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# Resolution of positional isomers by capillary electrochromatography

Darsha Hindocha[a], Norman W. Smith[b,*]

[a]*Development Chemistry 5, Chemical Development Division, GlaxoWellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts SG1 2NY, UK*

[b]*SKB/Zeneca Centre for Analytical Science, Imperial College of Science, Technology and Medicine, South Kensington, London SW7 2AY, UK*

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**Abstract**

Electroseparations have been very successful in increasing efficiencies and reducing analysis times. The analytical technique originally applied to open tube capillaries (capillary electrophoresis) has been used as a basis to develop renewed interest in electrochromatography. This paper describes the use of capillary electrochromatography to separate two positional isomers and describes a comparison between gas chromatography (GC), capillary electrochromatography (CEC) and nano-HPLC. The resolution of these isomers is quite crucial, since one of the isomers is the impurity in a pharmaceutically active drug. 

*Keywords:* Capillary electrochromatography; Positional isomers

## 1. Introduction

Capillary Electrochromatography is the term used to describe separations carried out in a capillary (usually fused silica) packed with a stationary phase where the flow of the mobile phase is generated by electroosmosis. In CEC as in HPLC and GC, the separation of a given mixture of neutral compounds is based on the partition coefficients of each component in this mixture between the stationary phase and the mobile phase. The stationary phase can be fixed on particulate material, commonly silica beads packed inside a capillary or fixed on the walls of an open tube. The potential of electroseparation in packed columns was first explored by Pretorius et al. [1] in 1974 this was miniaturised by Jorgenson and Lukas in 1981 when they used a 170 μm capillary packed with 10 μm partisil ODS-2 to separate 9 methyl anthracene from perylene [2]. The potential to use smaller capillaries packed with very small particles and reasonably low concentration buffers was demonstrated by Knox and Grant in 1987 and 1991 [3,4]. Knox also showed that as long as the operation conditions are kept within the boundaries of thermal effects a plug-like flow profile is generated [5]. Subsequent development and application of CEC has increased significantly [6–10]. The major advantage of CEC is that it demonstrates a plug-like flow profile seen in capillary electrophoresis. In CEC the flow is driven electrically therefore the flow profiles within the channels between the particles is plug-like (as in CE). In HPLC (pressure driven flow) the flow profiles within similar channels are parabolic leading to band broadening due to eddy

*Corresponding author. Tel.: +44-171-594-5841; fax: +44-171-594-5833.

*E-mail address:* n.w.smith@ic.ac.uk (N.W. Smith).

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diffusion. Hence efficiencies seen in HPLC are not as good as those obtained from CEC. It is believe that this advantage will aid better isomeric resolution when compared with HPLC. This example also shows the applicability of CEC as at least an orthogonal technique and maybe even a primary technique in the future analyses of pharmaceutical actives.

### *1.1. Resolution of isomers*

The usual approach to the separation of positional isomers in pharmaceutical actives using chromatographic techniques is either by HPLC (typically normal phase) [11] or GC [12]. For small volatile molecules, GC is often the preferred technique. The chemical structures of the two isomers (GW524889X and GW420867X) to be separated are shown in Fig. 1.

As mentioned above GC is often used for isomeric separations of small molecules and this was to be the case for the compounds shown below. Initial method development and analysis of GW524889X in GW420867X have been successfully achieved using a GC method, (see Fig. 8 in the results section). However problems were encountered during this analysis, with the GC column conditioning often taking 24 h. Long equilibration times were observed each time the column was installed in the instrument, irrespective of the age of the column. Investigation by normal phase HPLC resulted in no separation of the isomers. Therefore method development for the separation of the two isomers by capillary electrochromatography was instigated. At this moment in time it was crucial to develop a method which had a faster equilibration time and was suitable for the determination of low levels of GW524889X in GW420867X. The CEC method developed has an equilibration time of approximately half an hour. Therefore in order to maximise on efficiency of operation the CEC method was preferred. The use of deuterated solvents was also investigated and was found to offer only a limited improvement in resolution and efficiency in both CEC and nano HPLC.

