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Experimental design-based development of a rapid capillary electrophoresis method for determining impurities in the tetrapeptide H-Tyr-(D)Arg-Phe-Phe-NH_2

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Abstract

A capillary electrophoresis (CE) method has been developed and validated for separating the tetrapeptide H-Tyr-(D)Arg-Phe-Phe-NH_2 and nine related substances. The method was developed using experimental design in a four-step procedure, in which eight variables were investigated in a total of 47 experiments. The preferred background electrolyte (BGE) consisted of 0.1 M malonic acid at pH 2.5 with 7 mM heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (2,6-DM-β-CD). The separation of H-Tyr-(D)Arg-Phe-Phe-NH_2 and the related substances was accomplished within 15 min, with a resolution greater than 1.5 between all peaks. The method was then investigated with respect to its selectivity, linearity, precision, detection limit (LOD) and quantitation limit (LOQ). In addition, a system suitability test was performed and response factors were determined, essentially following International Conference of Harmonization guidelines for the validation of analytical methods. LOD and LOQ for the related substance H-Arg-Phe-NH_2 were found to be 0.3 and 0.8 μg/ml, respectively, at a target H-Tyr-(D)Arg-Phe-Phe-NH_2 concentration of 1 mg/ml. The method performed well with respect to all of the validation parameters.

Keywords: H-Tyr-(D)Arg-Phe-Phe-NH_2; Tetrapeptides

1. Introduction

Peptides are involved in the control and regulation of many vital processes in all living organisms, acting (for instance) as hormones, neurotransmitters, immunomodulators, coenzyme or enzyme inhibitors, toxins and antibiotics. The pharmaceutical industry continuously strives to develop new peptide-based drugs. Consequently, advanced methods are required to separate, prepare, characterize, and determine peptides. Efficient separation techniques such as high-pressure liquid chromatography (HPLC) and capillary electrophoresis (CE) are essential for this. To date, HPLC has been the most commonly used technique, and the method of choice for routine purposes. However, despite being reliable and well-established, HPLC has certain disadvantages (for instance, it requires relatively large samples, and it is relatively time-consuming). CE is a micro-scale technique offering a selectivity process that is orthogonal to chromatography and gaining favor as a potentially viable supplement or alternative to HPLC. The most frequently quoted advantages of the CE technique are its high efficiency, short analysis time and small injection volumes. Numerous papers on CE-based peptide separation have been presented [1–7].

Optimization of CE methods generally requires a lot of experience and skill. Furthermore, it is often necessary to perform a large number of experiments to obtain successful results. The main reason for these disadvantages is that many variables are involved in CE, and these variables have quite disparate and sometimes unexpected effects on the selected responses (usually the resolution and time of the last migrating compound). However, the method development process can be significantly shortened if experimental design is applied. Altria et al. published a review on this topic nearly a decade ago [8], which still more or less reflects the current state of the art. However, several papers since then have reported on the applicability of experimental design to

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CE method development [5,9–16]; just to mention a few of these papers.

Many factors influence the separation in CE. Buffer additives may be introduced to the background electrolyte (BGE) to enhance the solubility of a determinand to improve the separation capabilities of the analytical system. Addition of organic modifiers may change the viscosity of the BGE and the solvation conditions, thereby affecting the analysis time and peak efficiency. Increasing the conductivity of the BGE also increases both the current through the capillary and Joule heating [17]. Various complexing agents (most commonly a small quantity of cyclodextrin [18]) have been added to the BGE when separating peptides by CE in order to alter the mobility of the determinands and thus improve selectivity. The degree of complexation is governed by the binding constant between the determinand and the cyclodextrin (CD). For separations of positively charged peptides with similar masses, which are done at low pH, in the range 2–4, an uncharged cyclodextrin would be a suitable choice [3,4,19].

This paper reports the CE separation of the peptide H-Tyr-(D)Arg-Phe-Phe-NH_2, some of its degradation products, and several related peptides (10 peptides in all). The overall objective was to achieve full baseline separation between all the determinands. A four-step procedure for optimizing the CE separation was adopted, involving four series of experiments. The initial set of experiments was performed to establish basic requirements. This was followed by a Plackett–Burman design for variable selection, a reduced factorial design to estimate the significance of the selected variables, and, finally, a circumscribed central composite design, including axial points, to carefully investigate the influence of the retained significant variables on the selected responses, resolution and migration time of the last eluting peak. The validation of the final method basically followed International Conference of Harmonization (ICH) guidelines [20,21].

