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Separation and quantification of geometric isomers in a leukotriene antagonist using cyclodextrins in capillary electrophoresis Comparison to high-performance liquid chromatographic methods

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Abstract

CE is increasingly used in the pharmaceutical industry to separate and quantify enantiomers of chiral drugs. This article describes the use of CE towards other types of isomerism, namely geometric, that may be present in new drug substances. The ability of CE to separate and quantify four geometric isomers in a leukotriene antagonist is demonstrated and the resolving power and detectability of CE are compared to two HPLC methods.

Keywords: Geometrical isomers; Pharmaceutical analysis; Leukotriene antagonists

1. Introduction

The majority of applications that utilise cyclodextrins as run buffer constituents in CE are directed towards the separation of enantiomers. This particular technique has become the first choice for enantiomeric separations, especially in the pharmaceutical industry, and of the scores of references that are available, a few selected ones are cited here [1–6]. Porra et al. demonstrated the use of various cyclodextrins to resolve the enantiomers of both *ortho*- and *meta*-fenfluramine and specifically to quantify L-*meta*-fenfluramine in the presence of the D-enantiomer [7]. Werner et al. used β- and γ-cyclodextrins to resolve the enantiomers of a neuroactive piperazinecarboxylic acid after derivatization and to quantify to 0.1% of the undesired isomer [8].

There appears however, to be limited literature on the separation of geometric isomers and diastereomers. Clark et al. [9] managed to resolve and quantify geometric isomers of dothiepin using a β-cyclodextrin as a CE run buffer additive as well as determining related impurities in the same method. Bempong et al. studied the effect of acetonitrile, sodium dodecyl sulphate and α-cyclodextrin on the separation of 13-*cis*- from all-*trans*-retinoic acid [10]. Diastereomers have been successfully separated by CE with a mixture of β-cyclodextrin and a bile salt in the run buffer [11]; in this case the enantiomers of a serotonin agonist were converted to diastereomeric derivatives prior to injection into the capillary for separation with the cyclodextrin–bile salt mixture.

The advent of chiral HPLC and CE has enabled drug manufacturers to control levels of enantiomers to quite tight limits, typically less than 1%. Such close control of enantiomers is important because the antipodes of an enantiomeric pair can differ widely in their biological activity [12,13]. This can have important repercussions in patient health, if one isomer is more toxic than its optical twin, and in establishing good structure–activity relationships in early phase development. Equally, it can be argued that molecules which display isomerism other than

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optical isomerism, such as geometric should be subject to similar controls. For instance, the *cis* and *trans* isomers of a retinoic acid derivative may have different activity [14–16]. Historically, limits set by both the USP [17] and BP [18] for *cis–trans* isomers have been rather high; the *cis* isomer in dothiepin is limited to 7.5% and the *cis* isomer in doxepin has a range of 13 to 18.5%. The reasons for these high tolerances are not apparent, but may reflect the analytical technology available when these drugs were developed or a lack of knowledge on the relative biological activities of geometric isomers. The current trend in the pharmaceutical industry is to produce drugs containing a single isomer and so obviate any potential problems arising from differences in structural activity or metabolism. Current analytical technology such as CE makes this achievable by enabling the analyst to separate not only enantiomers, but also other isomeric species and to quantify undesired ones to low levels.

This paper describes the use of CE to resolve four geometric isomers of a leukotriene antagonist (LY170680) and its ability to control three of these isomers to below 1%. Previously, this separation was achieved using either of two HPLC methods [19]; the CE method is compared to the HPLC methods in terms of resolution, analysis time and detectability.

2. Experimental

2.1. Reagents

Acetonitrile, dichloromethane and methanol (all HPLC grade) were obtained from FSA Laboratory Supplies, Loughbrough, UK. Disodium hydrogenorthophosphate (anhydrous), orthophosphoric acid (85%, HPLC grade), disodium tetraborate, sodium hydroxide and trifluoroacetic acid (HPLC grade) were obtained from BDH, Poole, UK. α-Cyclodextrin was obtained from Sigma, Poole, UK. β-Cyclodextrin and hydroxypropyl β-cyclodextrin were obtained from Technicol, Stockport, UK. Distilled water was used for HPLC and distilled–deionised water was used for CE.

