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A generic approach to the impurity profiling of drugs using standardised and independent capillary zone electrophoresis methods coupled to electrospray ionisation mass spectrometry

Three standardised, capillary zone electrophoresis-electrospray ionisation mass spectrometry (CZE-ESI-MS) methods were developed for the analysis of six drug candidates and their respective process-related impurities comprising a total of 22 analytes with a range of functional groups and lipophilicities. The selected backround electrolyte conditions were found to be: 60/40 v/v 10 mM ammonium formate pH 3.5/organic, 60/40 v/v 10 mM ammonium acetate pH 7.0/organic and 10 mM piperidine, pH 10.5, where the organic solvent is 50/50 v/v methanol/acetonitrile. The coaxial sheath flow consisted of either 0.1% v/v formic acid in 50/50 v/v methanol/water, or 10 mM ammonium acetate in 50/50 v/v methanol/water, depending on the mixture being analysed. Factor analysis and informational theory were used to quantify the orthogonality of the methods and predict their complementarities. The three selected CZE-ESI-MS methods allowed the identification of 21 out of 22 of all the drug candidates and their process-related impurities and provided orthogonality with four established high-performance liquid chromatography-mass spectrometry (HPLC-MS) methods. These methodologies therefore form the basis of a generic approach to impurity profiling of pharmaceutical drug candidates and can be applied with little or no analytical method development, thereby offering significant resource and time savings.

Keywords: Capillary zone electrophoresis / Impurity profiling / Liquid chromatography / Mass spectrometry / Orthogonality
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1 Introduction

Over the past decade, significant investment and scientific effort have been directed to enhancing the efficiency of the drug discovery processes, with the result that high-throughput synthesis and screening technologies are now capable of producing very large numbers of drug candidates. The increased throughput required for the impurity profiling of drug candidates has highlighted the inefficiencies of the conventional approach to analytical method development. Time constraints mean that it is uneconomical to develop new analytical methods for separating impurities from each new drug candidate [1]. In addition, regulatory authorities place a significant emphasis on drug purity, requesting full characterisation and identification of any impurities, normally at the level of 0.1% and above [2]. Hence, strategies to improve the throughput of analytical methods applied to impurity profiling of drug candidates have the potential to save time and produce safer and purer batches of drugs for initial biological evaluation. Automation of the method development stage is unlikely to solve the problem since it is accepted that the complete resolution of component peaks in complex samples is not practically feasible using a single separation method [3]. For example, Giddings [4] predicted that approximately four million theoretical plates would be required to separate 82 of 100 components randomly distributed in a single separation, which is clearly not feasible. It is thus highly desirable to develop two or more complementary methods to ensure that all impurities and related substances are detected and identified [5]. To achieve the best resolution of all impurities, it is desirable that the separation methods should have a high degree of orthogonality, *i.e.*, the separation mechanisms underlying each method should be independent as possible, thereby leading to non-correlation of peak separation data between the different methods [6, 7]. For example, capillary zone electrophoresis (CZE) offers the potential for a separation mechanism which is orthogonal to the partitioning or hydrophobic interactions commonly encountered in reversed-phase high-performance liquid chromatography (RP-HPLC) [8–12]. Many biologically active molecules are fully or partially ionized at physiological pH and, often, biological activity is

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dependent on the presence of ionisable groups (typically amines or carboxylic acids) [13]. The development of separation methodologies by HPLC can be difficult for such compounds, due to various deleterious interactions with the ionisable silica stationary phase and chelating metal impurities often encountered in the base silica [14]. CZE is often a better choice, due to the electrophoretic nature of the separation mechanism, providing unique selectivity [15]. The poor concentration sensitivity and lack of specificity often encountered with CZE-UV methods may be overcome by coupling CZE with electrospray ionisation-mass spectrometry (ESI-MS). Interfacing CZE with ESI-MS also enhances the generic applicability of the technique and increases the potential for identification of unknown impurities or degradation products.

Multidimensional analysis was pioneered by Bushey and Jorgenson [16] using CE in combination with HPLC, and since then, publications have primarily referred to directly coupled 2-D systems, such as LC-LC or LC-CE [17, 18]. These systems have not yet been developed to a routine automated level because of the complexity of the hyphenating and data-handling processes. A combination of independent, orthogonal methods can also be used for a serial 2-D analysis, where a new sample is used for each separation. As opposed to multidimensional analysis, this approach does not provide increased peak capacities but complementary results, which improves the mapping of all the analytes present in a sample by minimising the probabilities of overlapping peaks. Hence, the use of orthogonal methods potentially enables an improved characterisation of the impurity profiles of pharmaceutical compounds.

A generic strategy aiming to speed up the process of pharmaceutical analysis with improved confidence was initiated with a systematic development of a series of orthogonal HPLC separation methods coupled with multiparallel MS analysis [19]. This approach eliminates the necessity to develop a new purity profiling method for each drug candidate and instead, relies upon the increased resolving power of a series of standardised, orthogonal separation methods. In the present research, a series of proprietary pharmaceutical drug candidates and their process-related impurities possessing varying physicochemical properties were used to develop a set of standardised CZE-ESI-MS methods. Numerical indices of separation were used to compare individual CZE methods and to establish the degree of orthogonality between the optimised CZE and established HPLC-based separation methods.

2 Materials and methods

2.1 Chemicals

All reagents used in the background electrolyte (BGE) were of high purity (HPLC-grade or better). Ammonium acetate, ammonium formate, ammonium trifluoroacetate, ammonium hydroxide, acetic acid, formic acid, trifluoroacetic acid (TFA), hydrochloric acid, methanol (MeOH), and far UV acetonitrile (MeCN) were purchased from Fisher Scientific (Loughborough, UK). Ammonium carbonate, ammonium hydrogen carbonate, and thiourea were purchased from Aldrich (Gillingham, UK). Piperidine and sodium hydroxide (NaOH) were purchased from Sigma Chemical (Poole, UK). Deionised ultrapure water (18.2 MΩ) was obtained from a USF Elga Maxima water purifier (USF services, Derbyshire, UK). A series of synthetic pharmaceutical compounds and their process-related impurities (22 analytes in total) was selected as being representative of typical drug candidates in terms of functional groups, log *P* and pK_a values, and the range of impurities. These compounds were obtained from Pfizer Global Research and Development (Sandwich, UK) and used without further purification. The pK_a and log *D* values of the selected drug candidates are presented in Table 1 and their functional groups are shown in Fig. 1.