2. Experimental

2.1. Chemicals

The peptides H-Tyr-(D)Arg-Phe-Phe-NH_2, H-Tyr-(D)Arg-Phe-Phe-OH, H-(D)Arg-Phe-Phe-OH, H-Tyr-(D)Arg-Phe-OH, H-(D)Arg-Phe-Phe-NH_2, and H-(D)Arg-Phe-OH were kindly provided by Biochem Immunosystems (Montreal, Canada). H-Tyr-(D)Arg-OH, H-Arg-Phe-NH_2, H-Phe-Phe-NH_2 and H-Phe-Phe-OH were purchased from Bachem (Bubendorf, Switzerland), while heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (2,6-DM-β-CD) and malonic acid were supplied by Sigma (St. Louis, USA). Sodium hydroxide, sodium chloride, phosphoric acid (85%), triethanolamine (TEA) and acetonitrile (ACN) were all of analytical grade (Merck, Darmstadt, Germany). Purified water was obtained from a Waters Milli-Q system (Watford, Herts, UK).

2.2. Solutions

A set of buffer solutions was prepared and tested in the concentration range 50–100 mM, covering pH values from 2.5 to 3.5. The total ionic strength was adjusted by adding NaCl. The buffer additives cyclodextrin, triethanolamine, methanol and acetonitrile were investigated in different constellations during the method development procedure. The peptide sample solution used in all experiments (if not stated otherwise) comprised 1.0 mg/ml H-Tyr-(D)Arg-Phe-Phe-NH_2 and 0.05 mg/ml of each of the nine related products dissolved in purified water (i.e. Milli-Q). All solutions were filtered through a 0.45 μm pore Millex-HV filter before use.

2.3. Instrumentation

All experiments were performed using a $HP^{3D}CE$ instrument equipped with a diode array detector and Chemstation software (version A.05.02) for data handling (Agilent Technologies, Waldbronn, Germany). The PVA-coated capillaries were also obtained from Agilent Technologies. These capillaries had an effective length (l_e) of 56.5 cm, a total length (l_t) of 64.5 cm, an inner diameter of 50 μm, and an outer diameter of 350 μm. The capillary was preconditioned prior to all runs by flushing with purified water, 0.1 M HCl, and BGE, each for 1 min. The temperature was varied in the range 15–40 °C and the applied potential between 15 and 30 kV. The sample introduction procedure entailed sequential hydrodynamic injection of sample and then BGE, by applying a pressure of 15 mbar for 5 s for each solution. Determinands were detected by monitoring UV absorbance at 200 nm (8 nm bandwidth).

The experimental design models were constructed and analyzed using Modde Software (Umetrics, Umeå, Sweden). The pK_a-values were calculated using the software ACD/pK_a DB, version 7.00 (Advanced Chemistry Development Inc., Toronto, Canada).

2.4. Choice of capillary

Peptides that are strongly basic and have similar mobility values often present considerable separation challenges. One of the most common problems is adsorption onto the capillary wall, particularly at low pH. This leads to poor efficiency, decreased resolution and asymmetric peaks. Hence, several organic additives have been used to coat the capillary wall in order to eliminate such adsorption [22]. In this study our choice fell on a permanently PVA-coated capillary that is commercially available offering a permanent, stable and reproducible surface. This type of capillary is suitable for the separation of basic peptides [22], and has previously been used for the successful enantiomeric separation of a tetrapeptide in CE [7].

2.5. Evaluation procedure

Two response functions were selected for this work, namely the resolution (R_s) and the migration time of the last eluting peak (in the following referred to as "the migration time"). The basic requirements were that these response functions should provide measures of the analysis time and baseline separation of H-Tyr-(D)Arg-Phe-Phe-NH_2 and its nine related products. The peak separation between H-Tyr-(D)Arg-Phe-Phe-NH_2 and H-(D)Arg-Phe-Phe-OH was identified as being the most critical, so the resolution, R_s, between these two peaks was chosen as one of the assessed responses throughout the investigation.

Peaks were integrated and the resolution was calculated using the Chemstation software, according to

$$R_s = 1.18 \times \frac{t_2 - t_1}{w_{1/2,1} + w_{1/2,2}} \quad (1)$$

where t_1 and t_2 are the migration times and $w_{1/2,1}$ and $w_{1/2,2}$ the peak widths at half peak heights. In this study, a resolution of at least 1.5 between two peaks is considered to give baseline separation.

