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Development and validation of a capillary electrophoresis method for ximelagatran assay and related substance determination in drug substance and tablets

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday.

Abstract

The advantages of simplicity, selectivity, versatility and ease of use of free solution capillary electrophoresis (CE) present an orthogonal and complementary separation technique to the established methods of liquid chromatography (LC) for pharmaceutical analysis. This work presents the development and performance of a suitable CE method for ximelagatran (formerly H 376/95) assay and related substance determination in both drug substance and tablets. The method employed was a low pH phosphate buffer, to which acetonitrile and hydroxypropyl-β-cyclodextrin were added, in order to facilitate the separation of ximelagatran and its related substances. An applied field of 350 V/cm was used and all compounds were resolved in ~20 min. Benzamidine hydrochloride was used as an internal standard in quantification. The data indicate that the performance of the validated method offers equivalent and complementary information, in terms of selectivity, sensitivity, accuracy, linearity and precision, to that of an established gradient LC method employed for similar purposes. Robustness of the method was investigated by experimental design and evaluated using multivariate calculations.

Keywords: Capillary electrophoresis; CE; Validation; Experimental design; Ximelagatran; Thrombin inhibitor; H 376/95

1. Introduction

Capillary electrophoresis (CE) is now a mature separation technique with free solution CE (FSCE) and micellar electrokinetic chromatography (MEKC), recognised as feasible and practical complementary alternatives to liquid chromatography (LC) for pharmaceutical and biological applications [1–6]. In addition to this, CE methods are now accepted in law courts, forensic analysis laboratories and pollution monitoring agencies [4] and recently, a general test chapter on CE has been added to both the US Pharmacopeia [7] and European Pharmacopoeia [8]. The acceptance of this technique within industry and regulated environments has arisen from the large number of

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publications indicating its general applicability for a wide range of analytes and equally important, the introduction of commercial instrumentation.

A number of electrophoretic separation methods have been developed and adopted within our laboratories for a diverse range of applications, ranging from inorganic ion quantification to separations of complex high molecular weight molecules, including the use of CE for the determination of physico-chemical parameters. Although LC is still the most widely employed separation tool for the majority of our pharmaceutical applications, we now consider electrophoretic methods, particularly FSCE as a result of its inherent simplicity, early in the development process with a view to choosing the most suitable technique at a later stage. Time and resources allowing, it can also be beneficial to apply both techniques in parallel, since they offer orthogonal and thus complementary mechanisms of separation, hence the characterisation of the drug substance or product gains another dimension which is important in its development. In most cases, however, the LC method will eventually be run in preference, since the separations achieved are adequate for the purpose, it offers a greater degree of robustness and analysts feel comfortable with its application. In some cases, however, a CE method may indicate clear advantages, very often selectivity, in which case it may be chosen as the method of choice. In this paper, a short description of a simple and selective FSCE method is presented for assay of a new direct oral thrombin inhibitor, ximelagatran (formerly H 376/95) (Fig. 1) [9,10] in drug substance and tablets. The same method is also used to separate and quantify known related substances and has been validated for these applications where the criteria applied are similar to those for assessing LC methods, as described previously [11,12].

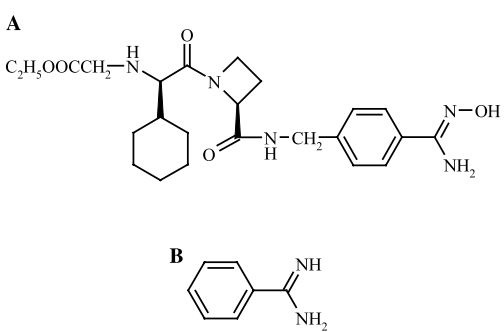

Fig. 1. The chemical structures of (a) the direct oral thrombin inhibitor, ximelagatran and (b) benzamidine, the internal standard used in these studies.