Table 1. Physicochemical properties of selected drug candidates

Candidates	pK_a acidic / basic		log *D* at pH 3.5[a)]	log *D* at pH 7.0	log *D* at pH 10.5	Known impurities
B1		9.2	0.2	0.4	3.0	2 bases
B2		4.8/11.6	0.5	6.5	6.5	5 bases, 1 zwitterion
Z1	3.6	7.7/9.6	−2.1	−1.3	−1.7	1 base, 3 zwitterions
Z2	9.0	2.0	1.1	2.7	1.2	1 zwitterion
A1	7.3		0.2	0.2	−2.8	2 acids
A2	4.7		5.7	3.4	1.5	1 neutral

a) log *D* values calculated using the Advanced Chemistry Development (ACD/pK_a DB v. 4.0) software

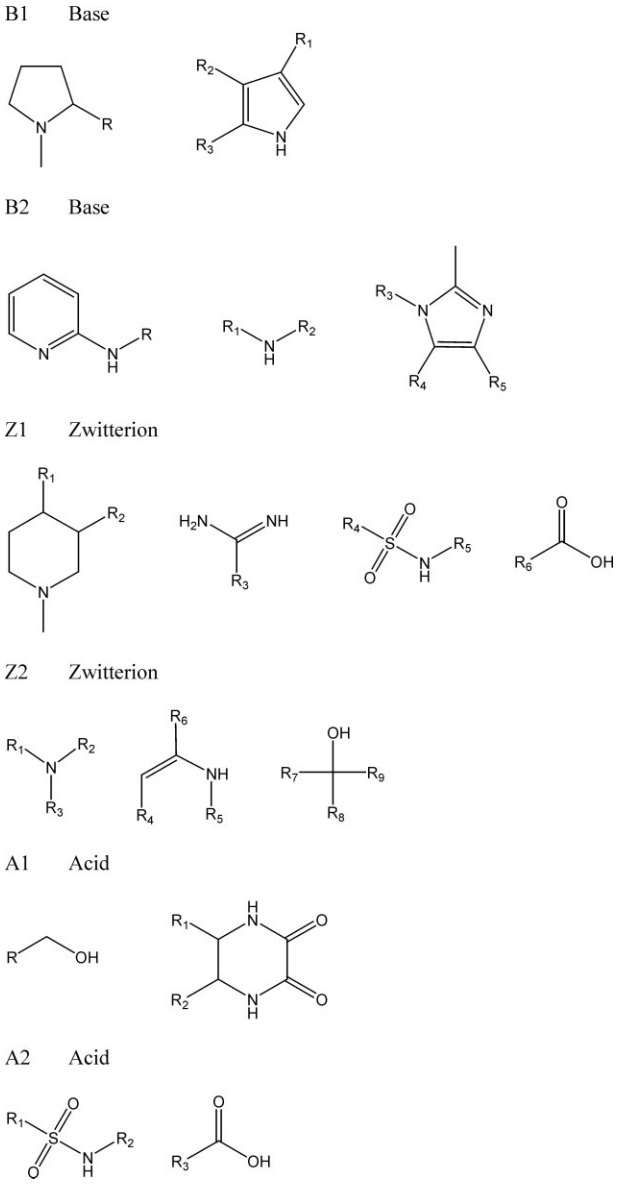

Figure 1. Functional groups of selected proprietary compounds.

2.2 Sample preparation

Stock solutions of each compound were prepared at a concentration of 0.8 mmol·mL^{-1} (between 0.3 and 0.6 mg·mL^{-1}) in 40/40/20 v/v/v MeOH/MeCN/H_2O. This ternary solvent provided the optimum solubility for all the compounds, according to initial studies. Test solutions were diluted from stocks to a concentration of 0.2 mmol·mL^{-1} (between 0.08 and 0.15 mg·mL^{-1}) in 50/40/10 v/v/v organic/H_2O/BGE buffer, where the organic solvent was 50/50 v/v MeOH/MeCN. Samples were filtered through a 0.45 μm filter (Gelman, Portsmouth, UK) and then degassed by ultrasonication.

2.3 Background electrolyte

BGE solutions were prepared at a final concentration of 10 mM by adding an appropriate volume of organic solvent to the pH-adjusted aqueous buffer solution. The organic solvent was a mixture of 50/50 v/v MeOH/MeCN. The pH was adjusted by titrating with the corresponding acid or base at the same concentration. The ammonium trifluoroacetate solution was adjusted to pH 2.5 with TFA, ammonium formate to pH 3.5 with formic acid, ammonium acetate to pH 5.0 with acetic acid and ammonium carbonate to pH 8.6 with ammonium hydrogen carbonate. Ammonium acetate was adjusted to pH 7.0 with 10 mM ammonium hydroxide and piperidine to pH 10.5 with 1 M hydrochloric acid. BGE solutions were filtered through a 0.45 μm filter (Gelman, Portsmouth, UK) and then degassed by ultrasonication.

2.4 CZE-UV analysis

CZE-UV analyses were carried out using an Agilent 3D-CE instrument (Waldbronn, Germany) interfaced with ChemStation software (Rev. A 08.03) and equipped with a diode-array detector. The experiments were performed using an uncoated fused silica capillary 48.5 cm × 50 μm ID (40 cm effective length) (Polymicro Technologies, Phoenix, AZ, USA). Hydrodynamic injection (25 mbar for 15 s) was used to avoid analyte discrimination. Separations were performed by applying a voltage of 20 kV in normal polarity mode. The temperature of the capillary was maintained at 25°C and the sample tray at *ca*. 10°C. Compounds were analysed individually and detected by UV absorbance (214 nm) in preliminary investigations to expedite the evaluation process and prior to coupling to MS detection. At the beginning of each run, the capillary was conditioned by flushing for 10 min with 0.1 M NaOH. During the run, the capillary was flushed before each injection with 0.1 M NaOH for 1 min, then with deionised water for 1 min and finally with BGE for 3 min. The EOF time was determined by observation of the UV baseline deflection for each sample injection and confirmed using thiourea at the beginning of each run. The electrophoretic mobility was reported as an average of three replicates and calculated as the difference between the apparent mobility of each analyte and the electroosmotic mobility [20].