2.6. Validation parameters

The purpose of validation is to establish whether or not an analytical method is acceptable for its intended purpose. The validation of this analytical method was performed according to the ICH guidelines [20,21], and the following parameters were studied: selectivity, linearity, sensitivity, accuracy, precision, and system suitability.

3. Results and discussion

The optimization procedure comprised four steps: a series of initial experiments followed by three sets of experiments based on different experimental designs.

3.1. Step 1: initial experiments

The aim of the initial experiments was to establish the basic analytical requirements of the method, such as the type of buffer and pH range of the BGE.

The pH value of the electrolyte solution is an important variable since it alters the charge of the determinands. Successful separations of basic peptides are normally achieved at low pH values [23]. According to the pK_a values presented in Table 1, the determinands have stable positive charges at pH values in the range 2–4, implying that the pH of the selected electrolyte solution should be in this range. Positively charged determinands move towards the detector in this analytical system. Three buffer systems, namely phosphoric acid/dihydrogen phosphate (pK_a 2.12), malonic acid/malonate (pK_a 2.84), and citric acid/citrate (pK_a 3.06) were tested, covering the pH range 2.5–3.5. The calculated pK_{a4} values seem to be high in comparison with the ones normally found for Arg residues in peptides.

The malonic acid/malonate buffer system at pH 2.5 proved to be the most suitable, since at least partial separation was obtained between all ten determinands. It also gave rise to the lowest current and the most stable baseline. An electropherogram obtained with 50 mM malonic acid buffer solution at pH 2.5 is shown in Fig. 1.

Consequently, this buffer was used in all subsequent experiments.

3.2. Step 2: experiments based on a Plackett–Burman design

A Plackett–Burman design was applied to identify variables that might influence the separation of H-Tyr-(D)Arg-Phe-Phe-NH_2 and its nine related substances. This screening design reduces the number of experiments required to test eight variables at two levels from 2^8 (256) to 12, and allows large amounts of information to be obtained about variables that have a significant influence on the selected response. Table 2 displays the eight variables tested here and their respective experimental domains. The

Table 1
Calculated pK_a-values for the determinands

Substance	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
H-Arg-Phe-NH_2	–	7.73 ± 0.35	–	13.4 ± 0.70
H-Tyr-Arg-OH	3.11 ± 0.22	8.32 ± 0.31	9.98 ± 0.15	13.5 ± 0.70
H-(D)Arg-Phe-OH	3.40 ± 0.10	8.40 ± 0.31	–	13.4 ± 0.70
H-(D)Arg-Phe-Phe-NH_2	–	7.73 ± 0.35	–	13.4 ± 0.70
H-Tyr-(D)Arg-Phe-OH	3.49 ± 0.10	7.62 ± 0.33	9.97 ± 0.15	13.4 ± 0.70
H-(D)Arg-Phe-Phe-OH	3.49 ± 0.10	7.74 ± 0.35	–	13.4 ± 0.70
H-Tyr-(D)Arg-Phe-Phe-NH_2	–	7.60 ± 0.33	9.96 ± 0.15	13.3 ± 0.70
H-Tyr-(D)Arg-Phe-Phe-OH	3.50 ± 0.10	7.60 ± 0.33	9.96 ± 0.15	13.3 ± 0.70
H-Phe-Phe-NH_2	–	7.69 ± 0.33	–	–
H-Phe-Phe-OH	3.41 ± 0.10	8.50 ± 0.31	–	–

pK_{a1}, pK_{a2}, pK_{a3} and pK_{a4} = pK_a values for the C-terminus carboxylic acid, the N-terminus amine, the tyrosine hydroxyl group and the arginine guanidine group, respectively.

