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Enantiomeric separation of TAPP, H–Tyr–(D)Ala–Phe–Phe–NH_2, by capillary electrophoresis using 18-crown-6-tetracarboxylic acid as a chiral selector

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Abstract

A capillary electrophoresis method for the enantiomeric separation of the tetrapeptide H–Tyr–(D)Ala–Phe–Phe–NH_2 (TAPP), has been developed and validated. The preferred background electrolyte (BGE) consisted of 0.1 *M* aqueous phosphoric acid adjusted to pH 3.0 with triethanolamine. The chiral selectors 18-crown-6-tetracarboxylic acid ($18C6H_4$) and heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (2,6-DM-β-CD) were compared and the crown ether $18C6H_4$ was found to be superior. The separation of TAPP and its enantiomer was accomplished within 30 min with a resolution greater than 3.5. The method was then investigated with respect to selectivity, linearity, accuracy, range, precision, detection limit (DL), quantitation limit (QL) and robustness, essentially following International Conference of Harmonisation (ICH) guidelines for the validation of analytical methods. The DL and QL for the TAPP enantiomer were found to be 0.3 and 0.8%, respectively, at the target TAPP concentration of 1 mg/ml. Robustness was tested using a full factorial design for the following four experimental variables varied at two levels: pH of the BGE, chiral selector concentration in the BGE, phosphoric acid concentration in the BGE, and temperature. The method showed good performance with respect to all of the validation parameters, and proved to be robust to changes in the experimental parameters within the tested domain.

Keywords: 18-Crown-6-tetracarboxylic acid; H–Tyr–(D)Ala–Phe–Phe–NH_2; Peptides

1. Introduction

Determination of enantiomeric purity is often essential in drug development and quality control since two enantiomers may have different biological activities. As interest in using peptides as potential drugs is increasing we aim to extend our ability to separate enantiomers of peptides. The tetrapeptide H–Tyr–(D)Ala–Phe–Phe–NH_2, TAPP, is a peripherally acting μ-agonist, known to have an analgesic effect [1]. It has four chiral centres and its enantiomer has the following configuration: H–(D)Tyr–Ala–(D)Phe–(D)Phe–NH_2 (subsequently referred to as ''the enantiomer''). The structures of TAPP and its enantiomer are shown in Fig. 1.

Cyclodextrins (CD) and their derivatives are currently the most commonly used selectors for chiral separation of peptides in capillary electrophoresis

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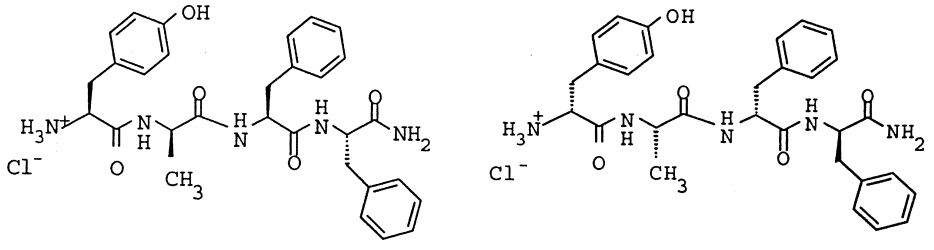

(a) *(b)*

Fig. 1. Chemical structures of (a) TAPP and (b) its enantiomer.

(CE). The inner cavity of the cyclodextrin, which is lined with hydrogen atoms and glycosidine oxygen bridges, is hydrophobic, while the outer part has hydrophilic properties [2]. Enantiomeric recognition is based on inclusion into the cavity and additional hydrogen bonding between hydroxyl or other groups around the conical CD opening and substituents of the guest molecule [3,4]. When charged CDs are employed ionic interactions also become important. Chiral separation can be achieved if there is complexation between enantiomers and the chiral selector, and if the mobility of the host–guest complex thus formed differs from that of the uncomplexed guest molecule [2]. In addition, the effective mobilities of the enantiomers have to be different. This occurs when the complex constants between the chiral selector and the respective enantiomer differ and/or when the enantiomer–selector complexes migrate with different velocity in the separation system [5–7]. Peptides containing at least one aromatic group have the ability to form an inclusion complex with the cyclodextrin [4].

