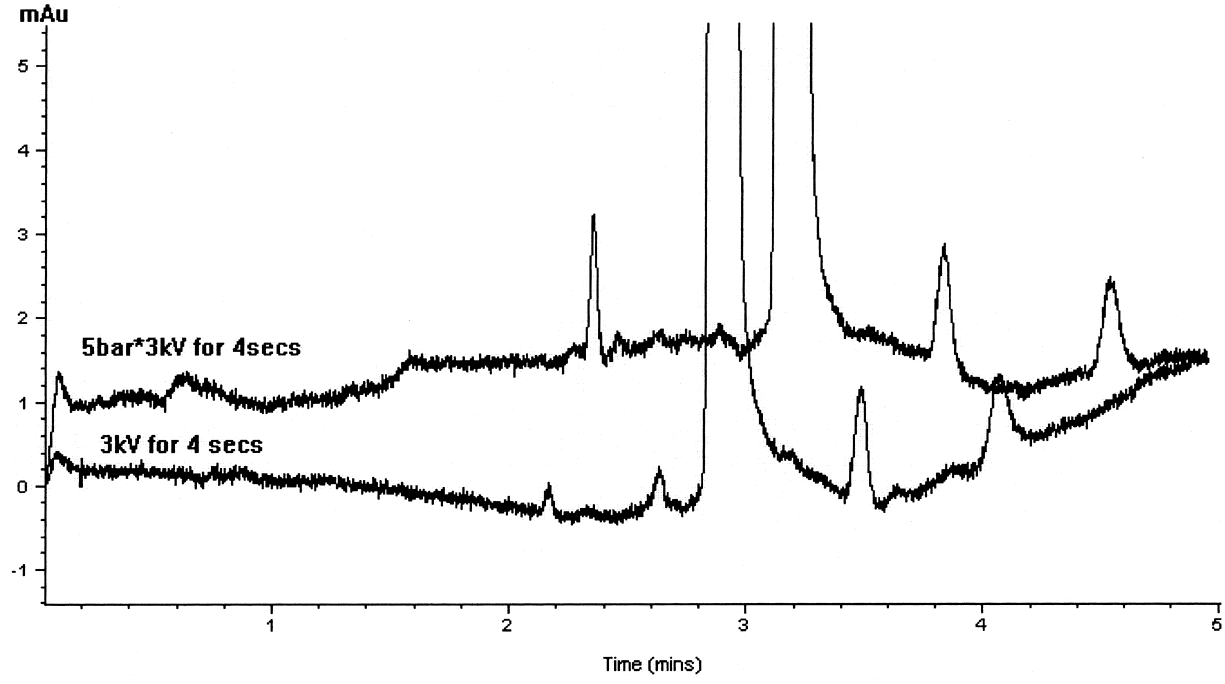

Fig. 6. Typical CEC electrochromatograms of LY300164 employing different injection techniques. (1) Pressurized electrokinetic injection, 5 bar×3 kV for 4 s, (2) electrokinetic injection 3 kV for 4 s.