2.5 CZE-ESI-MS analysis

CZE-ESI-MS experiments were performed on a 3-D CE system, interfaced with a 1100 series MSD Trap SL, equipped with a CE-MS adapter kit (G1603A), a CE-ESI-MS sprayer kit (G1607A), and an isocratic HPLC pump

(all Agilent Technologies). An Agilent MSD Trap software (v. 4.1) was used for MS instrument control, data acquisition, and data analysis. Separations were carried out in an uncoated fused-silica capillary (Polymicro Technologies) with 80.0 cm × 50 μm ID, 21.6 cm to the UV detector. The polyimide coating was removed from the end of the capillary (*ca.* 3 mm) to maintain a stable electrospray. The coaxial sheath liquid was delivered at 10 $\mu L \cdot min^{-1}$ by the pump, equipped with a 1:100 splitter. The ESI capillary was set at 4.0 kV, the skimmer at 40 V and the nitrogen nebulizing gas at 15 psi. The drying gas was delivered at a flow rate of 4.0 $L \cdot min^{-1}$ and a temperature of 325°C. The mass spectrometer was operated in "Smart" mode, therefore other acquisition parameters such as capillary exit offset voltage, octapole voltage, and trap drive were automatically optimised for each sample. The spectrometer was set to scan from 100 to 900 *m/z*; the target (number of ions × gain) was set at 30 000 and the accumulation time at 300 ms; 5 microscans per scan were accumulated in the full scan mass range. Each drug candidates and their respective process-related impurities were analysed as test mixtures. Test mixtures B1, B2, and Z1 were analysed in positive ion mode, with 60/40 v/v 10 mM ammonium formate, pH 3.5/organic as BGE (organic being 50/50 v/v MeOH/MeCN), and 0.1% v/v formic acid in 50/50 v/v MeOH/H_2O as sheath liquid. Test mixtures Z2, A1, and A2 were analysed in negative ion mode, with 10 mM piperidine, pH 10.5, as BGE and 10 mM ammonium acetate in 50/50 v/v MeOH/H_2O as sheath liquid. The capillary was flushed before each injection with BGE for 3 min. Other CZE conditions were as stated in the CZE-UV section.

2.6 HPLC analysis

The conditions of the four HPLC-MS methods developed in previous studies are summarized in Table 2. Briefly, the methods utilised four columns (Phenomenex, Manchester, UK) with different HPLC stationary phases, mobile phases, and gradient profiles. The retention time for each analyte, as well as the void time, were recorded and used to calculate indices of orthogonality.

2.7 Assessment of orthogonality

A normalised migration index (NMI) was defined as according to Eqs. (1) and (2) to allow the direct comparison of HPLC- and CZE-based methods. This transformation enabled the analysis time of each solute in each dimension to be compared on the same scale, *i.e.*, between 0 and 1.

Table 2. Generic HPLC-MS methods[a)]

Method	Stationary phase	Mobile phase
LC 1	Phenyl-hexyl, 5 μm (15 × 0.46 cm)	10 mM ammonium acetate, pH 6.8 (unbuffered)/MeCN (gradient to 95% v/v MeCN)
LC 2	C_{18}, 5 μm (15 × 0.46 cm)	10 mM ammonium trifluoroacetate, pH 3.0 (adjusted with TFA) / MeCN (gradient to 95% v/v MeCN)
LC 3	Aqua C_{18}, 125 Å (15 × 0.46 cm)	0.1% formic acid, pH 2.6 (unbuffered) / MeCN (gradient to 95% v/v MeCN)
LC 4	Cyano-propyl, 5 μm (15 × 0.46 cm)	10 mM ammonium acetate, pH 5.8 (adjusted with acetic acid)/MeCN (gradient to 50% v/v MeCN)

a) Experimental conditions: injection volume, 20 μL; temperature, 25°C; flow rate, 1.0 $mL \cdot min^{-1}$; UV detection, 210–290 nm; ionisation, ES^+; RF lens voltage, 300 V; extraction cone voltage, 3.0 V; gas flow rate, 50 $mL \cdot min^{-1}$; nebulisation gas, ~950 $L \cdot h^{-1}$

For HPLC experiments:

$$NMI_{HPLC} = \frac{t_r - t_o}{t_{last} - t_o} \quad (1)$$

where t_r is the retention time of the analyte, t_o is the retention time of an unretained component (*i.e.*, solvent front), and t_{last} is the retention time of the last eluting component in the dimension.

For CZE experiments:

$$NMI_{CZE} = \frac{\mu_e - \mu_{fastest}}{\mu_{slowest} - \mu_{fastest}} \quad (2)$$

where μ_e is the electrophoretic mobility of the analyte, $\mu_{fastest}$ is the electrophoretic mobility of the fastest migrating component in the dimension (*i.e.*, the most mobile cation), and μ_{last} is the electrophoretic mobility of the last migrating component in the dimension (*i.e.*, the most mobile anion). Orthogonality was firstly assessed using the geometric approach to factor analysis described by Liu and Patterson [21]. In this method, correlation coefficients (*C*), peak spreading angles (β), theoretical peak capacities (N_t), and practical peak capacities (N_p) were calculated as described in [20]. In addition, the resolving power (RP) of the 2-D separation systems was calculated as:

$$RP = (N_p/N_t) \cdot 100 \quad (3)$$

Results obtained from the factor analysis approach are a measure of the 2-D separation space utilisation. A measure of data overlap was evaluated using the informational

orthogonality, which utilises similarity and synentropy indices. This method is fully described by Massart and Kaufman [22] and was first applied to 2-D chromatographic systems by Slonecker *et al*. [6].

3 Results and discussion

3.1 Selection of CZE-UV separation conditions

A range of BGE solutions was evaluated; each contained an aqueous electrolyte at 10 mM (buffered at a range of pH values from 2.5 to 10.5) and an organic solvent (content between 0 to 60% v/v in BGE). Only volatile BGE buffer components were used at a concentration kept as low as practically possible (10 mM), in order to ensure sufficient MS compatibility whilst maintaining buffer capacity [23]. BGE solutions required the addition of a mixture of MeOH and MeCN (50/50 v/v) to ensure solubility of all the compounds. Peak distortion was observed at organic solvent content of 60% v/v in BGE, thus higher fractions were not evaluated. The use of organic solvents in the BGE was compatible with our strategy to develop a generic set of methods since it extends the range of compounds that can be analysed (in terms of poorly aqueous soluble drugs) and is advantageous for achieving high selectivities [24, 25]. Other solvents, such as formamide, *N*-methylformamide, *N,N*-dimethylformamide, and dimethylsulfoxide, although potentially advantageous, were not used because their high UV absorbance at the selected wavelength (214 nm) [26] hampered their initial evaluation by CE-UV. Initially, CZE separating conditions were assessed using UV detection for the six drug candidates analysed individually without impurities.

