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Quantitative determination of tryptophan enantiomers by capillary electrophoresis

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Abstract

A novel capillary electrophoretic method is reported which allows efficient detection of 0.1% L-tryptophan in the presence of the D-enantiomer. The optimised conditions employed a triethanolamine–phosphoric acid electrolyte containing α-cyclodextrin. The method is also capable of acceptable injection precision resulting from the incorporation of an internal standard. The care and maintenance of the separation capillary are discussed. Acceptable validation criteria for sensitivity, precision, linearity, repeatability and recovery are included. The importance of including instrument-to-instrument method transfer in method validation is stressed and demonstrated.

Keywords: Enantiomer separation; Tryptophan

1. Introduction

Tryptophan is an important and frequently used starting material in the chemical synthesis of a range of pharmaceuticals. Tryptophan possesses a chiral centre and is commercially available in the D-, L-, or racemic forms. There is a strong requirement to obtain data on the optical purity of input batches of tryptophan raw material prior to its use in synthesis. Conventionally enantiopurity determinations are performed using chirally selective HPLC methods. For example, trytophan has been chirally resolved by HPLC using an α-cyclodextrin bonded phase [1]. However, there is an increasing use of chirally-selective-optimised capillary electrophoretic (CE) methods in enantio-purity determinations [2]. Optimised CE methods offer robust, sensitive and reliable means for detection of trace (<1%) undesired enantiomer levels. A number of methods have been validated [3–5] for this purpose.

Underivatised tryptophan has been directly chirally resolved in CE by use of a variety of chirally selective mechanisms including use of α-cyclodextrin [6] and chiral crown ethers [7,8]. Derivatisation of tryptophan with chiral derivatisation agents such as 2,4-dinitrophenyl (DNP) [9], dansyl [10] and DBT [11] can be used to form diastereomers which do not necessarily require use of a chiral additive to the carrier electrolyte. Diastereomeric separations have been achieved through use of electrolyte containing PVP [11], β-cyclodextrins [9], β-cyclodextrins in combination with urea [10] and vancomycin (a macrocyclic antibiotic) [12].

Given the simplicity and robustness of cyclodextrin-based chiral separations it was decided to focus on this chiral selectivity mechanism. The objective of the study being to develop a method

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capable of quantifying 0.1% L-tryptophan present in D-tryptophan.

There is also a need to assay the tryptophan content in batches of tryptophan drug substances which requires a method that is capable of achieving an acceptable degree of precision. Generally, injection precision is worse in CE than for HPLC and use of an internal standard is required to eliminate CE injection volume related variabilities [13].

This paper reports optimisation and validation of a novel CE method which is capable of simultaneous assay and enantio-purity determination of tryptophan. Validation details include sensitivity, precision, linearity, recovery, robustness, repeatability and instrument-to-instrument method transfer. It is concluded that the novel method reported is suitable as a routine quality control method.

2. Experimental

AnalaR grade disodium hydrogenorthophosphate, α-cyclodextrin (α-CD) and phosphoric acid were purchased from Aldrich (Poole, UK). Triethanolamine–phosphoric acid buffer was obtained from AECS (Bridgend, UK). CE analysis was performed on both Beckman P/ACE 5100 (Fullerton, CA, USA) and ABI 270A (Foster City, CA, USA) CE instruments. The method details for the two instruments are given in Table 1. The second injection step in Table 1 enabled the capillary to be dipped into a separate vial containing electrolyte prior to the separation process. This step effectively removes any sample solution from the outside of the capillary prior to the capillary insertion into the vial containing the separation electrolyte. This simple procedure minimises sample carry-over effects which may occur when using relatively high sample concentrations.