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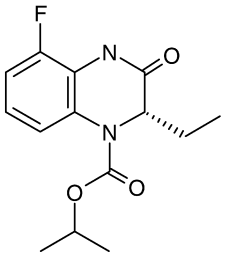


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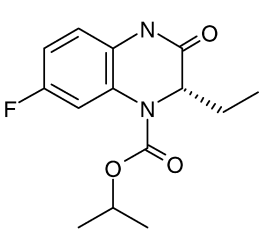


Fig. 1. Structures of the two isomers. Structure 1 is (s)-2-ethyl-5-fluoro-3-oxo-3,4 dihydro-2H-quinoxaline-1-carboxylic acid isopropyl ester, which will be referred to as GW524889X. Structure is 2 (s)-2-ethyl-7-fluoro-3-oxo-3,4-dihydro-2H-quinoxaline-1-carboxylic acid isopropyl ester, which will be referred to as GW420867X.

## 2. Experimental

### *2.1. Reagents and equipment*

HPLC grade acetonitrile (MeCN) and methanol (MeOH) were purchased from Fisher Chemicals, Loughborough, UK. Deuterated methanol ($CD_3OD$) was spectroscopic grade and was purchased from Euriso-top Cedex, France. Deuterium oxide ($D_2O$) and deuterated phosphoric acid ($D_3PO_4$) were purchased from Aldrich Gillingham, UK. Deionised water of 18.2 MΩ quality was produced by a Milli Q system and was used for all CEC experiments. Disodium hydrogen phosphate was AR grade purchased from Fisons Loughborough, UK.

Buffers solutions for the CEC experiments were prepared as follows: A 25 mM buffer solution was prepared by dissolving 0.9 g of AR grade disodium hydrogen phosphate in 100 ml of deionised water. The 50 mM buffer solution was prepared by dissolving 1.8 g of AR grade disodium hydrogen phosphate in 100 ml of deionised water. Both buffer solutions were adjusted to pH 7.0 using 0.1 M $H_3PO_4$. $D_3PO_4$ was used for pH adjustment in the $D_2O$ experiment.

Gas chromatography was performed on a HP5890, a fully automated integrated system. The column used was a 30 m×0.25 mm×0.25 μm HP-50 stationary phase consisting of 50:50 diphenyl/dimethylsiloxane. Both the instrument and the column were purchased from Hewlett Packard, Waldbronn, Germany.

Capillary Electrochromatography was performed on a $HP^{3D}CE$ system, Hewlett Packard, Waldbronn, Germany. Capillaries packed with 3 μm Waters Spherisorb ODS-1 and 3 μm Waters Spherisorb $C_6$/

SCX (38.5 cm×100 μm, 30 cm packed bed) were purchased from Innovatech Ltd., Stevenage, UK. These capillaries were packed using a high pressure packing method [13,14].

## 3. Results and discussion

### 3.1. Analysis by gas chromatography

The initial method development and optimisation was carried out using individual compounds and a 50/50 mixture of the two isomers. The mixture was prepared by weighing 300 mg of GW542889X and 300 mg of GW420867X into a 10 ml volumetric flask, diluting these to volume with HPLC grade methanol. Once suitable conditions were achieved, the method was used to analyse batches of GW420867X. Fig. 8 in the results section shows a typical batch chromatogram containing low levels of GW542889X.

Although the method gives good resolution of the two isomers and can detect very low levels of GW542889X in GW420867X, the temperature ramp causes difficulties during analysis. Often an unstable baseline is observed and conditioning the column takes too much time.

### 3.2. Analysis by capillary electrochromatography

The first CEC experiment performed, used a Waters Spherisorb ODS-1 capillary with 80/20 MeCN/25 mM disodium hydrogen phosphate pH7 (v/v) as the run buffer. Resolution between the two isomers was not achieved. Resolution of the two isomers was not achieved even at lower acetonitrile concentrations. The next CEC experiment was conducted using the $C_6$/SCX phase. This phase is less retentive than ODS-1, so the acetonitrile concentration was lowered to 60%, however no separation of the isomers was observed (see Fig. 2)

Since no separation was achieved, the fundamental rules used in HPLC method development were explored. In HPLC the interactions between the analyte and the stationary phase and the analyte and mobile phase are affected by hydrogen bonding. The separation in CEC will also depend upon the hydrogen bonding characteristics of the methanol. In