3.1.1 Effect of BGE composition on electrophoretic mobility of drug candidates

The effects of the BGE pH and organic solvent content on the electrophoretic mobility of the six drug candidates are shown in Fig. 2. Experimental pK_a values, which can be inferred from the mobility curves in the same manner as for titration curves, appeared to correspond well to the theoretical values given in Table 1. The strong base B1 (pK_a = 9.2) was ionised throughout the pH range studied, whereas the weak base B2 (pK_as = 4.8/11.6) comigrated with the EOF at pH ~7 and above. The zwitterion Z1 (pK_as = 3.6/7.7/9.6) behaved as a weak base at pH ~8.6 and below, and as a weak acid at pH ~8.6 and above. The zwitterion Z2 (pK_as = 2.0/9.0) behaved either as a weak acid or a weak base. The weak acid A1 (pK_a = 7.3) behaved as if ionised at pH ~5 and above, whereas the acid A2 was somewhat stronger (pK_a = 4.7), showing evidence of ionisation at pH ~3.5 and higher. A consistent observation for all the analytes was an apparent reduction in ionisation with increasing organic solvent content in BGE, *i.e.*, the analytes exhibited reduced electrophoretic mobilities. The most significant example is Z1, a zwitterion which should be negatively charged at pH 10.5, but whose ionisation (and any electrophoretic mobility) was apparently fully suppressed upon adding organic solvent to the BGE (Fig. 2). These observations are in agreement with previous reports. For example, Porras [27] indicated that, for the actual mobilities of aromatic cations, the organic solvents levelled the mobility range, possibly due to their lower degree of solvation compared to water, resulting in a certain consistency of the Stokes radii. Barron *et al.* [28] observed similar behaviours when analysing quinolones: the electrophoretic mobilities of the fully protonated and deprotonated species decreased (in absolute values) with increasing tetrahydrofuran content in the BGE solutions. Barbosa *et al.* [29, 30] have developed a model to predict quinolone mobilities in MeCN/H_2O mixtures as a function of pH, pK_a, and activity coefficients. Mathematical models have also been formulated to describe anion mobility in hydro-organic media [1, 31–35], but the explanation for this phenomenon is still rather unclear.

3.1.2 Effect of BGE composition on peak efficiencies of drug candidates

Porras *et al.* [35] have indicated that plate heights in CZE are dependent on the analyte's longitudinal diffusion, thermal broadening, electromigration dispersion and wall adsorption. Such variables are influenced by the pH/ionic strength/solvent composition of the BGE, in addition to analyte effects, and hence it can prove difficult to quantify the individual effects of each parameter on peak efficiency. The effect of BGE composition on efficiency was evaluated for each compound. An example of the results is shown in Fig. 3 for base B1 (pK_a = 9.2). This drug showed a general increase in efficiency as pH was increased, with this trend being more apparent with the higher organic% in the BGE. This could be explained by the suppression of potential electrostatic interactions between the amine of the analyte and ionised silanol groups of the capillary wall.

Improved solubility of hydrophobic compounds was obtained in hydro-organic media, leading to improved sensitivity. Higher solubility has also been reported to lead to less self-aggregation of the analytes, which improves the dispersion of the analyte in solution and hence the interactions with solvent molecules and supporting elec-