Fused-silica capillaries were obtained from Composite Metals (Hallow, UK). Data acquisition and peak integration was performed using a Hewlett-Packard (Bracknell, UK) laboratory automation system. Capillaries were initially pre-conditioned, prior to their first use, with a 30-min rinse with 0.1 *M* NaOH to regenerate surface silanols. Water was obtained from a Milli Q system (Waters, Watford, UK). Trytophan samples and aspartame internal standard were obtained from within Glaxo Wellcome Research and Development. Two analyses were performed prior to starting each analytical sequence to allow the system to settle and for samples and electrolyte solutions to reach a constant temperature on the autosampler tray.

3. Results and discussion

3.1. Method optimisation

The principal operating parameters optimised in a chiral CE separation are cyclodextrin type and concentration, electrolyte composition and temperature.

3.1.1. Cyclodextrin

(α-CD) had previously been shown to give resolution of tryptophan enantiomers [6]. This naturally occurring CD is commercially available in highly purified form. Chemically derivatised cyclodextrins

Table 1
Experimental conditions for the two instrument types used

Parameter	ABI	Beckman
Rinse 1	1 min (0.1 *M* NaOH)	1 min (0.1 *M* NaOH)
Rinse 2	2 min with electrolyte	2 min with electrolyte
Set temperature	30°C	30°C
Injection (1)	1 s vacuum sample	1 s pressure sample
Injection (2)	1 s vacuum electrolyte	1 s pressure electrolyte
Separation	+30 kV for 20 min	+20 kV for 20 min
Detection	200 nm	200 nm
Capillary dimensions	50 cm×50 μm	37 cm×50 μm

Sample solution: 1.5 mg/ml in electrolyte.

are often employed in chiral CE separations but the variability in the degree of substitution of the derivatised cyclodextrin can alter the chiral selectivity obtained [5,14]. This variability causes additional variation and robustness issues and therefore it is recommended to employ underivatised cyclodextrins if sufficient separation selectivity can be obtained. CD concentrations were varied over the range 25–100 m*M* α-CD. Resolution increased with increasing CD concentration. Acceptable performance was obtained for 50, 75 and 100 m*M*. The method concentration was set at 75 m*M* to ensure robustness of the separation to this parameter.

3.1.2. Electrolyte composition

Generally low-pH phosphate buffers have been employed for these separations [15]. However, it has been reported that improved peak symmetries and resolutions may be obtained using a triethanolamine–phosphoric acid buffer [16]. This reported improvement was verified in our work and the separation obtained is shown in Fig. 1 using 25 m*M* triethanolamine–phosphoric acid containing 75 m*M* α-CD. Higher electrolyte concentrations were evaluated but these generated higher currents with no increase in resolution.

3.1.3. Temperature

Since this method is to be routinely applied in a variety of laboratories worldwide, it was necessary to standardise the separation temperature at 30°C. This

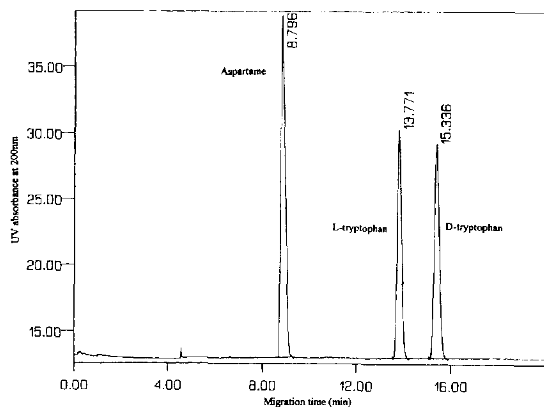

Fig. 1. Separation of a racemic tryptophan sample (0.1 mg/ml) with aspartame (0.1 mg/ml) as internal standard. For separation conditions see Table 1 (Beckman).

was necessary as some CE instruments employed cannot regulate subambient temperatures. Therefore, despite improvements in chiral resolution at lower temperature [17,18], it was necessary to utilise this relatively high temperature to allow successful method transfer.

3.1.4. pH

The optimal pH was found to be pH 2.5, resolution was maintained at pH 3.1 but lost at pH 4.9. The electrosmotic flow (EOF) decreases with pH thereby increasing analysis times; thus pH values lower than 2.5 were not assessed.