2.2. Apparatus

The HPLC system comprised a Milton Roy Constametric 3000 pump, a Waters Wisp 710B autosampler programmed to deliver 10 μl, a Pye-Unicam PU4020 ultra-violet detector set at 238 nm and Kipp and Zonen BD40 chart recorder. Chromatographic data were stored using a Hewlett-Packard HP1000 data system. HPLC columns used were: two 25 cm×0.46 cm I.D. Vydac C_{18} columns (part No. 218 TP 54) and a 10 cm×0.46 cm I.D. Hypercarb S column (Shandon Scientific, Cheshire, UK). pH adjustments to one of the mobile phases was monitored by a Beta 52 meter fitted with an EIL glass electrode.

For CE, a Spectra-Physics Spectraphoresis 1000 system (Thermo-Separation Products, Stone, UK) was used with a 70 cm×0.05 mm I.D. fused-silica capillary (62 cm to detector) maintained at 30°C. Electropherograms were stored using a Hewlett-Packard HP1000 data system.

2.3. Mobile phases

System 1: the following mobile phase was used to elute material from two coupled Vydac columns; 1 ml trifluoroacetic acid was dissolved in 600 ml water and the pH was adjusted to 2.4 with 5% (w/v) disodium hydrogenorthophosphate (anhydrous). 400 ml acetonitrile was then added. A flow-rate of 1 ml/min was used.

System 2: a mobile phase consisting of 680 ml methanol, 320 ml dichloromethane and 6.8 ml trifluoroacetic acid was used in conjunction with the Hypercarb column. A flow-rate of 1 ml/min was used.

2.4. Electrophoresis conditions

The running buffer was 0.1 *M* disodium tetraborate adjusted to pH 9 with orthophosphoric acid into which 0.5 mg/ml of α-cyclodextrin was finally dissolved. This was filled into the capillary for 2 min for each analysis. A run voltage of 30 kV with the anode as the injection end, was used for analysis. The capillary was washed both before and after each analysis with 0.1 *M* sodium hydroxide for 2 min, followed by water, also for 2 min.

A 1 s hydrodynamic injection was used to introduce sample into the capillary. Analytes were observed using a 238 nm detector wavelength.

2.5. Test solutions

For HPLC, 1 mg/ml solutions of LY170680 in methanol were injected.

For CE, 0.5 mg/ml solutions of LY170680 in run buffer were used.

3. Results and discussion

Fig. 1 shows the structure of LY170680 and three other isomeric arrangements of the conjugated diene system. Of these structures, the *trans–cis* isomer is the most active as a leukotriene antagonist, hence the synthesis is designed to produce mainly this isomer. Unfortunately, small quantities of the other species are produced as synthetic by-products which even after purification are still present at around the 1% level; therefore an analytical method was required to monitor these undesired contaminants.

Before the recent advent of CE instrumentation, methods to separate isomers centred on HPLC technology. The first useable method that was developed to assay the geometric isomer content of this drug used two coupled 25 cm Vydac C_{18} columns in combination with the mobile phase described under

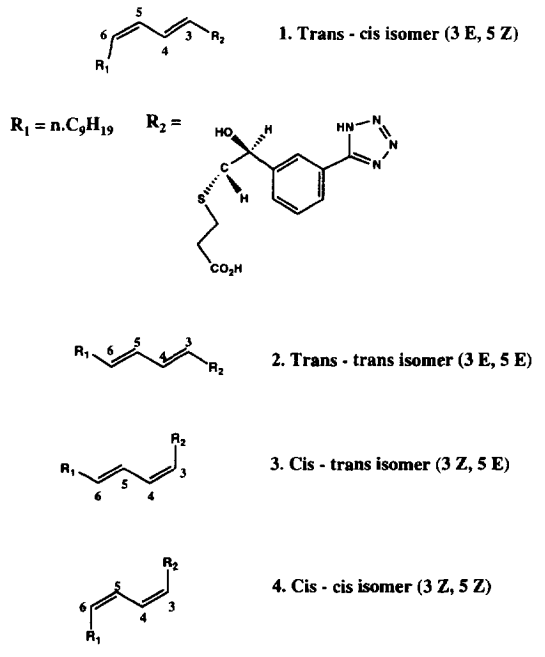

Fig. 1. Structure of LY170680 and isomers.