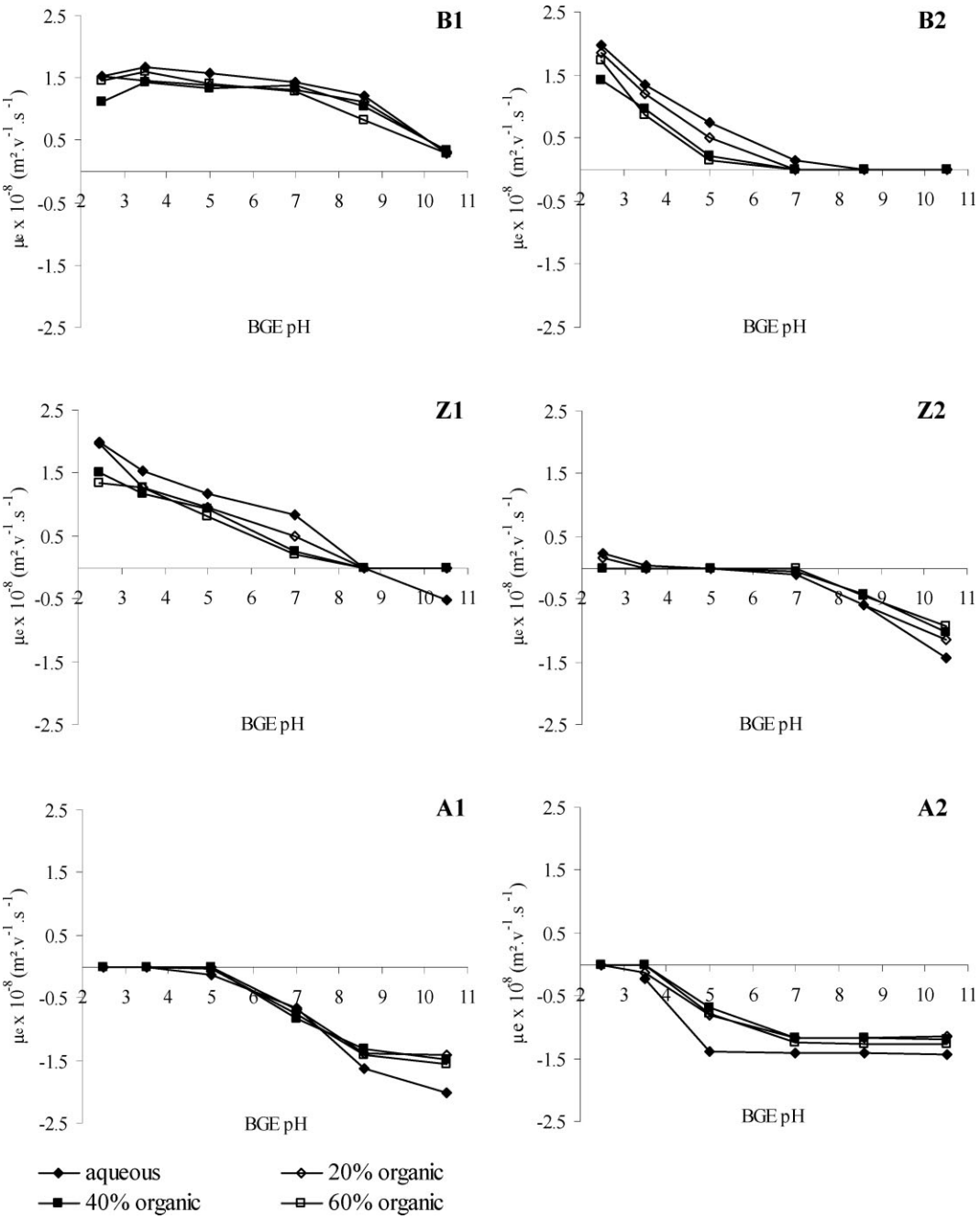

Figure 2. Effect of BGE composition on electrophoretic mobility of drug candidates. Experimental conditions: organic solvent, 50/50 v/v MeOH/MeCN; uncoated fused-silica capillary, 48.5 cm × 50 μm ID; sample concentration, 0.2 mmol·L^{-1}; injection, 25 mbar for 15 s, separation, +20 kV, capillary temperature, 25°C; n = 3.

trolytes [36]. This effect may have affected the observed changes in peak efficiencies of hydrophobic compounds (such as B2 or A2) upon varying the organic solvent content in BGE. Finally, a very unstable current and consequently unstable baseline was observed when using TFA as BGE buffer component, probably indicating a poor solubility of the sample plug in the electrolyte. TFA is also known for its marked suppressive effect on ESI efficiency in both negative and positive mode and may not be an ideal choice for buffer composition [37].

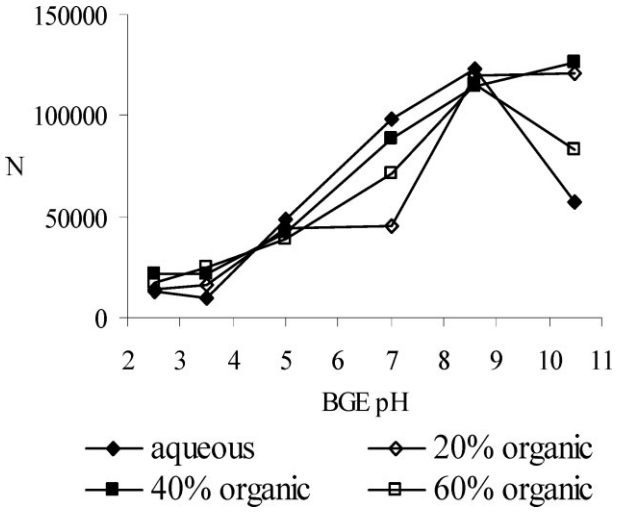

Figure 3. Effect of BGE composition on the peak efficiency of B1. Experimental conditions as stated in Fig. 2.

3.1.3 Selection of final CZE-UV methods

On the basis of the preliminary investigations, three BGE conditions were chosen in order to be able to analyse all the compounds with the full range of BGE conditions, *i.e.*, acidic, neutral, and basic conditions. At pH 3.5 and 7.0, a good compromise between solubility and efficiencies was found at an organic content of 40% v/v in BGE. Sufficient solubility in aqueous conditions was achieved at pH 10.5, which was chosen to avoid any reduction of the electrophoretic mobilities (especially for Z1) upon adding organic solvents to the BGE. Therefore, the three selected CZE conditions were: CZE Method 1 (CZE 1): 60/40 v/v 10 mM ammonium formate, pH 3.5/organic, CZE Method 2 (CZE 2): 60/40 v/v 10 mM ammonium acetate pH 7.0/organic, and CZE Method 3 (CZE 3): 10 mM piperidine, pH 10.5. The organic solvent was a mixture of 50/50 v/v MeOH/MeCN. All of the drug candidates and their process-related impurities were analysed individually using these three CZE methods and the complementary nature of the methods was assessed using orthogonality studies.

3.2 Orthogonality assessment

3.2.1 CZE *vs.* CZE orthogonality

A normalised migration index plot was used to display the correlation between the three CZE separation methods and orthogonality was quantified using the factor analysis and informational theory methods. CZE *vs.* CZE orthogonality plots are presented in Fig. 4 and orthogonality values in Table 3. Theoretically, separations can be truly orthogonal ($C = 0$, $\beta = 90°$, RP = 100%, similarity = 0, and synentropy = 0%) or truly correlated ($C = 1$, $\nu = 0°$, RP = 0%, similarity = 1, and synentropy = 100%), but in practice most of the separations lie between these two extremes [21]. The results (Table 3) indicate relatively modest degrees of orthogonality between each of the CZE methods, as expected with purely electrophoretic separation mechanisms. However, the potential utility of the approach was evident despite the small changes in selectivity observed. For example, the zwitterion Z2 and its impurity were only resolved using CZE 3, whilst the base B1 and its impurities were best separated using CZE 1, thereby indicating complementary methods (Fig. 4). Generally, CZE 1 was capable of separating basic compounds, whilst CZE 2 preferentially separated the strong from the weak bases and the strong from the weak acids. CZE 3 preferentially separated acidic compounds.

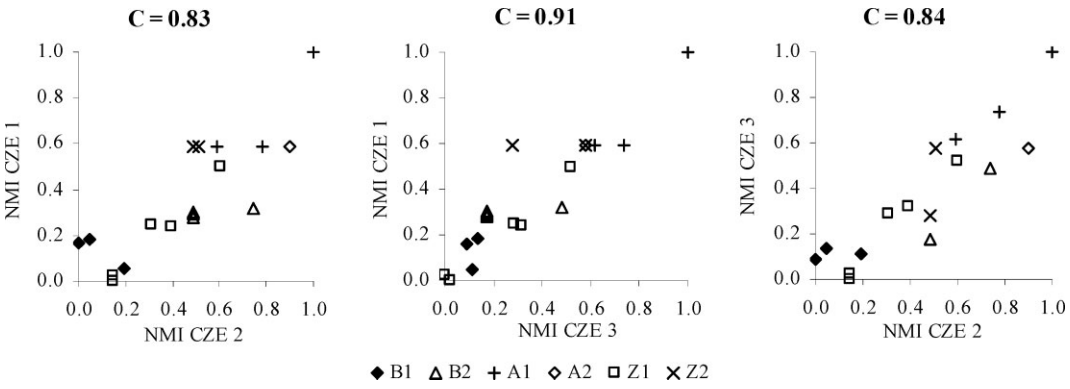

Figure 4. CZE *vs.* CZE orthogonality plots of drug candidates and impurities. CZE 1: 60/40 v/v ammonium formate, pH 3.5/organic; CZE 2: 10 mM ammonium acetate, pH 7.0/organic; CZE 3: 10 mM piperidine, pH 10.5. The organic solvent was 50/50 v/v MeOH/MeCN.