3.1.5. Sample loading effects

The sampling variables in CE are the sample concentration and injection time. A range of different sample concentrations and injection times were assessed using the resolution of a 1% spike of L-tryptophan from D-tryptophan as the measured response. It was experimentally observed that resolution was better when using short injections of high concentration samples. For example, resolution was maintained for a 1-s injection of a 1.5 mg/ml solution but lost for a 2-s injection of a 0.75 mg/ml solution. This effect is due to the length of the initial sample zone inside the capillary which is directly reflected in the width of the separated peaks when the sample is dissolved in electrolyte.

3.1.6. Capillary maintenance

It is essential to adopt good working practices [18] in order to maintain consistent migration times and resolution. Each capillary is dedicated solely to this method as this prevents 'memory effects' from adsorbed surface-active species such as phosphate [19] and surfactants, leading to non-repeatable separations. New capillaries are flushed with 0.1 *M* NaOH solution [20] for 30 min to ensure total rehydration of the capillary surface. It is also vital to ensure that the tip of the capillary used for injection purposes is cleanly cut. If the tip is poorly cut then perturbation of the baseline or loss in resolution can occur [21]. Fig. 2a shows the separation achieved using a poorly cut capillary and Fig. 2b shows the resolution to be restored after carefully re-cutting the tip of the same capillary. These problems can occur due to trapping of sample solution in the fractured

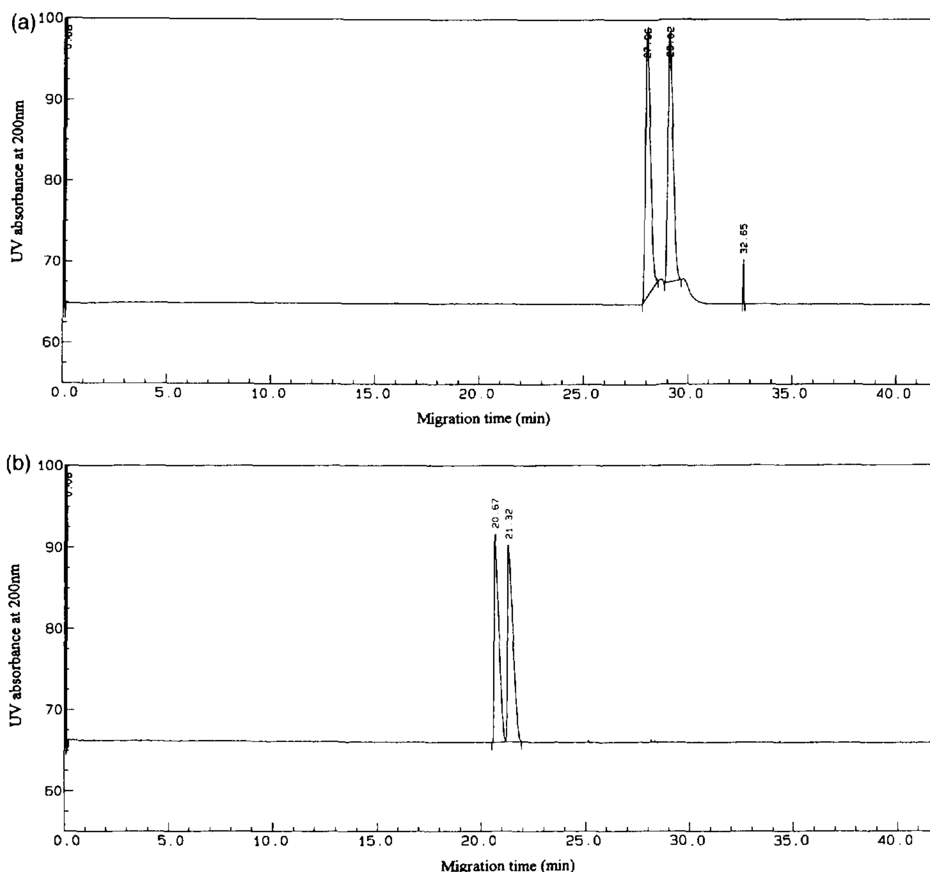

Fig. 2. Separation of a racemic tryptophan sample. (a) Poorly cut capillary lip; (b) correctly cut capillary lip. For separation conditions, see Table 1 (Beckman).