system 1. Fig. 2 shows a chromatogram from a development batch. The drawbacks with this method are quite apparent: a very long analysis time of 5 h 30 min is required to obtain even sub-optimal separation; the separation of *cis–trans* from the main peak is minimal and the *cis–cis* is eluted on the tail of the main peak. The accuracy and repeatability of peak area measurements would hence be compromised by this lack of total separation. High back-pressures of approximately 4000 p.s.i. were also a constant problem with the use of two 25 cm columns in series (1 p.s.i.=6894.76 Pa).

A considerable improvement in resolution and analysis time came about when an HPLC procedure based on the use of a porous graphitic carbon (PGC) column was developed. The properties of PGC as described by its co-inventor Knox et al. [20] were well suited to this type of problem and mobile phase 2 in combination with a 10 cm PGC column was found to produce baseline resolution of each geometric isomer in 35 min, (see Fig. 3). Higher sample throughput is now possible and peak areas would be more accurate, with subsequently more reliable estimates of the levels of the isomers present. For both HPLC systems, it was assumed that each isomer had the same response at the detection wavelength of 238 nm. Isomer content was calculated by the following procedure: a 100× dilution of the test solution was injected and the area of the major peak (*trans–cis*) was recorded. The 1 mg/ml test solution was also injected and areas of the minor isomer components were also recorded: ratios were then taken of each of these to the peak area from the 100× dilution to give a w/w percentage. This method was successfully used to monitor the isomer content of several development batches of this drug.

When CE became available in our laboratory, its application to this analysis was examined mainly to see if analysis time could be further improved upon. Method development was carried out on a specially prepared sample that contained approximately equal amounts of each geometric isomer. A survey of the literature revealed that cyclodextrins make excellent chiral selectors, so it was decided to investigate their use in resolving the four geometric isomers possible from the LY170680 structure. As stated in the Introduction (Section 1), cyclodextrins are mostly utilised as chiral selectors to resolve enantiomeric mixtures, but in our laboratory we have used these