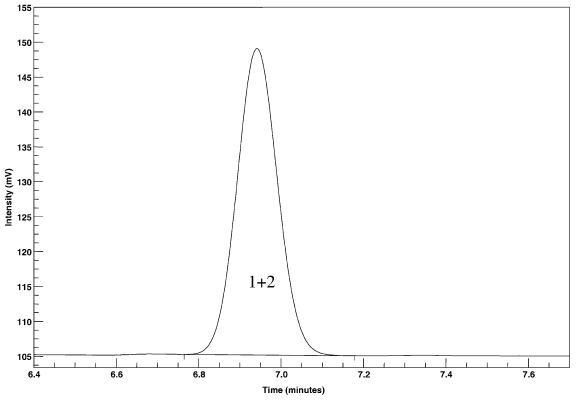


Fig. 2. Analysis of a mixture of GW420867X and GW524889X by CEC using 60/40 MeCN/25 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (30 cm packed bed); Voltage: 30 kV; Temperature: 30°C; Injection: Electrokinetic 10 s @ 10 kV; Detection: UV @ 230 nm; Sample: 1 mg of each GW420867X and GW524889X were dissolved and diluted in 1 ml of 70/30 MeCN/$H_20$ (v/v).

HPLC mobile phases methanol also displays different selectivity compared with acetonitrile [15]. As the fundamental separation mechanism of neutral compounds during HPLC and CEC analysis is the same, we investigated the use of methanol as the mobile phase modifier in the next experiment.

Using the same C6/SCX phase acetonitrile was substituted with methanol. The run buffer used was 80/20 MeOH/25 mM disodium hydrogen phosphate pH7 (v/v). A higher concentration of methanol was used to ensure that both components eluted within a reasonable runtime. Some separation of the isomers was now evident; see Fig. 3.

As expected methanol does show better selectivity for isomers by CEC as well as by HPLC. The elution order is as for the GC analysis with GW524889X eluting first followed by GW420867X. The poor separation is probably due to the high methanol concentration in the mobile phase; however, the baseline resolution of the two compounds should be achievable after further optimisation.

The method optimisation was progressed as follows. First the mobile phase was changed to 70/30 MeOH/25 mM disodium hydrogen phosphate pH7 (v/v). However, only a slight improvement in the separation was achieved. Although reducing the applied voltage from 30 kV to 20 kV further

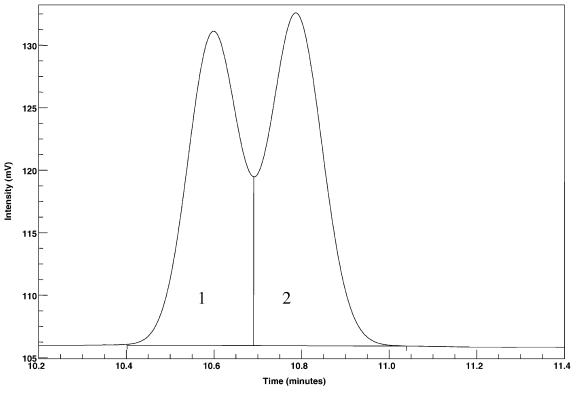


Fig. 3. Separation of GW420867X and GW524889X by CEC using 80/20 MeOH/25 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (30 cm packed bed); Voltage: 30 kV; Temperature: 30°C; Injection: Electrokinetic 10 s @ 10 kV; Detection: UV @ 230 nm; Sample: Sample: 1 mg of each GW420867X and GW524889X were dissolved and diluted in 1 ml of 70/30 MeOH/$H_2$0 (v/v).

improved the separation, further reduction of the voltage increased the runtime substantially without significant improvement in the resolution. Adequate separation was only achieved when the ionic strength of the run buffer was increased from 25 mM to 50 mM and the peak shape improved considerably, however the separation was still not baseline (Fig. 4). These conditions were tried on the ODS-1

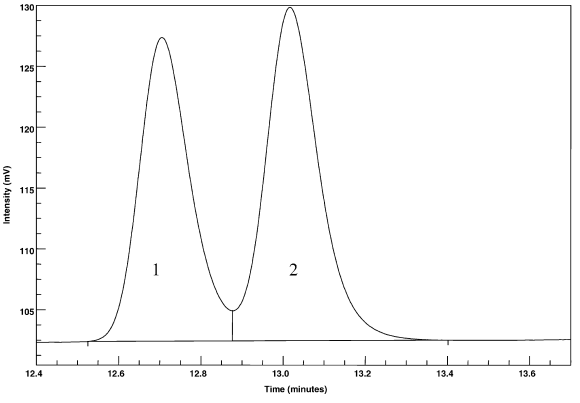


Fig. 4. Separation of GW524889X and GW420867X by CEC using 70/30 MeOH/50 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (30 cm packed bed); Voltage: 20 kV; Temperature: 30°C; Injection: Electrokinetic 10 s @ 10 kV; Detection: UV @ 230 nm; Sample: 1 mg of each sample was dissolved and diluted in 1 ml of 70/30 MeOH/$H_2$0 (v/v).

capillary, however both compounds were retained on the column even after a 40 min runtime.