end of a poorly cut capillary which effectively acts as a variable source of sample carry-over. The capillary tip should be cleanly cut with a laser, diamond cutter or ceramic stone. The injection end of the capillary can be lightly flamed or electrically heated to remove a few millimetres of the polyimide coating.

3.1.7. Precision optimisation

It was essential that an acceptable degree of precision could be obtained for assay purposes. Although use of a 1-s injection is highly beneficial for resolution purposes it is detrimental to the level of precision that can be obtained [13]. This imprecision is caused by the reduced ability of CE instruments to control injection volumes using extremely short injection times. The majority of instruments monitor the actual pressure during the injection process and automatically alter the injection time to compensate for fluctuations through a feedback system. Use of 1-s injection times does not allow sufficient time for repeatable feedback compensation and should be avoided when attempting high precision operations. Use of an internal standard can compensate for any imprecision relating to injection volume.

Aspartame was identified as a suitable internal standard as it is commercially available at high purity, has acceptable UV characteristics and has a suitable migration position with respect to

tryptophan. Fig. 1 shows separation of a racemic tryptophan sample with aspartame used as the internal standard.

3.1.8. Wavelength optimisation

Wavelengths between 190–230 nm were assessed for maximum response. Use of 200 nm gave the highest response for aspartame whilst the tryptophan response was relatively unaltered across the wavelengths investigated.

3.2. Validation

The validation aspects assessed are similar [22] to those evaluated for a chiral HPLC method and include performance parameters such as selectively, sensitivity, precision, linearity, response factors, repeatability of sample preparation, repeatability of separation on different instruments and recovery.

3.2.1. Selectivity

The migration order of the L-enantiomer before D-tryptophan was confirmed by spiking experiments.

3.2.2. Sensitivity

Fig. 3a and Fig. 3b show the separations obtained on two different CE instrument types of a sample containing 0.1% L-tryptophan spiked into the pure D-form. The signal-to-noise ratios and resolutions clearly indicate that lower limits of detection could be obtained if required.

3.2.3. Precision

Ten repeated injections of 1.5 mg/ml D-tryptophan dissolved in internal standard solution gave the data presented in Table 2. The use of the internal standard effectively eliminates the imprecision due to injection volume as shown by the dramatically improved precision value obtained using the internal standard (peak-area ratios rather than just peak area). The electrolyte system used gives reproducible migration times which can be improved by use of relative migration times. The use of two internal standards [23] allows sub 0.1% R.S.D. mobility data to be obtained on both an intra- and inter-day basis. Use of the migration time of one enantiomer as the internal reference for the other also permits sub 0.1% R.S.D. values to be obtained for relative migration times [24].

3.2.4. Linearity

The linearity of detector response (peak area) for D-tryptophan was assessed over the range 0.4–2.5 mg/ml. This represents 25–150% of the nominal method concentration. The correlation coefficient obtained was 0.9992 with an intercept value of +2.3% of the method value response. It is often typical in CE to obtain an appreciable positive intercept value due to an inadvertent injection resulting from capillary action that occurs when the capillary is initially dipped into the sample solution [25]. Use of an internal standard improves [26] both correlation coefficients (reduces scatter of points due to random error) and reduces intercept value (as it eliminates imprecision due to injection volume).