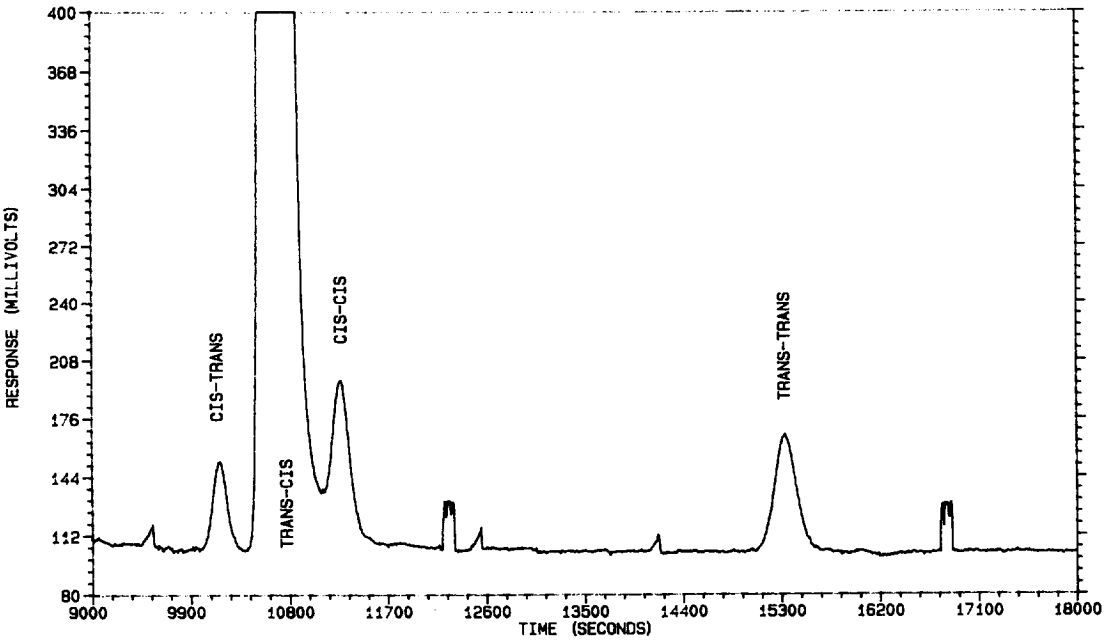

Fig. 2. Separation of LY170680 by Vydac columns. Mobile phase: 1 ml TFA in 600 ml water, pH 2.4 (5%, w/v, Na_2HPO_4), 400 ml acetonitrile. Flow: 1 ml/min. Column: 2×25 cm Vydac C_{18}. Detection: 238 nm.

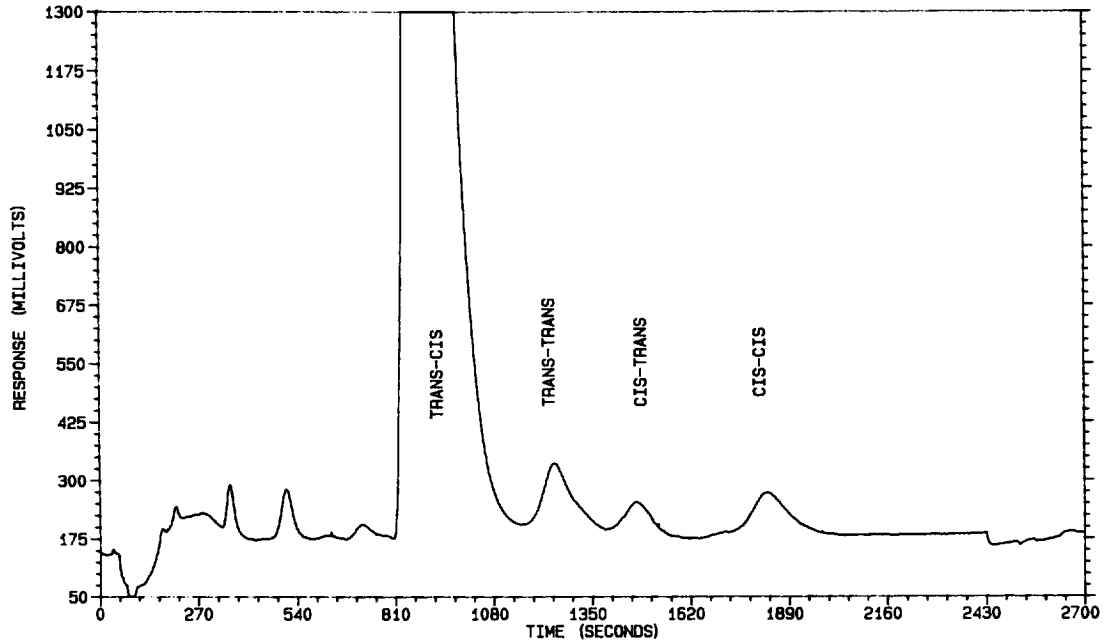

Fig. 3. Separation of LY170680 by carbon column. Mobile phase: 680 ml methanol, 320 ml dichloromethane, 6.8 ml TFA. Flow: 1 ml/min. Column: 10 cm Hypercarb S. Detector: 238 nm.