Crown ethers are macrocyclic compounds that were originally discovered in 1967, but only (+)-(18-crown-6)-2,3,11,12-tetracarboxylic-acid (18C6-H_4) has so far been used as a chiral selector in CE [8]. Kuhn and co-workers [9] were the first to report its use in this way. The simultaneous separation of eight stereoisomers of a tripeptide using the selector 18C6H_4 has been reported [10] and chiral separations based on this crown ether have been reviewed [11]. Kuhn and co-workers proposed two alternative chiral recognition mechanisms [12]. According to the first mechanism, the carboxylic acid pairs on the two sides of the chiral crown ether act like chiral barriers, dividing the space available for the substituents of the chiral atom adjacent to the primary amino functional group into two cavities. Diastereometric complexes with different equilibrium constants can be formed due to differences in the size and spatial arrangement of these substituents. The second proposed mechanism is based on ionic or hydrogen bonding interactions with polar carboxylic acid substituents on the polyether ring.

The existence of a primary amino group seems to be essential for enantiomeric CE separation with 18C6H_4. It is also advantageous for the substituents on the chiral carbon to be bulky rather than small [8]. TAPP meets both of these requirements. However, Steffeck et al. have found that separation of secondary amines is feasible using LC and a chiral stationary phase comprising 18C6H_4 [13].

In this paper we report on the successful separation of TAPP and its enantiomer by CE using 18C6H_4 as a chiral selector. 18C6H_4 was compared with heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (2,6-DM-β-CD) and was found to be superior. The proposed method has also been validated essentially following the International Conference of Harmonisation (ICH) guidelines on requirements for the registration of pharmaceuticals [14,15].

2. Experimental

2.1. Chemicals

Biochem Immunosystems (Montreal, Canada) kindly provided the tetrapeptide H–Tyr–(D)Ala–

Phe–Phe–NH_2 (TAPP) and its enantiomer H–(D)Tyr–Ala–(D)Phe–(D)Phe–NH_2. Heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (2,6-DM-β-CD) was purchased from Aldrich (Milwaukee, USA) and (+)-(18-crown-6)-2,3,11,12-tetracarboxylic-acid (18C6-H_4) from Sigma (St. Louis, USA). Phosphoric acid and triethanolamine were both of analytical grade (Merck, Darmstadt, Germany). Purified water was obtained from a Waters Milli-Q system (Watford, Herts, UK).

2.2. Solutions

A range of background electrolyte (BGE) solutions in 0.1 *M* phosphoric acid adjusted to pH 3.0 with triethanolamine (if not otherwise stated) plus various concentrations of each of the chiral selectors was prepared and tested. A solution containing approximately 1 mg/ml of TAPP and 0.01 mg/ml of its enantiomer was also made, and is referred to as the system suitability test solution (SST-solution). Working sample solutions were freshly prepared and filtered through 0.45 μm Millipore Teflon filters before use.

2.3. Instrumentation

All experiments were performed using a $HP^{3D}CE$ instrument (Agilent Technologies) equipped with a diode array detector and Chemstation software for data handling. The PVA-coated capillaries were obtained from Hewlett-Packard. These capillaries had an effective length (l_e) of 40.0 cm, a total length (l_t) of 48.5 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 0.1 *M* H_3PO_4 (5 min), purified water (5 min) and BGE (10 min). The temperature was varied in the range 15–25 °C. The applied potential (kept at a constant 20 kV throughout the process) induced electrophoretic motion towards the detector.

The sample introduction procedure entailed sequential hydrodynamic injection of sample then BGE, by applying a pressure of 15 mBar for 10 s for each solution. Direct UV detection was performed at 200 nm (8 nm bandwidth).