3.2.5. Response factors

As the enantiomers of the species being resolved are partially complexed into the cyclodextrin it is possible that each enantiomer may have a different UV response [27]. Therefore it is important to ascertain that the peak-area ratio of the two enantiomers equates to 1 (i.e. that the separation of a racemic sample gives peaks of equal peak area). It is important also that the peak areas of the peaks are normalised to their migration times [28] to compensate for their differential detector residence times. A racemic tryptophan sample was analysed and the D/L peak-area ratio obtained confirmed no significant difference between enantiomer responses.

3.2.6. Repeatability

Ten preparations of pure D-tryptophan were prepared and analysed. A precision of 1.45% R.S.D. was obtained for response factors. The separation and quantitation of 0.1% L-tryptophan in D-tryptophan was successfully repeated on different days, in different laboratories, using different capillary and reagent sources.

3.2.7. Transfer between instruments

This is a validation aspect that is peculiar to CE when compared to HPLC. In HPLC, methods can be transferred between equipment types with a minimum of modification, the most frequent being varia-

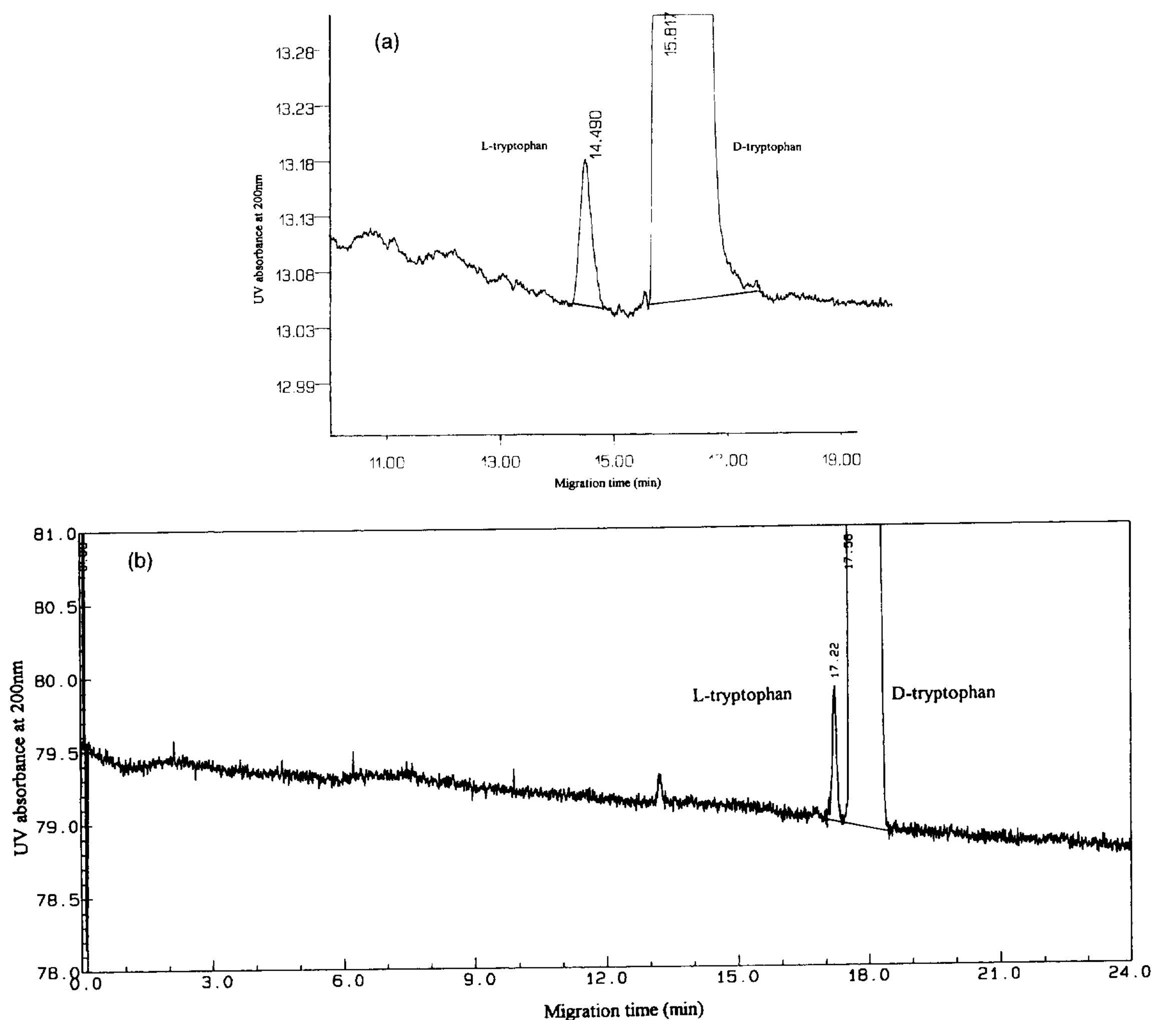

Fig. 3. Separation of 0.1% L-tryptophan present in D-tryptophan. (a) Beckman instrument; (b) ABI instrument. Separation conditions: see Table 1; 1.5 mg/ml tryptophan solution in electrolyte.

tions in gradient profiles to accommodate different pump dead-volumes. However, the present commercial CE instruments are generally first generation models and as such great differences exist between instrument types. Hydrodynamic injection procedures are different on various instruments, some relying on positive pressure, vacuum or gravity (siphoning) to inject the sample. The other major differences are related to the capillary lengths involved. It is often beneficial to employ short capillary lengths in order to use a lower voltage and limit the current. However, the minimum capillary lengths vary between instruments. The distance along the capillary at which the detector is located (L_d) also varies considerably. Peaks are detected sooner when

Table 2
Precision data ($n=10$) for repeat injections of D-tryptophan (on Beckman instrument)

Factor	R.S.D. (%)
Migration time	0.73
Relative migration time	0.60
Peak area	8.16
Peak-area ratio	0.80