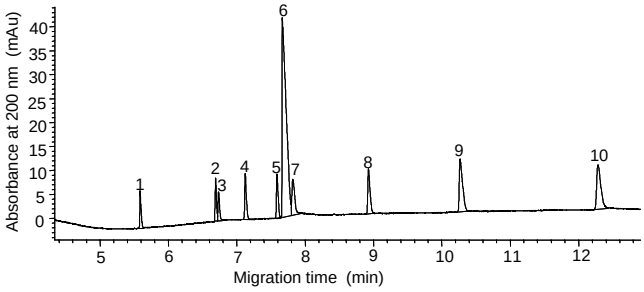

Fig. 1. An electropherogram of H-Tyr-(D)Arg-Phe-Phe-NH_2 and the nine related substances. BGE: 50 mM malonic acid, pH 2.5, with an applied voltage of 20 kV and at 25 °C. Migration order: 1. H-Arg-Phe-NH_2, 2. H-Tyr-(D)Arg-OH, 3. H-(D)Arg-Phe-OH, 4. H-(D)Arg-Phe-Phe-NH_2, 5. H-Tyr-(D)Arg-Phe-OH, 6. H-(D)Arg-Phe-Phe-OH, 7. H-Tyr-(D)Arg-Phe-Phe-NH_2, 8. H-Tyr-(D)Arg-Phe-Phe-OH, 9. H-Phe-Phe-NH_2, 10. H-Phe-Phe-OH.

concentration levels of the BGE solution components, i.e. the organic modifiers and the 2,6-DM-β-CD, followed recommendations from the literature [23]. The chosen concentration of triethanolamine corresponds to a constant pH of 2.5 for the BGE. Finally, the domains for the two physical parameters are directly related to the instrumental limits. A sample containing H-Tyr-(D)Arg-Phe-Phe-NH_2 and its nine related products was injected into the PVA-coated capillary according to the worksheet presented in Table 3.

The regression coefficients of the eight variables are presented in Fig. 2 using resolution (Fig. 2a) and analysis time (Fig. 2b) as response factors.

As can be seen, raising the concentration of 2,6-DM-β-CD in the buffer solution has a strong positive effect on the resolution, but a strongly negative effect on the analysis time. This is consistent with expectations, since addition of 2,6-DM-β-CD decreases the charge density of the determinand–CD complex and hence its migration time. The differences in complexation between the 2,6-DM-β-CD and the determinands contribute to the enhanced resolution.

Table 2
Selected variables and their respective domains in the Plackett–Burman design

Variable	Experimental domain	
	Low	High
Methanol (%)	0	15
Acetonitrile (%)	0	15
Triethanolamine (mM)	0	15
(2,6-DM)-β-CD (mM)	0	20
Buffer concentration (M)	0.05	0.10
Ionic strength	0.03	0.07
Temperature (°C)	15	40
Applied voltage (kV)	15	30

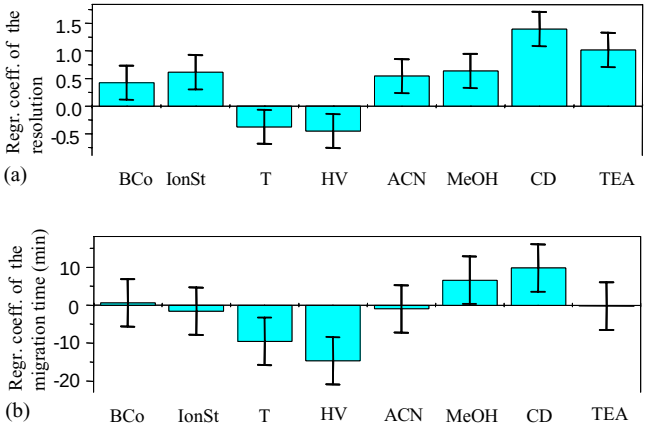

Fig. 2. Regression coefficients of the variables for (a) the resolution between H-(D)Arg-Phe-Phe-OH and H-Tyr-(D)Arg-Phe-Phe-NH_2 and (b) the migration time. Variables: buffer concentration (BCo), ionic strength (IonSt), temperature (*T*), applied high voltage (HV), acetonitrile addition (ACN), methanol addition (MeOH), addition of heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (CD) and addition of triethanolamine (TEA).