2. Experimental

2.1. Chemicals and reagents

Fused silica capillaries were purchased either as a 100 m roll from Polymicro Technologies Inc. (Phoenix, AZ) or ready to use from Agilent Technologies (Waldbronn, Germany). Sodium dihydrogen phosphate was purchased from Merck KgaA (Darmstadt, Germany). Ortho-phosphoric acid (85%) was purchased from Scharlau Chemie SA (Barcelona, Spain). Hydroxypropyl-β-cyclodextrin (HP-β-CD) and benzamidine hydrochloride (hydrate) internal standard were purchased from Sigma-Aldrich Sweden AB (Stockholm, Sweden). A second HP-β-CD was used during robustness testing and was purchased from Beckman Instruments Inc. (California). Sodium hydroxide (0.1 N) was purchased from Agilent Technologies (Waldbronn, Germany). The direct oral thrombin inhibitor, ximelagatran drug substance and tablets were from AstraZeneca R&D Mölndal (Mölndal, Sweden). Acetonitrile (MeCN) used throughout the study was of HPLC grade. Deionised water (18.2 MΩ) used throughout the study was taken from an Elga Maxima water purification system (High Wycombe, UK).

2.2. Instrumentation

Capillary electrophoresis was carried out using the Agilent Technologies ^{3D}CE system (Agilent Technologies). Data acquisition and signal processing were performed using the Agilent Technologies ^{3D}CE ChemStation (Rev. A.06.03, Agilent Technologies). GHP Acrodisc 0.45 μm filters were purchased from Pall Gelman Sciences (Ann Arbour, MI).

Table 1
FSCE method parameters for ximelagatran substance and product analysis

Capillary	Uncoated fused silica 75 μm × 56.0 cm (total length 64.5 cm)
Detection	230 ± 4 nm (reference wavelength at 350 ± 40 nm)
Temperature	Cassette set to 22 °C
Rise time	1.0 s
Background electrolyte (BGE)	MeCN/100 mM NaH_2PO_4 (pH 1.9):(22:78, v/v) containing 11 mM HP-β-CD (DS = 0.6, Rmm ≈ 1380)
Capillary preconditioning	1 min pressure with 0.1 M NaOH, 1 min pressure with BGE and 2 min voltage (206 V/cm)
Injection 1	Hydrodynamic injection of sample 10 mbar for 15 s (25 nl)
Injection 2	Hydrodynamic injection of electrolyte 5 mbar for 2.5 s (replenishment of this vial after each injection is preferred)
Voltage	350 V/cm applied over 6 s
Typical current	~115 μA
Replenishment	Replenishment of both separation vials and injection 2 vial is preferred after each injection
Capillary preconditioning (*new capillary only*)	Pressure flush with 0.1 M NaOH for 30 min followed by water for 30 min

2.3. Method conditions

The optimised method described in Table 1 is essentially a low pH phosphate buffer with additional amounts of organic modifier and cyclodextrin additives to fine tune selectivity, similar to that described previously for a range of basic drugs [13]. The primary aim when setting this method was that of simplicity, thus complex BGE and/or instrumental parameters were avoided where possible. The phosphate buffer was therefore prepared by combining 1 M NaH_2PO_4, 1M H_3PO_4 and water in a 10:15:75 v/v/v ratio which, when measured, was found consistently to be pH 1.9. A MeCN/phosphate buffer solution was then mixed in a 22:78 v/v ratio and used to prepare a 11 mM HP-β-CD solution which was used as BGE. The use of benzamidine (Fig. 1) internal standard, to increase injection-to-injection peak area and migration time precision [14], was chosen due to its similar structure to that of ximelagatran. This had a 2-fold advantage, namely (a) it had similar UV properties and (b) it had similar electrophoretic mobility characteristics to that of the main component. Moreover, it can be obtained from a commercial source with high purity and at a low cost. The method was designed to provide analytical data on both assay and related substance determination for both drug substance and proposed drug product (12, 24 and 36 mg tablets) and the following points were considered for validation: selectivity, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD), freedom from sample matrix interference, robustness and stability of analytical solutions.

Although not the primary method for confirming the identity of pharmaceutical substance in tablets, this CE method can also be applied. This may be useful when identity, assay and related substance determinations are required simultaneously (as is often the case), thus avoiding any additional spectroscopic determination. Positive identity of ximelagatran can be confirmed when the ratio between relative migration time (RMT) in the reference standard and ximelagatran in either a substance or tablet sample solution is 1.00 ± 0.02 in addition to the spectra being identical.