Table 3. CZE *vs.* CZE orthogonality

Attribute	CZE 1/ CZE 2	CZE 1/ CZE 3	CZE 2/ CZE 3
Correlation (*C*)	0.83	0.91	0.84
Peak spreading angle (β)	34°	24°	33°
Theoretical peak capacity (N_t)	3804	5669	2669
Practical peak capacity (N_p)	1893	2065	1258
Resolving power (RP)	50%	36%	47%
Similarity	0.94	0.97	0.99
Synentropy	79%	90%	91%

The peak capacities obtained (indicating the number of peaks which can fit in a separation window whilst maintaining a resolution of 1.0 [38]) were in the range of typical CZE separations: 90 for CZE 1, 42 for CZE 2, and 63 for CZE 3. Peak capacities in HPLC are in the range of 10–40, whilst higher values are usually obtained in capillary electromigration techniques due to the improved efficiencies that can be achieved. The practical peak capacities are lower than the theoretical values, since the correlation of the methods (due to the random rather than even distribution of peaks) reduces the separation space [39]. It should also be noted that the base B2 and its impurities, all hydrophobic compounds with similar structures, showed considerable overlap in their separation windows in all the CZE methods. In addition, the impurity of the acid A2 did not migrate under any CZE conditions, which was attributed to its poor solubility in the BGE solutions. This indicates that, while the three CZE methodologies are applicable for most compounds and are able to provide resolution of potential impurities, some "difficult" compounds are always likely to fall outside the chosen methods. Hence, we wished to examine the possibility of an improved degree of orthogonality for these sets of analytes if, as would be predicted by their different separation mechanisms, CZE and HPLC were chosen as analytical methods.

3.2.2 HPLC *vs.* HPLC orthogonality

The HPLC component of the development of this generic analytical strategy was achieved in previous studies [19] and it is beyond the scope of this paper to discuss the results in details. Briefly, HPLC *vs.* HPLC methods showed improved orthogonality compared to CZE *vs.* CZE systems ($0.26 < C < 0.91$ and $0.83 < C < 0.91$, respectively). This was expected since the BGE was the sole variable in the CZE methods, as opposed to the mobile and stationary phases being varied in the HPLC methods. Gray *et al.* have reported similar HPLC results for the evaluation of comprehensive 2-D systems, with *C* values being comprised between 0.26 and 0.99, and between 0.45 and 0.64 for the separation of 32 isomers [3] and 58 oligostyrenes [17], respectively. In this study, the possibility of an improved degree of orthogonality for this set of analytes using CZE and HPLC selected methods was examined.

3.2.3 CZE *vs.* HPLC orthogonality

Orthogonality results between the four HPLC and the three CZE methods are presented in Table 4 and Fig. 5. HPLC *vs.* CZE systems ($0.09 < C < 0.90$) provided improved orthogonality between each of the single dimensions than CZE *vs.* CZE ($0.83 < C < 0.91$) or HPLC *vs.* HPLC ($0.25 < C < 0.91$) systems based on the calculated correlation coefficient (*C*). Given that RP-HPLC and CZE have two mechanistically different separation processes (*i.e.*, partitioning *vs.* electrophoresis), it was expected that a greater orthogonality would be obtained when comparing methodologies using these two techniques than those using the same technique. This was further demonstrated with the comparison of correlation values of CZE and HPLC methods using similar BGE/mobile phase pHs. For example, CZE 1 (pH 3.5) showed its lowest correlation ($C = 0.29$) with LC 2 (pH 3.0), and its highest correlation ($C = 0.78$) with LC 4 (pH 5.8). These results confirmed the methods were orthogonal due to their different separation mechanisms.

The assessment of orthogonality obtained by the informational theory approach [6] were not consistent with the other approaches used. For example, although there were excellent correlations across all the CZE *vs.* LC pairs

Table 4. CZE *vs.* HPLC orthogonality

Attribute	CZE1 / LC1	CZE1 / LC2	CZE1 / LC3	CZE1 / LC4	CZE2 / LC1	CZE2 / LC2
C	0.52	0.29	0.52	0.78	0.60	0.25
β	59°	73°	59°	39°	53°	75°
N_t	2865	4449	4204	2667	1349	2095
N_p	2149	3817	3096	1542	906	1825
RP	75%	86%	74%	58%	67%	87%
Similarity	0.95	0.93	0.95	0.97	0.96	0.94
Synentropy	30%	46%	69%	39%	23%	64%
Attribute	CZE2 / LC3	CZE2 / LC4	CZE3 / LC1	CZE3 / LC2	CZE3 / LC3	CZE3 / LC4
C	0.44	0.65	0.75	0.09	0.33	0.90
β	64°	50°	42°	85°	71°	26°
N_t	1980	1256	2010	3122	2950	1871
N_p	1526	804	1162	2983	2454	761
RP	77%	64%	58%	96%	83%	41%
Similarity	0.96	0.97	0.97	0.96	0.97	0.98
Synentropy	57%	44%	20%	36%	46%	42%