Table 3
Worksheet for Plackett–Burman design

Exp. no.	MeOH (%)	ACN (%)	TEA (mM)	CD (mM)	Concentration (mM)	Ionic strength	Temperature (°C)	Voltage (kV)
1	0	0	15	20	0.1	0.03	40	15
2	0	0	15	0	0.1	0.07	15	30
3	0	15	0	0	0.05	0.07	40	15
4	15	0	0	0	0.1	0.03	40	30
5	0	15	0	20	0.1	0.07	15	30
6	15	15	15	0	0.1	0.07	40	15
7	15	0	0	20	0.05	0.07	40	30
8	0	15	15	20	0.05	0.03	40	30
9	15	15	15	0	0.05	0.03	15	30
10	15	15	0	20	0.1	0.03	15	15
11	15	0	15	20	0.05	0.07	15	15
12	0	0	0	0	0.05	0.03	15	15
13	7.5	7.5	7.5	10	0.075	0.05	27.5	22.5
14	7.5	7.5	7.5	10	0.075	0.05	27.5	22.5
15	7.5	7.5	7.5	10	0.075	0.05	27.5	22.5

The addition of either acetonitrile or methanol (MeOH) to the buffer affects the resolution in a positive way. However, acetonitrile has no significant influence on the migration time, whereas addition of methanol increases it. Hence, they were added in the further investigations.

The addition of triethanolamine had a positive and significant effect on the resolution between H-Tyr-(D)Arg-Phe-Phe-NH_2 and H-(D)Arg-Phe-Phe-OH. Therefore it was worth carrying out a more detailed investigation of the magnitude of triethanolamine's effect on the separation. No significant effect on the migration time was observed.

The capillary temperature (T) and the high voltage (HV) had significant and positive effects on the analysis time. This was expected since the magnitude of the high voltage influences the rate of movement of the determinands [18]. The temperature affects their mobility through changes in the viscosity of the BGE and by changes in the pK_a value of the determinands and the constituents of the BGE. When operating at high temperatures and high voltage levels a satisfactory baseline separation was still achieved, even though both variables had a negative influence on the resolution. Thus, in further experiments the physical variables were held constant at their high levels.

The ionic strength (IonSt) of the BGE was found to have a positive significant effect on the resolution between H-Tyr-(D)Arg-Phe-Phe-NH_2 and H-(D)Arg-Phe-Phe-OH, but no influence on the migration time. This was consistent with expectations, since increasing the ionic strength of the BGE improves the peak efficiency and thus also the resolution [17,18]. Even at a low ionic strength a resolution of at least three was obtained. Therefore, we saw no reason to increase the ionic strength above the value of 0.03 M.

The buffer concentration (BCo) had a significant and positive effect on the resolution. A secondary positive effect of a high buffer concentration is that it promotes stacking due to differences in conductivity between the sample and the BGE. This variable was therefore set to the high level in all further experiments.

Consequently, the following variables were further investigated in subsequent method optimization steps: 2,6-DM-β-CD, methanol, acetonitrile and triethanolamine.

3.3. Step 3: experiments based on a full factorial design

The full factorial design involved the four selected factors, comprising 16 experiments (2^4) and three repeated experiments at the center point. The obvious objective was to investigate possible interactions between the variables. The respective domain for each of the variables was maintained as in Table 2, and the temperature and the voltage were held constant at 40 °C and 30 kV, respectively, throughout all of the experiments. The BGE was based on 100 mM malonic acid.

The solution containing H-Tyr-(D)Arg-Phe-Phe-NH_2 and its nine related products was injected into the PVA-coated capillary according to the worksheet presented in Table 4.

Table 4
Worksheet for the full factorial design

Exp. no.	CD (mM)	TEA (mM)	ACN (%)	MeOH (%)
1	0	0	0	0
2	20	0	0	0
3	0	15	0	0
4	20	15	0	0
5	0	0	15	0
6	20	0	15	0
7	0	15	15	0
8	20	15	15	0
9	0	0	0	15
10	20	0	0	15
11	0	15	0	15
12	20	15	15	15
13	0	0	15	15
14	20	0	15	15
15	0	15	15	15
16	20	15	15	15
17	10	7.5	7.5	7.5
18	10	7.5	7.5	7.5
19	10	7.5	7.5	7.5

Only the concentration of 2,6-DM-β-CD was found to be a significant variable for both of the response functions. The addition of triethanolamine to the buffer had no effect, either on the analysis time or the resolution. Triethanolamine was therefore omitted as a BGE constituent in all further experiments. The addition of the organic modifiers only influenced the analysis time. In fact, a buffer containing acetonitrile reduced the time by 2 min, while a buffer with methanol increased it by 2 min. No significant variable interactions were observed.

In accordance with these findings, the effects of the presence of 2,6-DM-β-CD and acetonitrile in the BGE were included in the subsequent method development procedure.