In theory additional separation can be achieved in electroseparations by using deuterated solvents.

Eq. (1) below shows that solvent viscosity and dielectric constant affect the solute mobility ($\mu$):

$$\mu = \frac{\epsilon_0 \epsilon_r \zeta E}{\eta} \quad (1)$$

where $\epsilon_o$ = vacuum permittivity; $\epsilon_r$ = relative permittivity; $\zeta$ = zeta potential; $E$ = electric field strength; and $\eta$ = Viscosity of the solvent.

The viscosity and dielectric constants are only marginally higher for deuterated methanol, $CD_3OD$ (0.6 and 32.7 respectively) when compared with methanol, MeOH (0.544 and 32.63 respectively). This small difference might have an affect on the solute mobility and in turn the resulting separation. Literature shows that deuterated solvents have been used successfully in CZE to improve resolution of close eluting compounds. Okafo et al. [16] were able to increase the resolution of peptides and proteins by replacing water, $H_2O$ with deuterium oxide, $D_2O$. However the viscosity and dielectric constant differences between $D_2O$ (1.24 and 79.96) and water, $H_2O$ (1.0 and 79.24) are higher than those between $CD_3OD$ and MeOH. The viscosity and dielectric constant values have been taken from literature [17–19]. However it was decided to investigate the effect of deuterated solvents in CEC. Deuterated solvents can be used in CZE and CEC analysis because of the low buffer consumption, unlike HPLC where mobile phase consumption makes the use of such solvents expensive.

Initially just methanol in the run buffer was substituted with deuterated methanol, because this is the main constituent of the mobile phase. The difference in the physical properties of MeOH and $CD_3OD$ appears to be significant enough to increase the retention by a minute and give baseline resolution of a 50/50 mixture of the isomers (see Fig. 5).

Subsequent scanning through literature showed that some work had been conducted on relative electroosmotic mobilities of solvents without electrolytes [20]. The tabulated data in the referenced article shows that the mobilities of water and deuterium oxide are $7.6147\times10^{-8}$ and $6.5917\times10^{-8}$, this slight difference in mobilities may have an

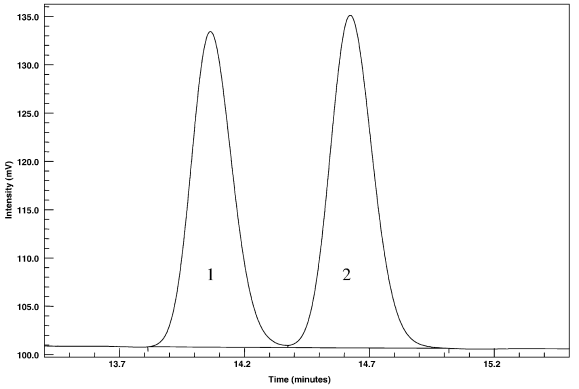


Fig. 5. The separation between GW420867X and GW524889X by CEC using 70/30 $CD_3OD$/50 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (30 cm packed bed); Voltage: 20 kV; Temperature: 30°C; Injection: Electrokinetic 10 s @ 10 kV; Detection: UV @ 230 nm; Sample: 1 mg of each GW420867X and GW524889X were dissolved and diluted in 1 ml of 70/30 MeOH/$H_20$ (v/v).

effect on the retention and resolution of the isomers. So in the next experiment the buffer (50 mM disodium hydrogen phosphate, pH adjusted using $D_3PO_4$) was prepared in $D_2O$ and the mobile phase prepared using this buffer and $CD_3OD$. The experimental conditions are listed with the resulting chromatogram in Fig. 6.