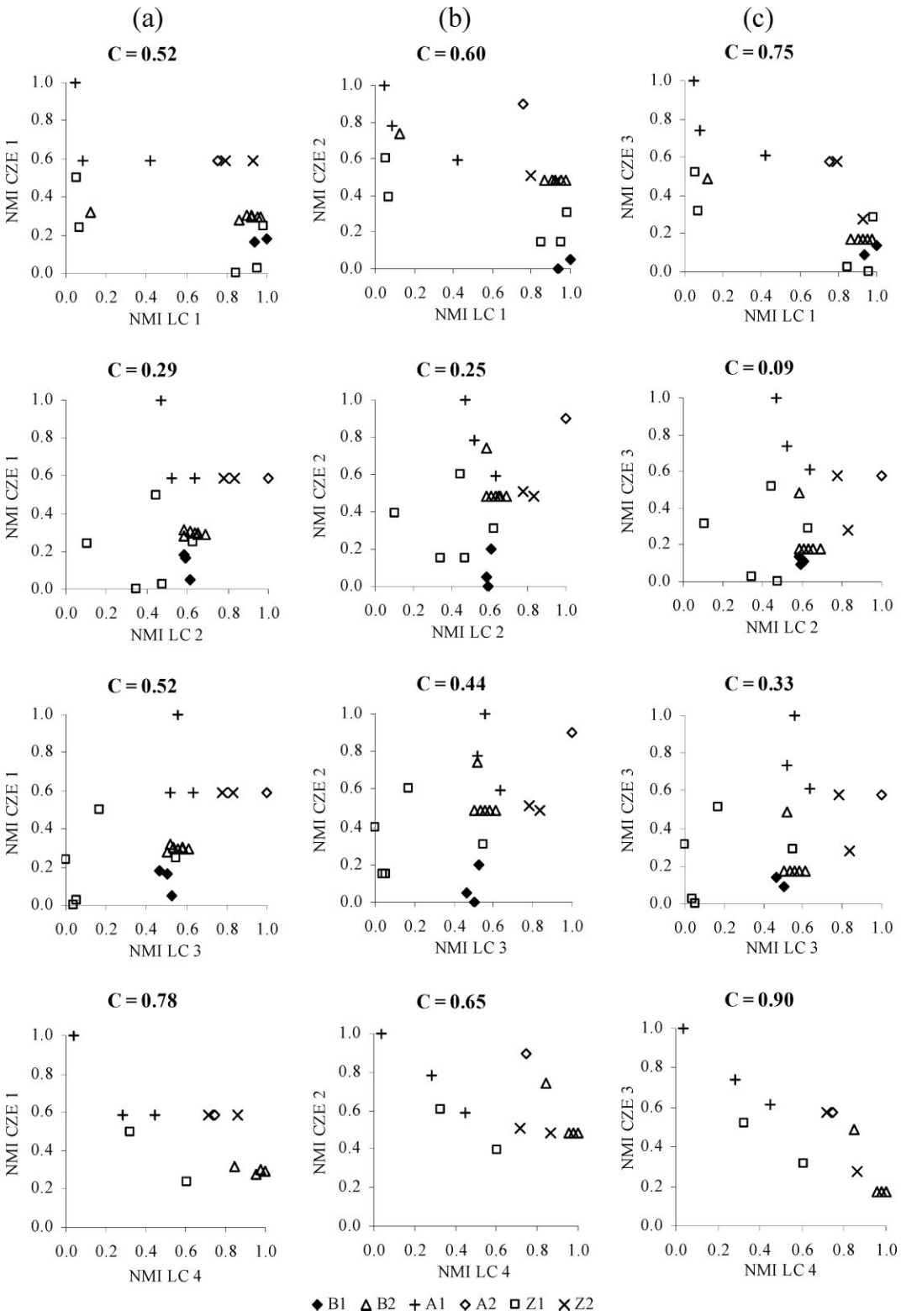

Figure 5. CZE *vs.* HPLC orthogonality plots of drug candidates and impurities. (a) CZE 1 *vs.* HPLC, (b) CZE 2 *vs.* HPLC, (c) CZE 3 *vs.* HPLC; LC 1: phenyl-hexyl, 10 mM ammonium acetate, pH 6.8/MeCN; LC 2: C_{18}, 10 mM ammonium trifluoroacetate, pH 3.0/MeCN; LC 3: aqua C_{18}, 0.1% v/v formic acid, pH 2.6/MeCN; LC 4: cyano-propyl, 10 mM ammonium acetate, pH 5.8/MeCN. CZE methods as stated in Fig. 4.

($R^2 > 0.96$) between correlation coefficient (*C*) and either peak spreading angle (β) or resolving power (RP), there was very poor correlation ($R^2 < 0.39$) between *C* and the indices of synentropy or similarity. The validity of our calculations of synentropy and similarity was checked by using one worked example given by Massart and Kaufman [22] and our results were in agreement with those published. Hence, in this study, the informational orthogonality method did not enable an adequate representation of data scatter on the 2-D plots, and factor analysis was a better approach to quantify the orthogonality of two methods.

The results also indicated that HPLC retention was decreasingly correlated with CZE migration in the following order: cyano-propyl, pH 5.8; phenyl-hexyl, pH 6.8; aqua C_{18}, pH 2.6; and C_{18}, pH 3.0; this order being observed for the correlation with all three CZE methods. The order of increasing hydrophobicity of the stationary phase of the HPLC columns is: cyano-propyl, phenyl-hexyl, aqua C_{18}, C_{18}, which corresponds to the order of decreasing CZE *vs.* HPLC correlation and hence increasing orthogonality. This suggests that any decrease in hydrophobicity of the HPLC stationary phase tends to a give a separation order more comparable with that observed in CZE. With a more hydrophilic stationary phase, it would be expected that adsorption or hydrogen-bonding type mechanisms may be primarily responsible for retention but it is difficult to see how such mechanisms would correlate with the electrophoretic separation mechanism of CZE.

It is also worthy of note that the base B1 and its two process-related impurities, as well as three impurities of the zwitterion Z1, did not elute using LC 4. Generally, mixture B1 was best analysed by CZE compared to HPLC, as can be predicted when analysing relatively strong bases. Nevertheless, the impurity of the acid A2, which was not detected using any of the CZE methods, eluted under the four HPLC conditions. In addition, the base B2 and its impurities, which showed poor resolution in CZE, were best separated by HPLC (but still not baseline-resolved). This emphasises that the developed CZE and HPLC methods are orthogonal and complementary, thereby improving the mapping of impurity profiles of the selected drug candidates.