2.4. System suitability and sample preparation

System suitability testing is an integral part of any analytical method and is usually performed prior to any samples examined. Guidelines pertaining to setting and the implementation of appropriate system suitability tests (SSTs) for CE methods, based on those applied in LC, have been presented and applied [11]. The SST parameters for the ximelagatran assay method were thus as follows: migration time for ximelagatran and benzamidine should be 11–12 and 8.5–9.5 min, respectively; standard correlation test for two

standards should be 1.000 ± 0.015; R.S.D. values (at least $n \geq 6$) for RMT and relative corrected peak area (RCA) [15] of a standard solution should be $\leqslant 1.5$ and 1.5%, respectively. An additional point regarding the resolution ($= 1.8$) between a related substance peak and the ximelagatran peak was added in the related substance method. Furthermore, an indication of the expected current generated from the method is also a good inherent SST [12]. Normalisation of peak areas for migration time [15] is used throughout and thus calculations are performed using corrected peak areas (CPAs). For related substances, the corrected peak area percent (CPA%) of the total peak area in the electropherogram (not including the internal standard) is reported.

Sample concentration was chosen so that sufficient sensitivity was possible to allow adequate quantification of all related substances at the levels required by the regulatory bodies. This process was found to be a trade off with sample solubility and peak shape. Peak sensitivity was further facilitated, however, through sample stacking [16] by employing a sample solvent of lower conductivity to that of the BGE, which also improved peak shape. To obtain a final concentration of 1.45 mg/ml of ximelagatran, the reference standard, drug substance or tablets were dissolved initially in 10% of the final sample volume using 0.1 M HCl to which the benzamidine internal standard was added (final concentration 1.0 mg/ml) and diluted to volume with water. All samples were filtered through a 0.45 μm membrane.

3. Results and discussion

3.1. Method development

Since ximelagatran and its related substances are basic molecules, a low pH phosphate buffer electrolyte without any additives was initially evaluated and yielded good success. Separation was not achieved for all known related substances, however, until a small optimisation procedure was undertaken. The effect of additional organic modifier type and concentration to the above low pH electrolyte was investigated and the addition of hydroxypropyl-β-cyclodextrin to fine tune even further the selectivity was employed. Having obtained suitable separation conditions, the final step was to optimise additional instrumental parameters, including capillary length, applied voltage, analytical wavelength and rise time, temperature and injection parameters, to yield the method described in Table 1. During this stage of development, it was demonstrated that both voltage pre-conditioning of the capillary prior to separation [17] and an injection of a small electrolyte plug subsequent to injection of sample [18] improved the precision and accuracy of the method. Although the use of 200 nm detection is widely recommended and used in published methods [13], 230 nm was used in preference, in order that data could be compared with those obtained with the existing LC methods. During CE method development within our laboratories, power (current $\times$ applied field/capillary length) is always evaluated in order that final methods have a value approximating that of the recommended 1.0 W/m [19]. Although typical values for system current observed for this method were ≈ 115 μA, which translate to ≈ 4.0 W/m, no adverse effects were observed and acceptable reproducibility was obtained (shown below). An Ohm's law plot [20] was calculated and shown to be linear, although starting to deviate, at the applied electric field (350 V/cm). Lowering the voltage, using a longer capillary or a smaller ID capillary were not considered an option, since these would have resulted in significantly longer analysis times and lower detection limits, respectively. It has been demonstrated, however, that acceptable CE can be accomplished at very high power values, 7 W/m [21], if buffer depletion issues are considered, as they are in this method.

3.2. Sample preparation

The CE sample preparation for both substance and tablets are similar. The former is simply dissolved in 10% of the final volume with 0.1 M hydrochloric acid to a final concentration of 1.45 mg/ml and placed in an ultrasonication bath for 15 min. The tablets are similarly prepared at the

same concentration, but placed on a shaking table for 30 min. The internal standard is then added from a stock solution (5 mg/ml) to yield a final concentration of 1.0 mg/ml and the solution made to volume with water. A comparison of this sample preparation was carried out to that of a validated LC method in order to verify its accuracy using 10, 12, 20, 24 and 40 mg immediate release (IR) tablets (in duplicate). The results in Table 2 show that the variance between LC and CE sample preparation methods is $<2\%$ and considered acceptable for assay and no significant difference observed in the related substances quantified. The nominal differences in related substance values between the 10, 20 and 40 mg and the 12 and 24 mg tablets, outlined in Table 2, can be attributed to improvements/changes in drug substance purity during the development process.