using shorter L_d values. Therefore, it is necessary to adjust the voltage on the various instruments to achieve the required migration times.

The amount of sample injected per second (in nanolitres) is also specific to each instrument type fitted with a capillary of set dimensions. Charts are available from instrument suppliers which detail exact injection volumes for capillary dimension–injection time combinations. Therefore, it is possible to inject equivalent volumes into the capillary using different instruments.

Few reports have considered method transfer between instrument suppliers. The most comprehensive being a series of method transfer exercises between seven independent pharmaceutical companies [24,29,30].

It is necessary to demonstrate that the method can be successfully repeated on any of the instrument types available within the laboratories concerned where testing may be performed. In some instances due to capillary length, restrictions or inadequate sensitivity it may be impossible to repeat the separation on another instrument type. Fig. 3 shows the required detection of 0.1% L-tryptophan in the presence of the D-enantiomer achieved on two equipment types.

3.2.8. Recovery

Levels of L-tryptophan (0.1 and 1.0%) were spiked into pure D-tryptophan and quantified. The area/area ratio (normalised to migration time) confirmed good recovery data (0.12 and 1.04%, respectively).

4. Conclusion

This report has shown that a capillary electrophoresis method can be successfully validated for the determination of 0.1% D-tryptophan present in L-tryptophan. Use of aspartame as an internal standard allowed suitable precision to be obtained for assay purposes. The optimised conditions consisted of 75 mM α-CD dissolved in a triethanolamine–phosphoric acid electrolyte.

It was observed that optimal resolution was obtained using short injection times coupled with high sample concentrations. The detrimental effect of a damaged capillary tip was highlighted by loss of resolution and baseline perturbation.

Successful validation was achieved including suitable assessments of selectivity, sensitivity, linearity, response factors, sample preparation repeatability and method repeatability. The issues relating to instrument-to-instrument method transfer were discussed in detailed.

It is concluded that the reported operating conditions are suitable for the routine assay and enantiopurity determination of tryptophan.

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