substances to resolve the two diastereomers as well as the enantiomer of a new drug containing two chiral centres. The appropriate buffer was initially decided upon: a pH 2.5 phosphate and a pH 9 borate buffer were tried both with 1 mg/ml methyl-β-cyclodextrin. The pH 9 buffer yielded two peaks and pH 2.5 yielded only one, so work proceeded with pH 9 buffer. This decision to concentrate efforts on pH 9 after minimal experimentation was justified on two counts: (1) varying the level of methyl-β-cyclodextrin with pH 9 buffer produced even more peaks and (2) from theoretical considerations. At pH 9, LY170680 is a carboxylate anion and will be attracted towards the anode which is the injection end of the capillary. This electrophoretic flow will oppose the electro osmotic flow which is towards the cathode at high pH values and which will eventually sweep the analyte past the detector. With these two types of flow opposed to each other, there exists a greater possibility that separation can be achieved once the isomer selector (in this case cyclodextrin) is added. Further studies with different levels of α-cyclodextrin, methyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin rapidly lead to optimum conditions. Using 0.5 mg/ml of α-cyclodextrin in pH 9 buffer produced the electropherogram shown in Fig. 4 from the special isomer mixture. Peak assignment was straight forward; injection of a typical development batch identified the *trans–cis* isomer which is the required form, injection of small-scale batches whose synthetic routes had been modified to produce an enhanced level of each isomer readily identified the other peaks. The theory behind the resolution of isomers by cyclodextrin additives is well established. When an analyte becomes included into the apolar cavity of the cyclodextrin, its mobility is greatly reduced and the extent of inclusion is a function of the stability constant. At the high pH used for this separation, the analyte with the higher stability constant is detected first as the electro osmotic flow that sweeps all species in the capillary towards the detector is opposed by the electrophoretic flow of the unbound analyte. For enantiomer separations it is postulated that one enantiomer complexes more favourably with the stereo-specific environment provided by the cyclodextrin cavity. In the case of *cis–trans* isomer separations, a similar mechanism could exist where-

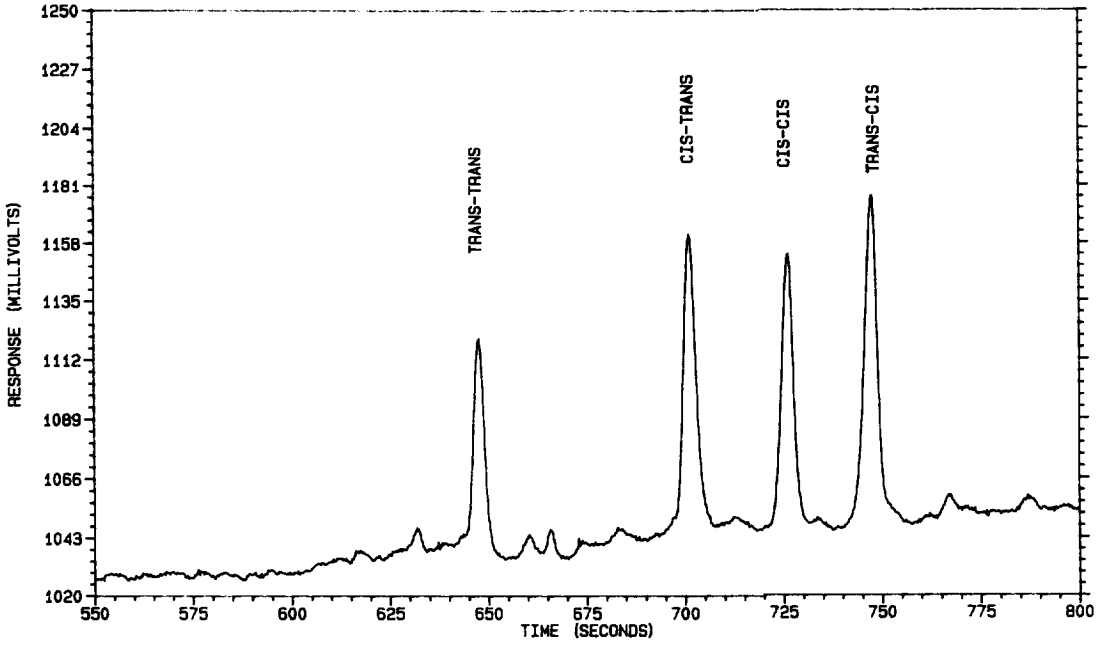

Fig. 4. Isomer mixture separation by CE. Capillary: 70 cm×0.05 mm I.D. fused-silica (62 cm to detector), 30°C. Run buffer: 0.5 mg/ml α-cyclodextrin in 0.1 *M* disodium tetraborate, pH 9. Voltage: 30 kV. Detector: 238 nm.