2.4. Evaluation procedure

The resolution and efficiency were selected as the primary response functions when evaluating the performance of the separation system. As a secondary response function we also recorded the total migration time. The amount of the enantiomer detected was expressed in terms of its corrected peak area, as a percentage relative to that of the main component, TAPP, if not otherwise stated. The corrected area value was calculated as the measured area of a peak divided by its migration time.

2.4.1. Resolution

Peaks were integrated and the resolution was calculated using Chemstation software (version A.05.02), according to

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

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

2.4.2. Efficiency

This response factor is related to the shape of the peaks. The efficiency was also calculated by Chemstation software, according to:

$$N = 5.54\left(\frac{t}{w_{\frac{1}{2}}}\right)^2 \quad (2)$$

where t is the migration time of the peak and $w_{\frac{1}{2}}$ is the temporal peak width at half its height.

3. Results and discussion

3.1. Choice of BGE

Most papers on CE separations of peptides employing 18C6H_4 as a chiral selector report on the use of TRIS/citrate when composing the BGE solution [11,16]. However, Kuhn et al. found that TRIS forms a weak complex with 18C6H_4 [17]. A BGE composition involving this compound was therefore

avoided. A pH value of 3 of the BGE was preferred due to the enhanced complexation degree observed at this pH in comparison with the complexation degree at lower pH values [18]. It has previously been shown that another basic tetrapeptide can be successfully separated from its enantiomer using 0.1 *M* phosphoric acid adjusted to pH 3.0 with triethanolamine [19]. Consequently, this pH was selected for all the experiments in this study. Adjusting the pH with a tertiary amine like triethanolamine is preferable since this compound exhibits low interaction with $18C6H_4$ in comparison with other buffer constituents, e.g., TRIS [18]. TAPP has two pK_a values, 7.2 at the N-terminus and 9.8 at the phenol group on the tyrosine. This means that the amine group will be fully protonated, and therefore positively charged, at this pH.

3.2. Choice of capillary

We compared bare fused-silica and PVA-coated capillaries using $18C6H_4$ as the chiral selector. The efficiency of the peaks and the resolution were found to be very similar for the two capillary types. However, the migration times were about 20% longer when using the bare fused-silica capillary in comparison with the PVA-coated capillary. This is probably due to interactions between the inner surface of the capillary tube and the analyte. These interactions seem to be less pronounced in the PVA-coated capillaries. Furthermore, the TAPP peak exhibits tailing when using the bare silica capillaries. Thus, we concluded that the PVA-coated capillary was the most appropriate choice for our application, its main advantage being the shorter analysis time it allowed.

3.3. Choice of chiral selector

The suitability of the chiral selector 2,6-DM-β-CD was first tested for the enantiomeric analysis of TAPP, at three different concentrations: 1, 10 and 20 m*M*. As shown in Fig. 2a, 10 m*M* CD gave poor separation between TAPP and the enantiomer, which appeared just before the TAPP peak and was hardly distinguishable from it. However, spiking experiments confirmed its identity. For the CD concentrations 1 and 20 m*M* the two peaks co-eluted completely. We found these results quite unexpected since successful enantiomeric separation of another tetrapeptide has been reported using this chiral selector [19]. No negatively charged CDs were examined as selectors in this work since they will migrate towards the anode thereby increasing the migration times to a large extent in comparison with the neutral CD [20].

Consequently, we decided to investigate the possibility of using the chiral crown ether $18C6H_4$. The results obtained with this selector are presented in Table 1. As can be seen, we achieved good separation at all three tested concentrations of $18C6H_4$. Even though the resolution is satisfactory with a buffer concentration of only 0.5 m*M* $18C6H_4$, the efficiency of the enantiomer is higher when using a concentration of 1.0 m*M*. High efficiency is very desirable when the analytes are present at low concentrations since it enhances the accuracy of the integration. The highest selector concentration, 1.5 m*M*, gave the highest resolution, but it also gave lower efficiency compared to 1 m*M* $18C6H_4$. A high selector concentration also increases the total migration time. Taking all these factors into account we conclude that the final separation buffer should consist of 1.0 m*M* $18C6H_4$ in 0.1 *M* phosphoric acid adjusted to pH 3.0 with triethanolamine. A representative electropherogram obtained under these conditions is depicted in Fig. 2b.