3.3 CZE-ESI-MS analysis of test mixtures

In order to assess the compatibility of the three selected CZE-UV methods with ESI-MS, each drug candidates and their process-related impurities were analysed as test mixtures by CZE-ESI-MS. All the drug candidates and their process-related impurities (except the impurity of A2 which was not soluble under any CZE conditions) were identified, despite some of these not being fully resolved. An example of separation and identification is shown in Fig. 6. The successful coupling of CZE with ESI-MS confirmed the generic applicability of our selected CZE methods, as well as the high selectivity and versatility of ESI-MS compared to UV detection. This study complements previous work demonstrating that CZE-ESI-MS is a complementary or alternative technique to chromatography for impurity profiling of drugs. For example, Pluym *et al.* [40] have reported the full profiling of domperidone impurities using a combination of CZE and HPLC, whereas the individual methods did not resolve all the impurities. Similarly in our studies, CZE methods best separated polar analytes (such as mixture B1), while HPLC methods best resolved hydrophobic compounds (such as mixture B2).

4 Concluding remarks

CZE-ESI-MS analysis using the selected methods allowed the identification of almost all the drug candidates and their process-related impurities from a set of typical pharmaceutical compounds, despite some of these not being fully resolved. The successful coupling of CZE with ESI-MS enabled detailed structural information to be recorded and confirmed the generic applicability of our CZE methods. This study also confirmed the utility of CZE-ESI-MS as an analytical technique for the impurity profiling of drugs. The type of 2-D orthogonality studies described here, which compares the separations of the same analytes under two different but standardised conditions, was used to predict the complementarities of the methods. For our set of compounds, factor analysis was a more suitable approach for orthogonality quantification than using informational theory. It was demonstrated that the electrophoretic separation mechanism underlying CZE provided highly orthogonal separations of the test compounds when compared to HPLC. Three CZE-ESI-MS methods were selected to complement the established HPLC-MS methods, which together form the basis of a generic approach to impurity profiling of pharmaceutical drug candidates. These methods can be applied with no or minimal analytical method development, thereby allowing the profiling of large numbers of organic impurities related to pharmaceutical compounds in limited numbers of analyses. This generic strategy offers significant time and resource savings and will speed up the process of pharmaceutical analysis, while maximising patients' safety. An important further advance would be to evaluate a series of additional separation techniques, including gas chromatography, supercritical fluid chromatography, nonaqueous capillary electrophoresis, and capillary electrochromatography, in order to assess whether it is possible to extend the versatility of the approach.

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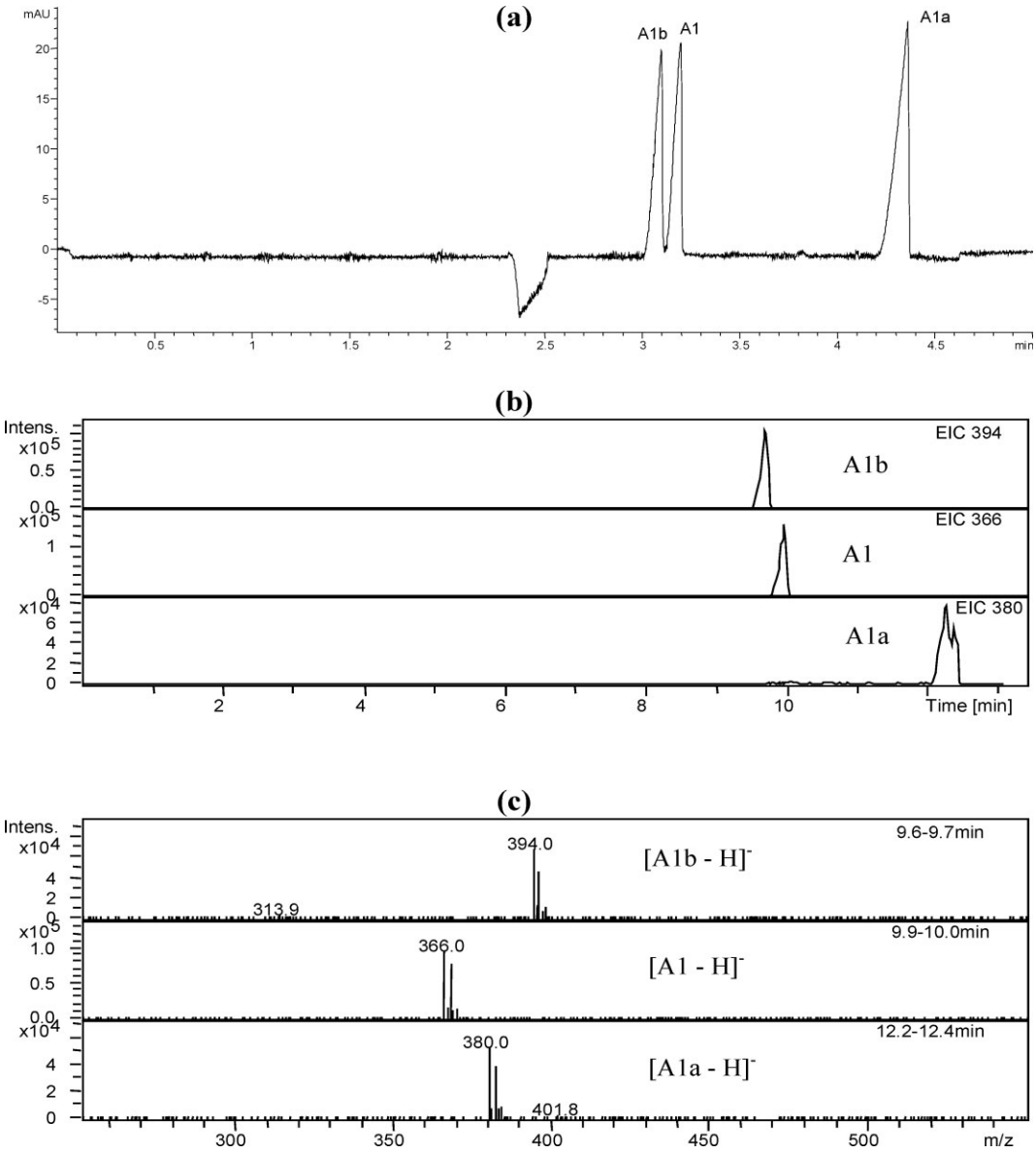

Figure 6. CZE-ESI-MS analysis of A1 and its impurities. (a) CZE-UV electropherogram, (b) CZE-ESI-MS extracted ion electropherograms, (c) ESI mass spectra. BGE, 10 mM piperidine, pH 10.5; sheath liquid, 10 mM ammonium acetate in 50/50 v/v MeOH/H_2O. Other conditions as stated in Fig. 2.

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