3.4. Step 4: experiments based on a circumscribed central composite design

The last step in the method development strategy was to use a circumscribed central composite design including axial points. Such a design investigates quadratic interaction terms and yields results in response surfaces that are easy to interpret. The two remaining factors, 2,6-DM-β-CD and acetonitrile, were studied in 11 experiments in which the center point was randomly repeated three times. The concentration of 2,6-DM-β-CD was altered between 10 and 25 mM and the concentration of acetonitrile in the range 5–20%. Table 5 shows the worksheet laying out the central composite design.

A graphical response surface for each of the two responses was constructed, see Fig. 3a and b. No distinguishable optimum could be found for any of the responses within the tested domain. The results indicate that the presence of acetonitrile in the BGE does not improve the analysis time, and only the CD concentration significantly influences both the resolution and the migration time. The results yielding the response surface depicted in Fig. 3a indicate that a

Table 5
Worksheet for the circumscribed central composite design

Exp. no.	CD (mM)	ACN (%)
1	10	5
2	25	5
3	10	20
4	25	20
5	6.9	12.5
6	28.1	12.5
7	17.5	1.9
8	17.5	23.1
9	17.5	12.5
10	17.5	12.5
11	17.5	12.5

CD concentration of 10 mM gives a resolution of about 4.5, which gives scope for further adjustment, since "baseline separation" of the peaks is considered to have occurred if the resolution is at least 1.5. Furthermore, the axial point in the design allows some variables to be tested outside the given domain, and the potential value of including such points is illustrated here, since the best results in this series were obtained from an axial point experiment where the CD concentration was just 7 mM. In this experiment all ten peaks were well separated, with a resolution of at least 2.7 and an analysis time of 14 min. An electropherogram of the main component and its nine related substances under these conditions is shown in Fig. 4. Using these conditions a method validation study was performed.

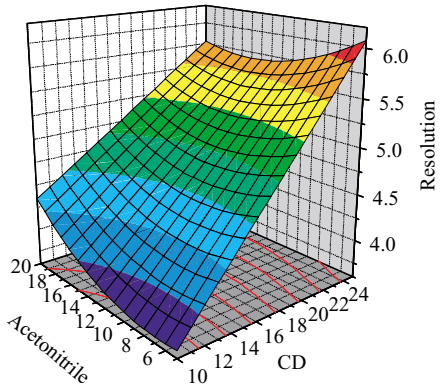

Fig. 3. Response curves for (a) the resolution between H-(D)Arg-Phe-Phe-OH and H-Tyr-(D)Arg-Phe-Phe-NH_2 and (b) the migration time.

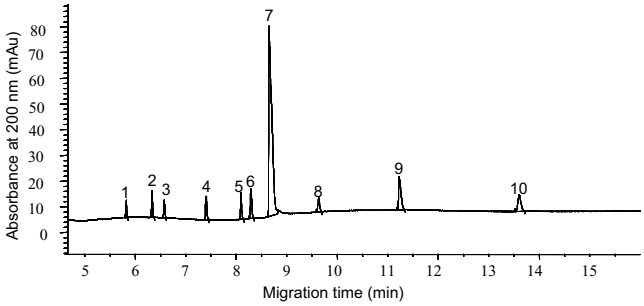

Fig. 4. An electropherogram of H-Tyr-(D)Arg-Phe-Phe-NH_2 and the nine related substances with 100 mM malonic acid buffer containing 7 mM heptakis(2,6-di-*O*-methyl)-β-cyclodextrin, operating at 30 kV, 40 °C. Migration order: 1. H-Arg-Phe-NH_2, 2. H-Tyr-(D)Arg-OH, 3. H-(D)Arg-Phe-OH, 4. H-(D)Arg-Phe-Phe-NH_2, 5. H-Tyr-(D)Arg-Phe-OH, 6. H-(D)Arg-Phe-Phe-OH, 7. H-Tyr-(D)Arg-Phe-Phe-NH_2, 8. H-Tyr-(D)-Arg-Phe-Phe-OH, 9. H-Phe-Phe-NH_2, 10. H-Phe-Phe-OH.

3.5. Validation

3.5.1. Selectivity

To evaluate the selectivity of the method the test solution described in Section 2.2 was injected. The relative migration times for all impurities are given in Table 6 and a representative electropherogram is depicted in Fig. 4. As can be seen, the resolution between all the involved compounds is excellent. The method proved its ability to baseline separate all nine of the related substances from the main component; thus fulfilling the selectivity requirements.