These results show that the substitution of water

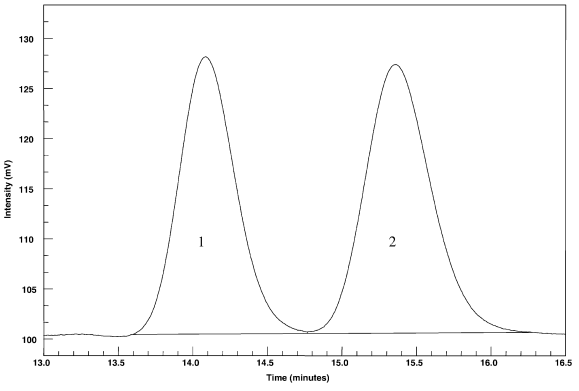


Fig. 6. The separation between GW420867X and GW524889X by CEC using 70/30 $CD_3OD$/50 mM disodium hydrogen phosphate pH7* (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (30 cm packed bed); Voltage: 20 kV; Temperature: 30°C; Injection: Electrokinetic 10 s @ 10 kV; Detection: UV @ 230 nm; Sample: 1 mg of each GW420867X and GW524889X were dissolved and diluted in 1 ml of 70/30 MeOH/$H_20$ (v/v). *The buffer was prepared using $D_2O$ and pH adjusted using $D_3PO_4$.

with deuterium oxide increased the retention time slightly, however the peak width for both peaks also increased hence overall improvement was not observed. The same was found to be true in an experiment conducted by Professor Jinno [21] where the separation factors of aromatics were compared in $CD_3OD$/water and $CD_3OD/D_2O$.

### *3.3. Comparison of data from the GC and initial CEC methods*

The resolution and efficiency data for all experimental data was calculated using standard Eqs. 2 and 3 shown below:

$$Rs = \frac{2(t_{r2} - t_{r1})}{w_1 + w_2} \tag{2}$$

$$N = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2 \tag{3}$$

Although the GC resolution of 5 is significantly better, the separation has been conducted on a 30 M column and therefore the overall separation efficiency, 2280 plates/m is not as good as the efficiency seen during the CEC analysis which is 191 834 plates/m with a resolution of 2.

### *3.4. Analysis of batches of GW420867X using the GC and CEC methods*

Four batches of GW420867X have been analysed using both the GC and the CEC methods. The data generated showed that the methods are comparable, unfortunately only one batch contained 'quantifiable' levels of GW524889X. This batch contained 0.06% area of GW524889X. Both methods detected 0.06%. The limit of quantification (LOQ) of the CEC method (Fig. 7) is 0.06% area, whereas the GC method (Fig. 8) has an LOQ of 0.02%. Chromatograms from these analyses are shown in Figs. 7 and 8 respectively.

### *3.5. Comparison of short-end CEC and nano-HPLC*

It is likely that if a separation is achieved by CEC then at least partial separation is feasible by re-

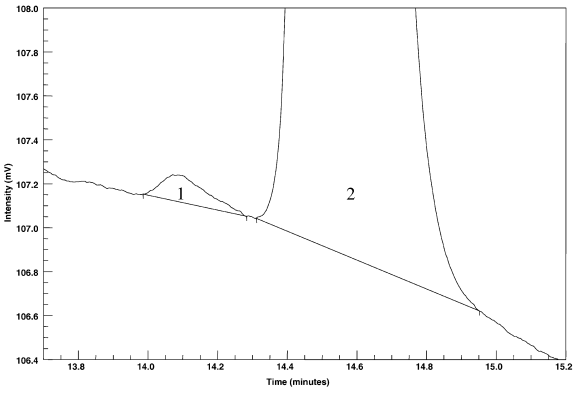


Fig. 7. Analysis of GW420867X batch 4 by CEC using 70/30 $CD_3OD$/50 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (30 cm packed bed); Voltage: 20 kV; Temperature: 30°C; Injection: Electrokinetic 10secs @ 10 kV; Detection: UV @ 230 nm; Sample: 1 mg of GW420867X batch 4 was dissolved and diluted in 1 ml of 70/30 MeCN/$H_20$ (v/v).

versed-phase HPLC. However because cost prohibits the use of deuterated solvents with conventional HPLC, the best option was to use a form of miniaturised HPLC where C6/SCX packing material and deuterated methanol can be used in small quantities.

In order to compare our CEC findings with nano-HPLC under the same conditions we repeated the CEC experiments using a short packed bed to ensure that pressure flow could be achieved for the corresponding nano-HPLC experiments. All experiments were conducted using the same capillary where the short-end (8 cm) was packed with 3 μm Spherisorb $C_6$/SCX packing material. The volume and mode of injection was kept constant during all four experiments, and this was using electrokinectic injection of −3 kV for 5 s.