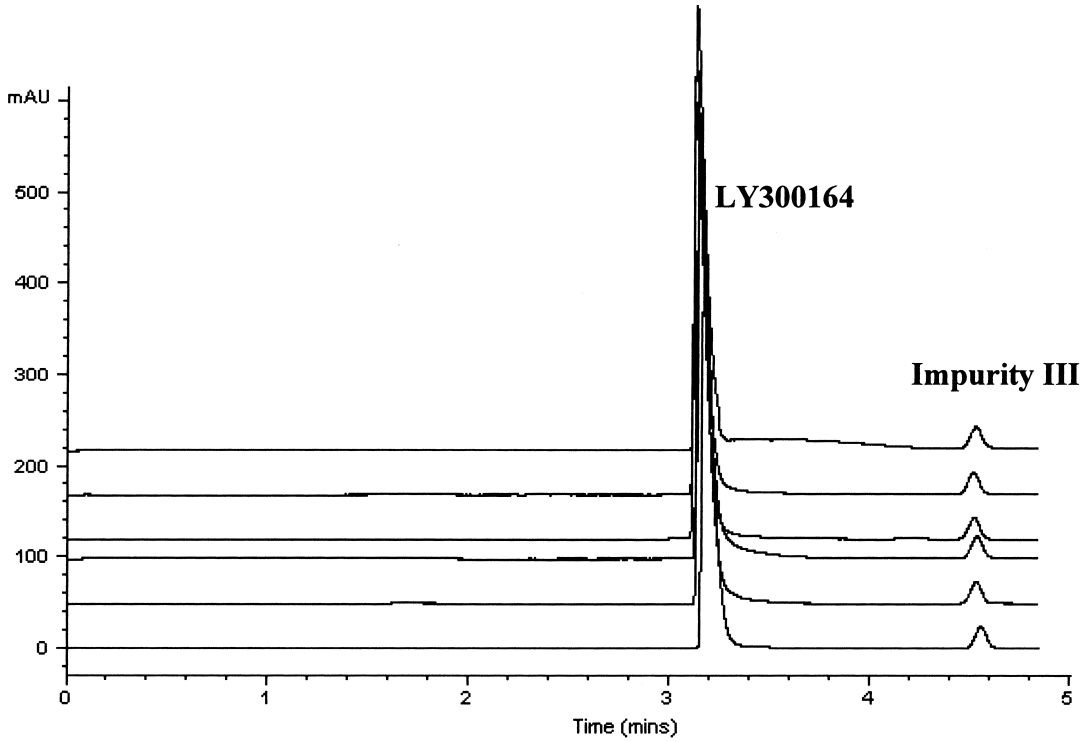

Fig. 7. Use of internal standard impurity III in LY300164 sample.

analysis there would be several analogues of the main drug under investigation, and it would be likely that one of these analogues would be selected as the internal standard. In the case of LY300164, a process impurity was employed as the internal standard, which had not been detected in LY300164.

In comparison to earlier investigations on injection parameters, pressurized electrokinetic injection was found to provide the best reproducibility and precision in peak migration and peak area without the use of the internal standard.

The electrochromatogram of LY300164 and its process-related impurities shows the same elution profile as for HPLC but in a much reduced run time of 6 min compared to 30 min for HPLC. LY300164 elutes first, followed by impurities I, III and then II, as shown in Fig. 8.

The highest individual impurity had earlier been identified as impurity '**x**' [32]. Impurity '**x**' had been fraction collected by HPLC, this was then re-injected on the CEC system and was found to be the last peak as observed on HPLC.

3.3.3. CEC linearity

A calibration was set-up for LY300164 covering the range 50–5000 μg ml^{-1} (0.25–25% of sample concentration). The linearity was determined from four repeat injections of five different concentrations of LY300164. The corrected area for LY300164 was plotted against the concentration in μg ml^{-1} linearity ($y=18.783x+0.1094$, $r^2=0.999$) was obtained. CEC clearly shows linearity of three orders of magnitude.

4. Discussion

4.1. Cross-comparison of percent total impurity and impurity 'x' values

Qualitatively CEC and HPLC traces look similar for the two development batches of LY300164, although CZE electropherograms are quite different. Quantitatively when five injections of a sample from both batches are compared in Table 1 for percent impurity values, it is seen that all techniques show

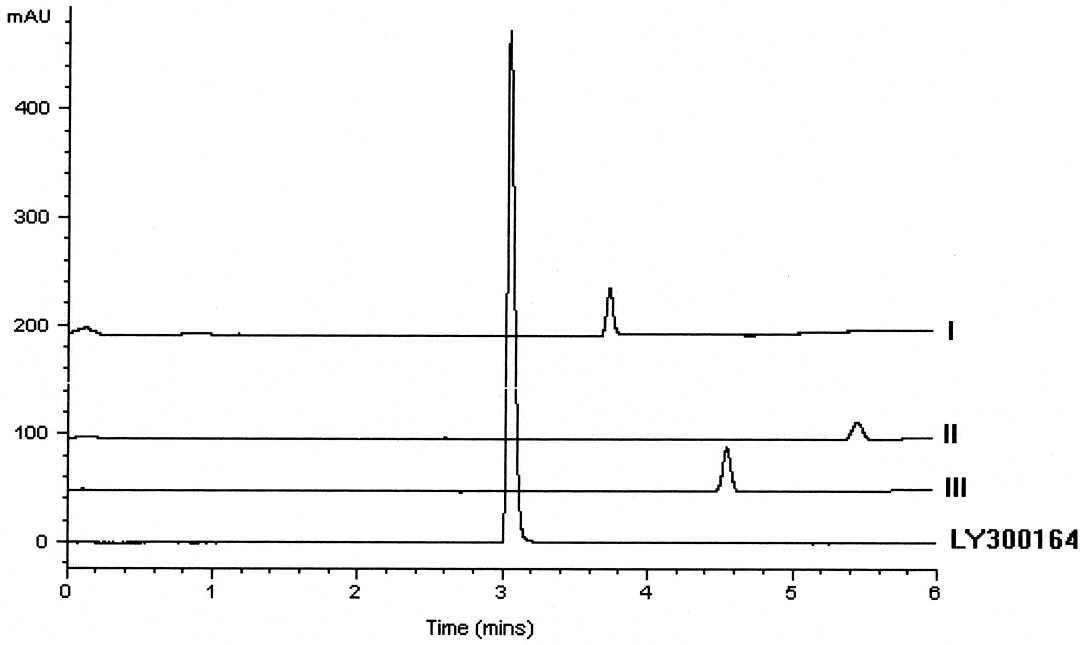

Fig. 8. CEC electropherogram of LY300164 and process-related impurities.