3.3. Selectivity

These terms are often used interchangeably, although they are, in principle, not identical, which often leads to confusion [22]. The method indicates a significant degree of selectivity, since the main peak is well separated from all seven known related substances that may (or may not) be expected to be present, shown in Fig. 2. Migration order confirmation for each known related substance was carried out by analysing spiked standard solutions and additionally, by analysing standards of each individually. Detection was carried out at 230 nm and all related substances, with one exception, had response factors within an acceptable range (0.8–1.2) to allow direct quantitation (CPA%) of each individual related substance. As mentioned above, one related substance had a value outside the acceptable range, but was not considered a problem since (a) the value was higher and thus its CPA% value was effectively overestimated and (b) it resulted from a synthetic precursor that was shown never to increase with time and essentially remained constant.

3.4. Linearity

A calibration curve for ximelagatran assay was prepared using a placebo tablet solution with the addition of different volumes of a standard stock solution to obtain 50, 75, 100, 125 and 150% of the intended concentration (1.45 mg/ml). A calibration curve for the internal standard was similarly prepared to contain 50, 75, 100, 125 and 150% of the intended concentration of benzamidine (1.0 mg/ml). The correlation between the concentration of substance with corrected peak area for both ximelagatran and the internal standard benzamidine was very good (Table 3). Based on these linearity data, standard solutions prepared at a single concentration are considered sufficient for accurate quantification. A study was also carried out to determine the linearity of related substances by examining solutions of each between a concentration approximating the limit of quantification (LOQ) up to 200% of the concentration which is permitted for each in the specifications (Table 3).

Table 2
Comparison of assay and related substances data for five ximelagatran tablet strengths obtained using the CE method described herein and a validated 'in-house' LC method

Tablet strength (mg)	LC assay (%)	CE assay (%)	Assay difference (%)	LC related substances (%)	CE related substances (%)
10	97.3	97.6	0.3	2.82	2.53
12	94.2	93.6	0.6	0.63	0.58
20	99.3	97.6	1.7	2.72	2.50
24	97.9	98.9	1.0	0.68	0.64
40	100.0	100.3	0.3	1.88	1.60