3.4. Method validation

3.4.1. Selectivity

The SST solution was analysed according to the proposed method and the two peaks were baseline-separated within 30 min (see Fig. 2b).

3.4.2. Linearity

The linearity of the response was investigated for both TAPP and its enantiomer. Six standard TAPP solutions were prepared, spanning the range 0.3–1.4 mg/ml, covering 30–140% of the targeted TAPP concentration, 1.0 mg/ml. Three enantiomer standard solutions were also prepared covering the interval 3–10 μg/ml, bracketing the limit of quantitation value for this analyte (see Section 3.4.7). Double injections were made at each level. Regression analysis of plots of the corrected area versus the

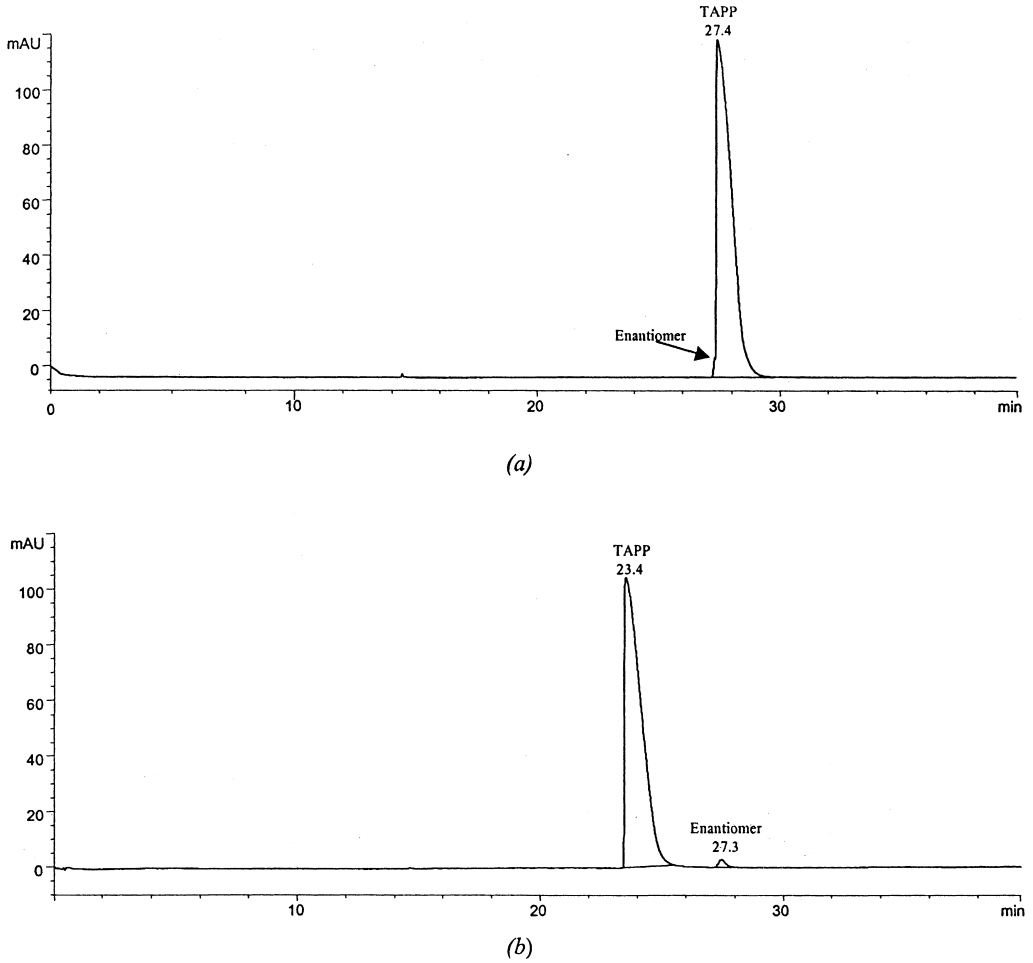

Fig. 2. Separation of TAPP and its enantiomer using a BGE consisting of 0.1 *M* phosphoric acid adjusted to pH 3.0 with triethanolamine and (a) 10 m*M* 2,6-DM-β-CD or (b) 1.0 m*M* $18C6H_4$ as chiral selector using a PVA-coated capillary with the following parameters: 40.0 cm (l_e), 48.5 cm (l_t) 50 μm I.D., 350 μm O.D., 15 °C, 20 kV, wavelength 200 nm, bandwidth 8 nm. Test solution: 1 mg/ml of TAPP and 0.01 mg/ml of the enantiomer.

Table 1
Separation parameters with $18C6H_4$ as chiral selector

Concentration $18C6H_4$ (m*M*)	Resolution	Efficiency TAPP (plates/m)	Efficiency enantiomer (plates/m)	Total migration time (min)
0.5	3.7	6230	33300	23.2
1.0	4.3	5250	42900	27.3
1.5	9.9	9140	33800	43.8

All runs were performed with 0.1 *M* phosphoric acid adjusted to pH 3.0 with triethanolamine, in a PVA-coated capillary with the following parameters: 40.0 cm (l_e), 48.5 cm (l_t) 50 μm I.D., 350 μm O.D., 15 °C, 20 kV, wavelength 200 nm, bandwidth 8 nm.

different standard concentrations resulted in the equations $y = (3.55 \pm 0.25)x - (0.25 \pm 0.23)$ and $y = (0.21 \pm 0.02)x - (0.043 \pm 0.12)$ for TAPP and the enantiomer, respectively, ±-values refer to 95% confidence limits. The correlation coefficient, r^2, values for the curves were 0.990 and 0.997 for TAPP and the enantiomer, respectively. Both analytes showed good linearity within their tested ranges, indicating that the corrected peak area values are directly proportional to the concentration values.

3.4.3. Range