3.5.2. Linearity

The linearity of the response was calculated for the main component, H-Tyr-(D)Arg-Phe-Phe-NH_2. To do this, seven standard solutions were prepared in the concentration range 0.18–1.38 mg/ml, with H-Tyr-(D)Arg-NH_2 as an internal standard (IS). The quotient between the corrected peak area of the main component and the corrected area of the IS was used in the regression analysis (y) versus the standard concentrations (x), yielding the equation $y = 2.1926x + 0.0674$ and a correlation coefficient, r^2, of 0.999. The method shows good linearity within the tested range.

3.5.3. Precision

The precision study was based on six repetitive injections of a test solution containing 1.0 mg/ml H-Tyr-(D)Arg-Phe-Phe-NH_2 and either 25 μg/ml (0.17 corrected area%) or 0.8 μg/ml (0.07 corrected area%) H-Arg-Phe-NH_2, respectively. The R.S.D. of the corrected area was 3.5% for H-Tyr-(D)Arg-Phe-Phe-NH_2. For 0.17 corrected area% H-Arg-Phe-NH_2 the R.S.D. was 1.4%, and for 0.07 corrected area% H-Arg-Phe-NH_2 the R.S.D. was 1.7%.

Table 6
Separation data for the substances in the mixed sample

Substance	Concentration (mg/ml)	Migration time (min)	Relative migration time	Resolution, R_s	R.S.D. corrected area (%)	Efficiency (Plates/m)	Response factor
H-Arg-Phe-NH_2	0.05	5.85	0.67	44.7	4.3	218000	0.6
H-Tyr-Arg-OH	0.05	6.61	0.76	37.6	3.4	451000	0.7
H-(D)Arg-Phe-OH	0.05	6.78	0.77	34.3	3.8	487000	0.4
H-(D)Arg-Phe-Phe-NH_2	0.05	7.51	0.85	20.7	5.0	524000	0.6
H-Tyr-(D)Arg-Phe-OH	0.05	8.29	0.94	7.57	2.0	489000	0.7
H-(D)Arg-Phe-Phe-OH	0.05	8.48	0.96	5.54	3.2	474000	0.7
H-Tyr-(D)Arg-Phe-Phe-NH_2	0.55	9.09	1.00	–	–	127000	1.0
H-Tyr-(D)Arg-Phe-Phe-OH	0.05	9.80	1.11	12.3	3.4	369000	0.8
H-Phe-Phe-NH_2	0.05	11.5	1.29	30.3	3.1	280000	1.0
H-Phe-Phe-OH	0.05	13.8	1.55	48.8	1.7	252000	0.8

The R.S.D. values for the corrected peak areas were all less than 4%: a typical and acceptable level for a CE-instrument.

3.5.4. Quantification and detection limits

The substance with the lowest absorbance at 200 nm, H-Arg-Phe-NH_2, was selected for the determination of the method's quantification limit (LOQ), and detection limit (LOD).

The LOQ is here defined as the concentration giving a signal to noise ration of 10:1. For a solution containing 1.0 mg/ml H-Tyr-(D)Arg-Phe-Phe-NH_2, the LOQ of H-Arg-Phe-NH_2 was found to be 0.8 μg/ml, corresponding to 0.07% of the main peak using internal normalization. A repeatability study at the LOQ concentration was also performed (see Section 3.5.3).

The LOD of H-Arg-Phe-NH_2 was calculated to be 0.3 μg/ml in a solution containing 1.0 mg/ml H-Tyr-(D)Arg-Phe-Phe-NH_2. The LOD corresponds to a signal to noise ratio of 3:1.

3.5.5. System suitability test

To assess the performance of the system a system suitability test (SST) was developed. The SST prescribes limits for the resolution between peaks, in our case between the H-Tyr-(D)Arg-Phe-Phe-NH_2 and the H-(D)Arg-Phe-Phe-OH peaks, which must be at least 1.5. The test solution comprised 0.55 mg/ml H-Tyr-(D)Arg-Phe-Phe-NH_2 and 0.05 mg/ml H-(D)Arg-Phe-Phe-OH. During the method validation procedure the R_s between these two peaks was greater than 3, and the R.S.D. for the R_s obtained from six repeated injections of this solution was 2%. This R.S.D. value is of the same magnitude as the one obtained for the precision study performed at the LOQ level of H-Arg-Phe-NH_2 indicating that the system maintains its suitability even at this low level.