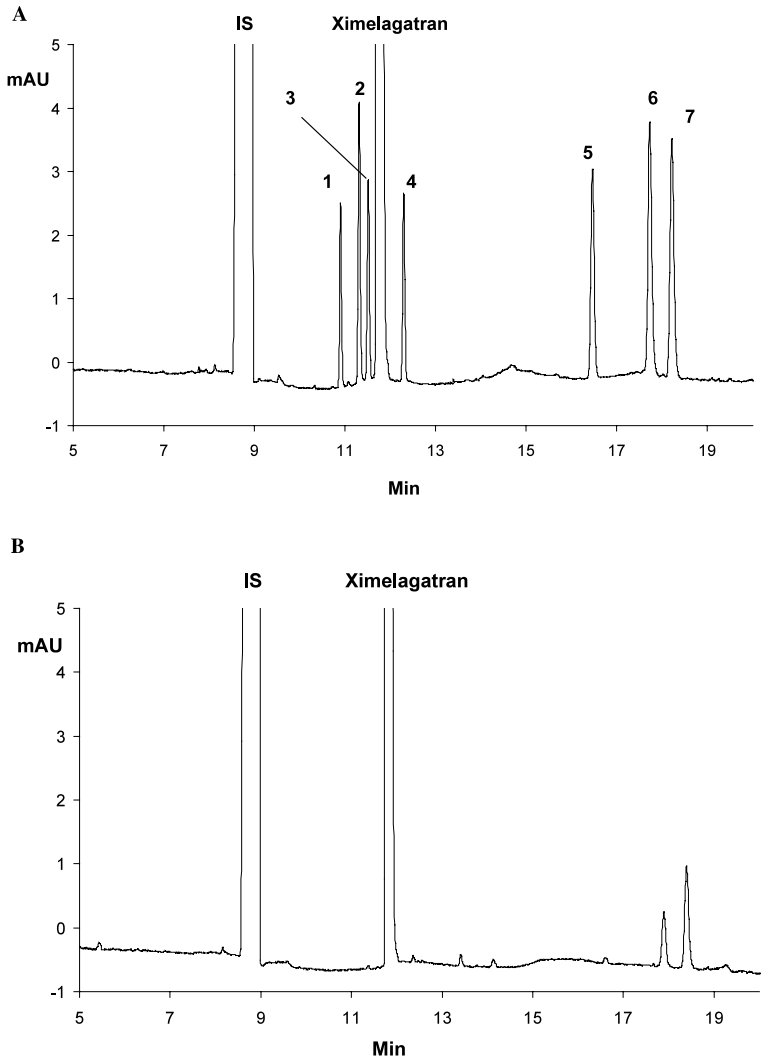

Fig. 2. CE separation of (a) a ximelagatran solution, where the main peak and the internal standard benzamidine are present at 1.45 and 1.0 mg/ml, respectively and the seven known related substances are spiked at ≈ 0.6 CPA% and (b) an analysis of 24 mg ximelagatran tablets. Conditions are as outlined in Table 1.

3.5. Accuracy

Accuracy data were obtained by performing a comparison of data from this CE method with data from the 'in-house' validated LC method that was shown to be accurate through recovery studies conducted at 80, 100 and 120% of the label claim. The data in Table 2 was thus evaluated and considered to determine the accuracy of the method. These data show that the percentage difference between the validated LC method and CE method is $< 2\%$ for assay and that there is good agreement between the two methods with respect to related substances. A larger difference between the LC and CE methods for assay of 20 mg tablets was observed, however, which is difficult to explain, but thought not to adversely effect the overall conclusions, since no trend is observed and results at either side of this concentration are acceptable.

3.6. Repeatability and intermediate precision

Precision was determined in two steps, namely the repeatability and the intermediate precision. Repeatability was determined by assessing R.S.D. in percent of (a) assay of 24 mg IR tablet preparations ($n = 8$) and (b) assay of ximelagatran in drug substance at 100% of the test concentration ($n = 8$). In addition to these, RMT and RCA were also used to assess repeatability. As shown in Table 4, the repeatability of the method for assay of both tablet and substance is acceptable. Intermediate precision was examined and assessed similarly to that of repeatability but by two analysts, on 3 consecutive days, using two different instruments, in two separate laboratories and shown to be acceptable (Table 5). It was difficult to rationalise the unusually high R.S.D. value for RCA of substance assay (3.1%), but is believed to be dependant on one low value, that of analyst B on day two. One cannot ignore this result, since this is the nature of the experiment, but if R.S.D. values were recalculated without this value it would agree to a greater extent with the corresponding value for tablets (1.5 and 1.1%, respectively).

3.7. Limit of quantitation (LOQ) and limit of detection (LOD)

LOQ and LOD values were estimated by preparing a solution with the seven related substances at a concentration approximately equivalent to 0.05 CPA% at the working concentration

Table 3
Linearity data obtained for ximelagatran, the internal standard benzamidine and all known related substances

Substance	Conc. range (mg/ml)	Cross correlation coefficient (R^2)	Slope	Intercept	Intercept at 95% C.I.
Ximelagatran	0.725–2.175	0.9997	1.5897	−0.0311	−0.08–0.07
Benzamidine	0.50–1.50	0.9994	3.2581	0.0104	−0.13–0.16
1	0.00074–0.015	0.9997	1.105	−0.00002	−0.0003–0.0004
2	0.00074–0.015	0.9996	1.145	−0.0001	−0.0006–0.0005
3	0.00077–0.015	0.9998	1.300	0.0002	−0.0004–0.0006
4	0.00071–0.014	0.9999	1.561	0.0001	−0.0004–0.0006
5	0.00074–0.092	0.9992	1.102	0.0006	−0.0017–0.0019
6	0.00072–0.014	0.9999	2.016	0.0003	−0.0002–0.0008
7	0.00072–0.029	0.9999	1.407	0.0001	−0.0005–0.0006

Table 4
Repeatability obtained using the CE method for ximelagatran substance and tablets