The specified range for any analyte (i.e., the range for which the analytical procedure has been shown to provide an acceptable degree of linearity, accuracy and precision [15]) can be derived from a linearity study. In this case, the linearity data show that the specified range for the TAPP concentration is 0.3–1.4 mg/ml, spanning the target sample concentration of 1 mg/ml, and the linear range for the enantiomer is estimated to be 0.3–1.0% (in terms of relative peak area) of the TAPP target concentration.

3.4.4. Accuracy

The accuracy of the method was determined by analysing samples of known composition. Two standard solutions were analysed, containing 1 mg/ml TAPP with either 1.17 or 0.524% of the enantiomer. The recovery, defined as the percentage ratio between the theoretical and determined amounts of the enantiomer, was calculated in each case, and the results are presented in Table 2. The obtained recovery (≥94%) is sufficient for this application.

3.4.5. Precision

3.4.5.1. Repeatability. The repeatability was investigated by injecting the SST solution six times. The relative standard deviation, RSD, was calculated in terms of both the corrected peak area of the enantiomer (as a percentage of the TAPP peak area) and the migration times of TAPP and the enantiomer. Calculations based on the corrected peak area of the enantiomer resulted in an RSD of 0.41%. The RSD for the migration time was 1.5% for TAPP and 3.4% for the enantiomer. These results show that the method provides good repeatability.

Table 2
Recovery data for the enantiomer

Sample	Theoretical concentration (%)	Determined concentration (%)	Recovery (%)
1	1.17	1.16	99.1
2	0.524	0.495	94.5

3.4.5.2. Intermediate precision. Within-laboratory variations were tested in studies covering different days as well as two different CE instruments. The SST solution was analysed and the results were evaluated by comparing the resolution between TAPP and its enantiomer. Three different SST solutions were prepared and analysed on three different days on two separate Hewlett-Packard^{3D}CE instruments. Calculations of the resolution of these analyses resulted in an RSD of 0.82%. This shows that there was no significant difference in the results between days or between instruments.