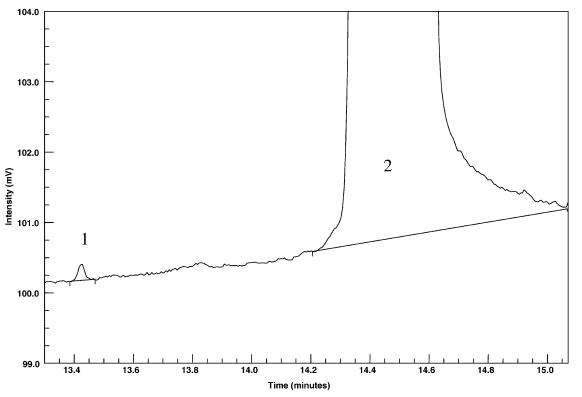


Fig. 8. Analysis of GW420867X batch 4 by GC using column: 30 m×0.25 mm×0.25 μm HP-50; Carrier gas: Helium, Flow rate: 1 ml/min (head pressure ~10 p.s.i.). Injector type: split @ split ratio 50:1; Injector temperature: 250°C; Loading: 1 μl of ~30 mg/ml of GW420867X batch 4 dissolved in MeOH, Detection: FID @300°C; Oven temperature program: Initial 150°C Ramp 5°C/min Final 260°C.

### *3.5.1. CEC*

Two CEC experiments were performed, one using methanol/50 mM disodium hydrogen phosphate pH 7(60:40 v/v), the other using deuterated methanol/50 mM disodium hydrogen phosphate pH 7 (60:40 v/v). Note the methanol and deuterated methanol concentrations had to be reduced in order to achieve sufficient retention for the required resolution. The column temperature was kept at 30°C and the voltage was reduced to −3.5 kV. This was again due to the shorter column length used.

### *3.5.2. HPLC*

As a comparison two similar nano-HPLC experiments were performed using exactly the same mobile phase systems and temperature as for the two CEC experiments. Flow was achieved by applying 12 bar to the inlet side of the capillary following sample injection. Because only 8 cm of the capillary was packed, sufficient flow was achieved to elute the compounds in a reasonable time.

As expected the data generated shows similar chromatography when we compare the short-end CEC with nano-HPLC. At a glance chromatograms from all four experiments show little difference in the resolution and efficiency of the systems (see Figs. 9 and 10). However the calculated resolution and efficiency values show that in both the nano-HPLC and the CEC experiments, deuterated methanol generates slightly better separation and efficiencies than ordinary methanol.

The values for the CEC experiments are:

Methanol: Resolution 1.34 Efficiency 162 351 plates/m.

$CD_3OD$: Resolution 1.47 Efficiency 187 534 plates/m.

The values for the nano HPLC experiments are:

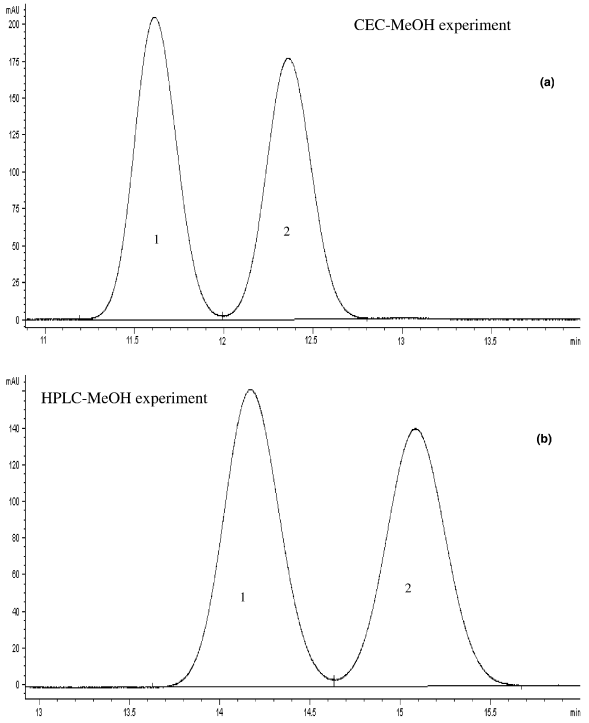


Fig. 9. (a) The separation between GW420867X and GW524889X by CEC using 60/40 $CH_3OH$/50 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (8 cm packed bed); Voltage: −3.5 kV; Temperature: 30°C, Injection: Electrokinetic 5 s @ −3 kV; Detection: UV @ 230 nm; Sample: 1 mg of each GW420867X and GW524889X were dissolved and diluted in 1 ml of 70/30 MeOH/$H_20$ (v/v). (b) The separation between GW420867X and GW524889X by nano-HPLC using 60/40 $CH_3OH$/50 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (8 cm packed bed); Pressure 12 bar; Temperature: 30°C; Injection: Electrokinetic 5 s @ −3 kV, Detection: UV @ 230 nm; Sample: 1 mg of each GW420867X and GW524889X were dissolved and diluted in 1 ml of 70/30 MeOH/$H_20$ (v/v).