Analysis No.	Assay of ximelagatran substance			Assay of ximelagatran 24 mg IR tablets		
	RMT	RCA	Assay (%)	RMT	RCA	Label claim (%)
1	1.343	0.742	99.72	1.315	0.641	94.97
2	1.341	0.741	99.62	1.317	0.643	95.27
3	1.342	0.740	99.43	1.317	0.644	95.49
4	1.340	0.753	101.23	1.314	0.645	95.59
5	1.342	0.739	99.28	1.315	0.644	95.48
6	1.340	0.739	99.32	1.311	0.644	95.40
7	1.341	0.735	98.79	1.316	0.643	95.36
8	1.337	0.731	98.32	1.318	0.645	95.66
Mean	1.341	0.740	99.46	1.315	0.644	95.40
R.S.D. (%)	0.131	0.851	0.85	0.167	0.202	0.225

Table 5
Intermediate precision obtained using the CE method for ximelagatran substance and tablets

Analyst	Inst.	Day	Assay of ximelagatran substance			Assay of ximelagatran 24 mg IR tablets		
			RMT	RCA	Assay (%)	RMT	RCA	Label claim (%)
A	1	1	1.342	0.734	98.78	1.309	0.667	96.71
B	2	1	1.335	0.735	99.02	1.321	0.644	95.40
A	2	2	1.333	0.736	97.14	1.332	0.650	96.06
B	1	2	1.333	0.695	100.00	1.327	0.653	97.11
A	1	3	1.314	0.763	98.78	1.327	0.653	96.17
B	2	3	1.336	0.747	98.97	1.333	0.648	97.46
Mean			1.332	0.735	98.78	1.325	0.652	96.49
R.S.D. (%)			0.713	3.079	0.934	0.613	1.095	0.781

Table 6
Calculation of LOD and LOQ values

Related substance	Height (mAU)	Noise (mAU)	S/N	CPA (%)	LOQ (%)	LOD (%)	CLOQ (μg/ml)	CLOD (μg/ml)
1	0.224	0.023	9.72	0.041	0.043	0.013	0.62	0.19
2	0.176	0.023	7.66	0.033	0.044	0.013	0.63	0.19
3	0.272	0.023	11.81	0.048	0.040	0.012	0.59	0.18
4	0.155	0.023	6.73	0.035	0.052	0.016	0.75	0.22
5	0.218	0.023	9.46	0.040	0.042	0.013	0.61	0.18
6[a]	0.182	0.023	7.93	0.061	0.076	0.023	1.11	0.33
7	0.250	0.023	10.87	0.047	0.043	0.013	0.62	0.19

[a] Related substance 6 is not corrected for response factor and is thus overestimated.

of 1.45 mg/ml. The concentration and thus the CPA% of each related substance were confirmed using a standard injection of ximelagatran at a known concentration. The baseline noise for a time period exceeding 1 min was calculated by Agilent Technologies ChemStation software and signal to noise (S/N) values determined by dividing each peak height by the noise. LOQ and LOD values were then determined from ten and three times this value, respectively. The absolute LOQ and LOD values were thus determined to be 0.59–1.1 and 0.18–0.33 μg/ml, respectively, (Table 6). It is shown that all related substances can be quantified with certainty at 0.05 CPA%. The repeatability was additionally assessed by examining the R.S.D. values for RMT and CPA% for both substance and 24 mg IR tablets spiked with related substances at the LOQ. These data are shown in Table 7 and once again indicate the suitability of this method for determination of related substances at the required levels.

3.8. Robustness

The robustness of the method was examined using an experimental design [23]. The following parameters were examined at exaggerated levels to determine their effect on the method: MeCN content, H_3PO_4 volume content when preparing electrolyte (this influences pH), cyclodextrin concentration, cyclodextrin supplier, voltage, temperature, filtration (this was to determine if filtering the electrolyte had a positive or negative effect on the method), injection time and capillary conditioning time. These factors were assessed on the following responses which were considered appropriate for estimating their influence: the resolution between peak 2 and 3 (although the lowest and

critical separation, its value is 2.5), the resolution between peak 3 and ximelagatran, in addition to RMT for ximelagatran, RMT for peak 5 and RCA for ximelagatran. The factor levels and responses attained are shown in Table 8. The response data was first evaluated by multi-linear regression (MLR) resulting in acceptable models for both resolution responses, but poor models for the remaining responses, indicating that the changes occurring in these were only noise. Since two of the factors were qualitative, we suspected therefore that the less widely accepted partial least squares projection on latent surfaces (PLS) modelling would result in more relevant models. The PLS models showed an improvement over the MLR models, but temperature, injection time and preconditioning times only showed insignificant contributions and were therefore excluded from the final models. Regression (R^2) and cross-correlation coefficient (Q^2) values are listed in Table 8.