3.5.6. Response factor determination

To determine the response factors, R_f, for the test substances, solutions were injected containing H-Tyr-(D)Arg-Phe-Phe-NH_2 and the selected impurities at known concentrations. The response factors, R_f, were calculated according to Eq. (2) and results are presented in Table 6.

$$R_f = \frac{\text{conc.}_{\text{main.comp.}} \times \text{area}_{\text{impurity}}}{\text{conc.}_{\text{impurity}} \times \text{area}_{\text{main.comp.}}} \quad (2)$$

Correction for the difference in R_f-values is appropriate when calculating the purity of H-Tyr-(D)Arg-Phe-Phe-NH_2 if $R_f < 0.8$ or >1.2.

4. Conclusions

This paper demonstrates that experimental design is a powerful tool for optimizing analytical procedures involving a large number of variables. The stepwise approach for the optimization enables swift identification of the significant variables through application of a Plackett–Burman design scheme. Further investigative steps of these significant variables, based on full factorial and circumscribed central composite designs, then reveal the optimum conditions for the separation and elucidate the robustness of the method, with a limited number of experiments. The choice of response function is normally critical when applying experimental design. In this case, only two response functions were considered: resolution and migration time. Both were useful parameters for optimization, but sufficient resolution is an absolute requirement, whereas the migration time is desirable but not essential.

The analysis time for the CE method (14 min) is much shorter than the corresponding time for the HPLC method (35 min), making CE an attractive technique for peptide separation. The CE technique is often affected by problems associated with the very short light path due to on-line detection, which make the detection limits relatively high. However, the detection limits for CE and HPLC analysis of peptides are comparable.

References

[1] P.G. Righetti, Biopharm. Drug Dispos. 22 (2001) 337.
[2] H.J. Issaq, T.P. Conrads, G.M. Janini, T.D. Veenstra, Electrophoresis 23 (2002) 3048.

[3] M.T.W. Hearn, Biologicals 29 (2001) 159.
[4] V. Kašička, Electrophoresis 24 (2001) 4139.
[5] Y.L. Loukas, S. Sabbah, G.K.E. Scriba, J. Chromatogr. A 931 (2001) 141.
[6] V. Kašička, Electrophoresis 24 (2003) 4013.
[7] H. Brunnkvist, B. Karlberg, I. Granelli, J. Chromatogr. B 793 (2003) 343.
[8] K.D. Altria, B.J. Clark, S.D. Filbey, M.A. Kelly, D.R. Rudd, Electrophoresis 16 (1995) 2143.
[9] S. Mikaeli, G. Thorsén, B. Karlberg, J. Chromatogr. A 907 (2001) 267.
[10] A. Sun, Y. Lin, J. Liq. Chromatogr. 24 (2001) 2051.
[11] M.M. Rogan, K.D. Altria, D. Goodall, Chromatographia 38 (1994) 723.
[12] R. Ragonese, M. Macka, J. Hughes, P. Petocz, J. Pharm. Biomed. Anal. 27 (2002) 995.
[13] M.G. Vargas, Y. Vander Heyden, M. Maftouh, D.L. Massart, J. Chromatogr. A 855 (1999) 681.
[14] V. Harang, M. Tysk, D. Westerlund, R. Isaksson, G. Johansson, Electrophoresis 23 (2002) 2306.
[15] K. Persson, O. Åström, J. Chromatogr. A 697 (1997) 207.
[16] K. Stubberud-Persson, O. Åström, J. Chromatogr. A 789 (1998) 307.
[17] B. Chankvetadze, Capillary Electrophoresis in Chiral Analysis, Wiley, 1997, Chapter 1, p. 5.
[18] K.D. Altria (Ed.), in: Chromatographia CE Series, vol. 2, Vieweg Press, 1998.
[19] C.E. Sänger-van de Griend, J. Chromatogr. A 782 (1997) 271.
[20] ICH Topic Q2A, Validation of analytical procedures: text on validations of analytical procedures, CPMP/ICH/381/95.
[21] ICH Topic Q2B, Validation of analytical procedures: methodology, CPMP/ICH/281/95.
[22] M.G. Khaledi (Ed.), in: Chemical Analysis, vol. 146, Wiley-Interscience, 1998, Chapter 19, p. 637.
[23] G.M. McLaughlin, J.A. Nolan, J. Liq. Chromatogr. 15 (1992) 961.