similar values for the total percent impurity values and also percent impurity '**x**' values. The differences between the values can possibly be attributed to differences in separation and injection modes and also integration parameters. It is interesting to note that the CZE percent impurity values look to be very similar to HPLC and CEC techniques although it has been shown previously in Section 3.2 that CZE is unable to detect the neutral process impurities that may be present. Hence it looks likely that the majority of the impurities in the two batches are positive in nature. The 'with' and 'without' pressure injections for CEC analysis on batch 2 are quoted and can be shown to give very similar results. The differences between the impurity values for all three techniques are not significant for an early phase pharmaceutical method with limited validation.

4.2. Precision data for five injections on each technique

When five injections of a sample from both batches are compared in Table 2 for precision, it is not a surprise that HPLC gives the best precision on peak areas, this is the essential parameter for quantification purposes. However, CEC is shown to show similar precision values to CZE, and with the use of an internal standard, R.S.D. values below 2% for peak areas. It is interesting also to note that when the CEC system has been conditioned well it can

Table 1
Cross-comparison of percent impurity values

Batch	Impurity '**x**' (%, w/w)			Total impurity levels (%, w/w)		
	HPLC	CZE	CEC	HPLC	CZE	CEC
1	0.39	0.49	0.40[a]	0.71	0.74	0.87[a]
2	0.61	0.50	0.34[a]0.35[b]	1.03	0.89	0.82[a]0.93[b]

[a]Without pressure electrokinetic injection 3 kV for 4 s.
[b]With pressure injection electrokinetic 5 bar×3kV for 4 s.

Table 2
Precision data for five injections on each technique

Batch	Peak areas (R.S.D., %)			Migration/retention times (R.S.D., %)		
	HPLC	CZE	CEC	HPLC	CZE	CEC
2	0.5	2.6	2.3[a]1.5[b]	1.3	0.6	0.2

[a]Without internal standard.
[b]With internal standard.

perform as well as HPLC in respect to retention/migration time variance.

4.3. Efficiency and limit of detection for each technique

Table 3 illustrates the differences in efficiencies and limit of detection of each technique. CZE for LY300164 shows by far the best efficiency values, whilst HPLC gives the best LOD. CEC shows good efficiencies of nearly 200 000 theoretical plates although the limit of detection is higher than in CZE and HPLC. However, efficiencies are also dependent upon buffer concentration for both CZE and CEC. Since CZE employed a running buffer concentration of 100 m*M* and 20% acetonitrile in the diluent, there was also some contribution from sample stacking. The high buffer concentration and stacking effect would also expect to enhance peak height and improve sensitivity compared to CEC.

5. Conclusions

For several years in the pharmaceutical Industry, HPLC has been complemented by the use of TLC for the determination of possible impurities in drug substances. Although this is a limited study, the data presented on one bulk pharmaceutical drug suggests the alternative use of CEC as a purity assay for pharmaceuticals. The elution order for LY300164 and its impurities is the same on CEC as HPLC using a similar packing. The analysis times much shorter for CEC than for HPLC, although there is a marked difference in limit of detection for the two techniques. CZE was deemed useful in respect to the analysis for impurity '**x**', although for LY300164 it cannot be deemed as an alternative to HPLC for total impurity analysis. MECC with further method development would probably be a suitable alternative; however, it would not be suitable for MS confirmatory analysis of related impurities. HPLC is the method of choice, although CEC has opened the doors to a rapid technique for screening process impurities compared to HPLC.

CEC offers the versatility of CZE and HPLC coupled with high column efficiencies. The technique permits separation of both neutral and weakly charged species. CEC as a separation technique is not routinely used in the pharmaceutical industry; however, this paper does show that the same validation requirements as HPLC can easily be addressed by CEC. The full benefit of CEC will only be realised when inherent technical issues such as the fragility of columns and air bubble formation can be fully resolved.

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Table 3
Efficiency and Limit of detection for each technique

Batch	Efficiency (plates/m)			LOD (mg/ml)		
	HPLC	CZE	CEC	HPLC	CZE	CEC
2	126 000	436 000[a]	197 000	3.7×10^{-05}	1.7×10^{-03}[a]	2.5×10^{-03}

[a]Achieved using stacking and a high buffer concentration.

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