Resolution between the critical pair, peak 2 and peak 3, was maintained throughout the factor space around the method conditions, with MeCN and CD manufacturer being the only factors

Table 7
Repeatability (R.S.D., $n = 8$) of RMT and CPA (%) for quantification of each of the related substance spiked at the LOQ in both substance and 24 mg IR tablets

Related substance	RMT (%)	Corrected area (%)	RMT (%)	Corrected area (%)
	Substance		Spiked tablets	
1	0.16	13.4	0.14	7.4
2	0.17	12.4	0.09	10.9
3	0.17	7.8	0.08	13.5
4	0.16	9.6	0.10	3.1
5	0.17	9.4	0.13	5.7
6	0.21	6.8	0.38	2.1
7	0.21	20.6	0.28	3.6

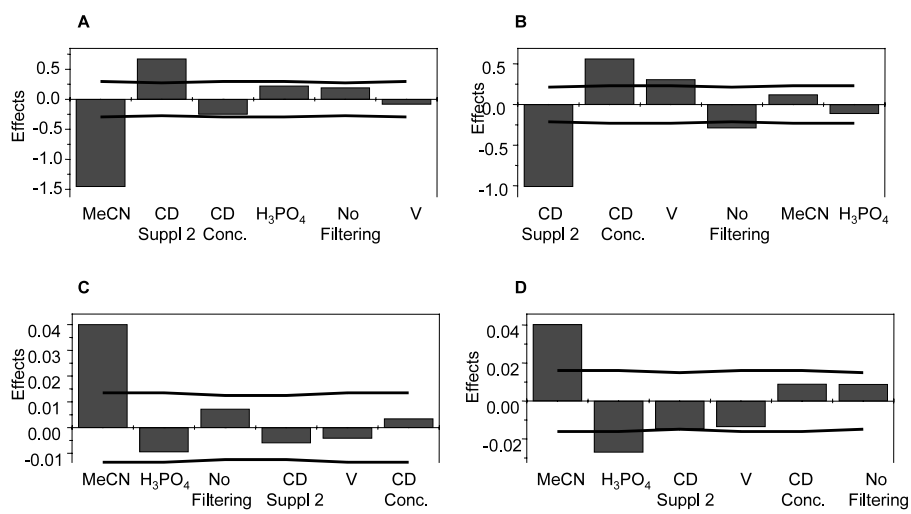

Fig. 3. Partial least squares models describing the robustness of the method against the factor space changes described in Table 8 for the responses: (a) resolution between peaks 2 and 3; (b) resolution between ximelagatran and peaks 3; (c) RMT of ximelagatran; and (d) RMT of peak 5.

Table 8
Experimental design and results obtained for the five responses evaluated during robustness testing of the method

H_3PO_4 (ml)	MeCN (%)	CD supplier	CD (mM)	Voltage (V/cm)	Temperature (°C)	Filter	Injection (s)	Cond. (s)		Rs‡	Rs*	RMT†	RMT #	RCA+
Experimental factor space										Responses evaluated				
160	20	1	10	335	20	No	15.5	3		4.20	0.93	1.76	1.30	0.65
160	24	2	12	335	20	Yes	15.5	3		1.48	2.59	1.82	1.35	0.62
140	24	1	10	365	20	Yes	14.5	3		2.14	1.48	1.81	1.34	0.63
160	20	1	12	335	24	Yes	14.5	1		3.74	1.49	1.76	1.30	0.67
160	24	2	12	365	20	No	14.5	1		1.66	2.65	1.82	1.35	0.60
160	24	1	10	365	24	Yes	15.5	1		2.26	1.47	1.78	1.33	0.62
140	24	1	12	335	24	No	14.5	3		2.01	1.57	1.84	1.36	0.61
140	20	1	12	365	20	No	15.5	1		3.76	1.65	1.77	1.30	0.66
140	20	2	12	365	24	Yes	15.5	3		2.46	3.12	1.80	1.32	0.65
160	20	2	10	365	24	No	14.5	3		3.10	1.99	1.76	1.31	0.64
140	24	2	10	335	24	No	15.5	1		1.81	2.04	1.84	1.36	0.63
140	20	2	10	335	20	Yes	14.5	1		2.82	1.91	1.80	1.31	0.65
150	22	2	11	350	22	Yes	15	2		2.40	2.74	1.79	1.32	0.66
150	22	2	11	350	22	Yes	15	2		2.49	2.70	1.77	1.30	0.63
150	22	2	11	350	22	Yes	15	2		2.43	2.79	1.80	1.32	0.64
									R^2	0.96	0.96	0.88	0.87	0.78
									Q^2	0.68	0.82	0.67	0.64	0.47