3.4.6. Detection limit

The detection limit (DL) is the lowest concentration that can be detected with certainty. A signal-to-noise ratio of three is considered acceptable for the DL. Calculations of the signal-to-noise ratio resulted in a DL of 0.3% for the enantiomer when injecting a sample solution containing 1 mg/ml TAPP. Three replicate injections of a solution containing the enantiomer at a concentration close to the DL, 0.4%, were performed and the RSD value for the corrected peak area of the enantiomer (relative to TAPP) was found to be 2.8%. The RSD values for the migration times were 0.74 and 2.5% for TAPP and its enantiomer, respectively. These results are quite satisfactory.

3.4.7. Quantitation limit

The quantitation limit (QL) is defined as the minimum level at which the analyte can be reliably quantified with acceptable accuracy and precision. A signal-to-noise ratio of 10 is considered acceptable for the QL. Calculations of the signal-to-noise ratio resulted in a QL of 0.8% of the enantiomer when injecting a sample solution containing 1 mg/ml TAPP. Three replicate injections were performed at the QL and the RSD for the corrected peak area of

the enantiomer (relative to TAPP) was found to be 0.89%. The RSD for the migration times were 3.7 and 4.0% for TAPP and the enantiomer, respectively. This is an acceptable result.

3.4.8. Robustness

Systematic variations in method parameters were tested to assess the reliability of the method, i.e., to determine its robustness, using an experimental design. For this, four factors were varied at two levels according to a full factorial design (2^4), resulting in 16 experiments. The addition of three centre points gave a total of 19 experiments. The factors investigated were the pH of the background electrolyte, the concentration of phosphoric acid, the concentration of $18C6H_4$ and the temperature. To evaluate the results we used the resolution between TAPP and the enantiomer, R_s, and the total migration time, TM_t, as responses. The factor domains are shown in Table 3. These domains were chosen to reflect possible variations occurring during the laboratory procedure.

The resolution was found to be higher than 3.5 in all 19 experiments thereby exceeding the minimum required value of 1.5.

3.4.9. System suitability test

System suitability testing (SST) is based on the concept that the equipment, electronics, analytical operations and sample to be analysed constitute an integral system and can be evaluated as such [15]. For our purpose, namely an enantiomeric separation, the SST is basically a determination of the resolution. We injected the SST solution containing 1 mg/ml TAPP and 0.01 mg/ml of the enantiomer (approximately its quantitation limit, QL), and then evaluated the electropherogram with respect to the resolution between TAPP and the enantiomer. The method is considered suitable when a resolution of at least 3.5 between TAPP and the enantiomer is obtained.

Table 3
Experimental domains for the investigated factors

Factor	Low level[a]	High level[a]
pH	2.8	3.2
$18C6H_4$ concentration, m*M*	0.5	1.5
H_3PO_4 concentration, m*M*	0.08	0.12
Temperature, °C	15	25

[a] The factorial variation around the method settings.

4. Conclusion

To our knowledge, this is the first report of the successful chiral CE separation of a tetrapeptide using $18C6H_4$ as a selector, although it has previously been used for enantiomeric separations of amino acids [21,22], and also both di- and tri-peptides [10,23–25]. The data obtained during the validation of the method for the tetrapeptide TAPP show that an effective CE separation scheme can be developed using this selector at pH 3. $18C6H_4$ has four pK_a values in a narrow range close to this pH, at pH 2.1, 2.8, 4.3 and 4.9 [26]. Nevertheless, the developed method has been shown to be robust.

The most commonly used chiral selectors for peptides and amino acids in other cases are various types of CDs. However, we have also achieved considerably better CE separations using the $18C6H_4$ selector as the background electrolyte rather than the CD alternative in preliminary enantiomeric separations of a peptide other than TAPP. Clearly, the chiral separation ability of the crown ether $18C6H_4$ deserves further examination to elucidate its full analytical potential.

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