Methanol: Resolution 1.28 Efficiency 113 539 plates/m.

$CD_3OD$: Resolution 1.40 Efficiency 139 893 plates/m

Fig. 9a and b show the resolution obtained from short-end CEC and nano-HPLC using methanol/buffer as the mobile phase.

The slightly improved resolution of the deuterated methanol/buffer system from both short end CEC and nano-HPLC are shown in Fig. 10a and b.

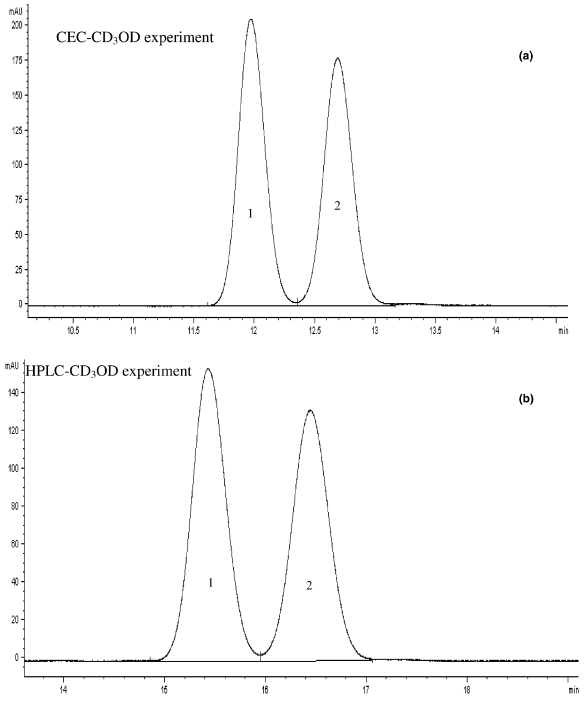


Fig. 10. (a) The separation between GW420867X and GW524889X by CEC using 60/40 $CD_3OD$/50 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (8 cm packed bed); Voltage: −3.5 kV; Temperature: 30°C, Injection: Electrokinetic 5 s @ −3 kV; Detection: UV @ 230 nm; Sample: 1 mg of each GW420867X and GW524889X were dissolved and diluted in 1 ml of 70/30 MeOH/$H_20$ (v/v). (b) The separation between GW420867X and GW524889X by nano-HPLC using 60/40 $CD_3OD$/50 mM disodium hydrogen phosphate pH7 (v/v); Column: 38.5 cm×100 μm 3 μm Waters Spherisorb $C_6$/SCX (8 cm packed bed); Pressure 12 bar; Temperature: 30°C; Injection: Electrokinetic 5 s @ −3 kV; Detection: UV @ 230 nm; Sample: 1 mg of each GW420867X and GW524889X were dissolved and diluted in 1 ml of 70/30 MeOH/$H_20$ (v/v).

## 4. Conclusions

The data generated shows that all three techniques allow for the determination of small quantities of GW524889X in GW420867X. The results from these experiments show that CEC can be applied to low level impurity quantification. Baseline resolution of a 50/50 mixture of the two isomers is achieved using methanol or deuterated methanol as the organic modifier; however the substitution of water with deuterium oxide did not further improve the res-

olution. The use of deuterated solvents produced only a marginal improvement in separation over conventional solvents in both the CEC and nano-HPLC modes. Their use is therefore considered to offer no advantage for the analysis of these compounds.

Advantages of the CEC method over the GC method are that the equilibration times were typically in the range of an hour hence offering a major advantage over the GC method. The main disadvantage is of course the lack of experienced analysts in the use of CEC. The advantages of the GC method are good resolution and trained personnel to carryout the analysis. The data represented shows that the two methods are complementary to each other, and although both methods are currently used to analyse the pharmaceutically active GW420867X and confirm the accuracy of the results generated, CEC is considered more efficient because of the overall conservation of time of analysis. This is crucial especially in a production environment.

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