‡ Resolution between peak 2 and peak 3.
* Resolution between peak 3 and ximelagatran.
† RMT for ximelagatran.
# RMT for peak 5.
+ RCA for ximelagatran.

showing significant effects. A higher resolution was obtained when using the second cyclodextrin supplier, but resolution was still maintained throughout. The total effect of the MeCN content was a decrease of 1.5 U when changing from a low to a high amount (Fig. 3a). These changes in MeCN are quite exaggerated and would correspond theoretically to an electrolyte preparation error ten times that of what could be expected.

The resolution between peak 3 and ximelagatran was also maintained throughout the factor space around the method conditions (Fig. 3b). The CD manufacturer was once again an important factor, but here a lower resolution was obtained when using the second cyclodextrin supplier. The applied voltage and filtering showed slight effects around the 95% confidence interval, but the cyclodextrin concentration showed a significant effect of 0.6 U. These changes in cyclodextrin concentration were, similar to that of MeCN, quite exaggerated compared to the changes expected when the method was to be used routinely. The effect observed routinely would be < 0.05 U.

The specific RMTs chosen, ximelagatran and peak 5 (Fig. 3c,d, respectively), were chosen since they fully represent all others present in the electropherogram. When examined over the factor space, the RMT values for peak 5 were lower with an increase in acid concentration. This would indicate that migration for peak 5 is faster (at lower pH) relative to that of the internal standard, which implies that peak 5 obtains a greater degree of charge on lowering pH. The migration time of all peaks increased as expected with an increase in MeCN, but these changes were quite small, especially when considering the large factor space chosen and therefore considered robust with respect to migration times.

The method is equally robust to deliberate parameter changes with respect to RCA with an effect of only 0.03 U observed over the entire MeCN factor space, which is not considered a problem since the estimated error when applying the method routinely is < 0.005 U. Filtering or not filtering the electrolyte was found to have no advantageous or deleterious effect on baseline noise, respectively.

3.9. Freedom from interference

To demonstrate freedom from interference, a 24 mg ximelagatran placebo tablet was analysed under identical conditions to those described above. No peaks were observed as expected, since no excipient carries either a charge under the conditions employed or has an inherent UV absorbance at the wavelength employed.

3.10. Stability of analytical solutions

The stability of both the separation electrolyte and benzamidine internal standard solutions was examined. The electrolyte was found to be stable when stored in a tightly closed container at ambient temperature for 2 weeks. It was considered important to investigate the stability or lifetime of the electrolyte at ambient temperature so that it could be stored in the replenishment bottle. The study was carried out by examining the R.S.D. values for ximelagatran and CPA and RMT values for related substances in a system suitability test solution, which were all found to be below 1.3%. The samples were analysed prior to and after storage at relevant temperature and time intervals. The benzamidine internal standard stock solution (5 mg/ml) was found to be stable when stored in a tightly closed container at 4 °C for 2 weeks.

4. Conclusions

A simple CE method has been developed and validated for the assay of the new oral thrombin inhibitor, ximelagatran and its potential related substances. The method is applicable for both drug substance and tablets. Acceptable method performance has been demonstrated for all validation points. Potential related substances can be reliably verified and quantified at 0.05 CPA% of the bulk drug and the precision of the method for both peak migration times and areas are satisfactory. In general, the results demonstrate that CE can be applied as a complementary or alternative technique to LC for applications carried out within a pharmaceutical R&D environment.

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