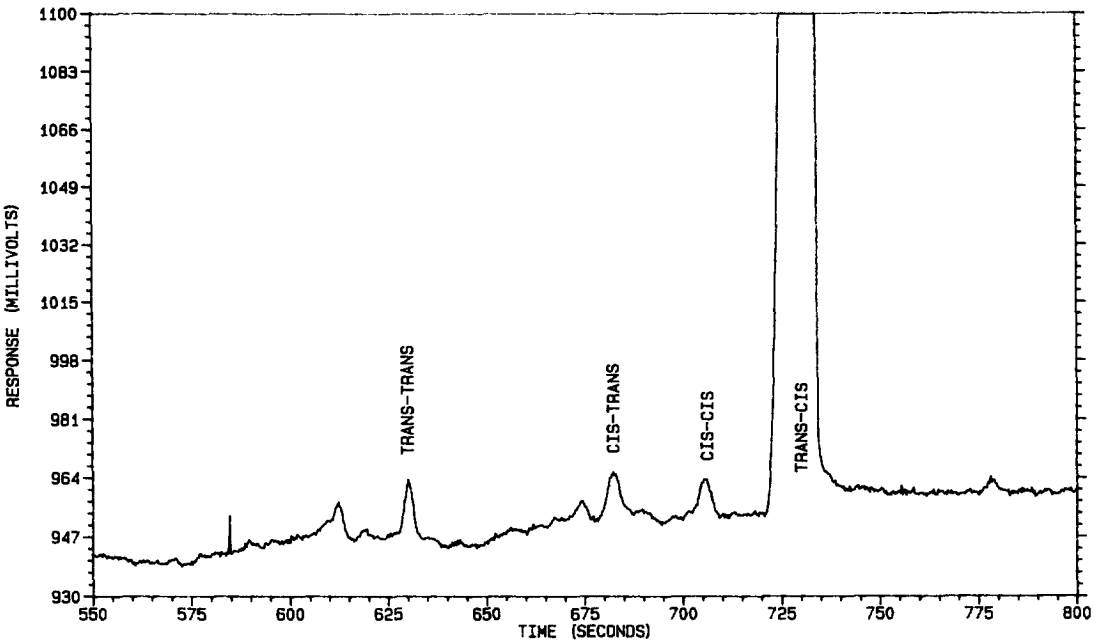

Fig. 5. Separation of LY170680 by CE. Capillary: 70 cm×0.05 mm I.D. fused-silica (62 cm to detector), 30°C. Run buffer: 0.5 mg/ml α-cyclodextrin in 0.1 *M* disodium tetraborate, pH 9. Voltage: 30 kV. Detector: 238 nm.

by the different geometries of each of the isomers complex to different degrees with the apolar cyclodextrin core. Fig. 5 is the electropherogram from a development batch and it is very interesting to compare this separation to the best that HPLC could produce (Fig. 3, carbon column). Analysis time has now been reduced to 13 min with greater baseline resolution of each isomer; this represents a marked improvement on the carbon column method as far as separation is concerned. To quantify the isomers, each peak area was firstly divided by the migration time in order to compensate for the different velocities with which each isomer passes the detector. A similar calculation to that used in the HPLC methods was then used to calculate the isomer content of the LY170680 batch. Accurate quantification in CE is highly dependent on the injection type and on the injection matrix e.g., ionic strength. Hydrodynamic injection was selected in preference to electrokinetic injection to avoid loading faster-migrating species at the expense of slower ones, which would introduce a bias to the integrations. The sample was dissolved in the separation buffer so that it was in a solution of similar ionic strength. It was considered that these measures would give the best chance of producing results for the isomeric species that were representative of their levels in LY170680. The best approach to quantify the isomers was considered to be by total peak area normalisation as previously described, because this should yield more repeatable results by eliminating injection variability. The alternative method of using external standards of each isomer was not possible because suitable standards were unavailable.

Results obtained by each HPLC and the CE method on the same LY170680 batch are displayed in Table 1. Close examination of baseline noise from the electropherogram and chromatograms from the two HPLC methods show that noise is worse in the

Table 1
Comparison of results from three methods (percent of total isomer recovered)

	Vydac HPLC	Carbon HPLC	CE
cis–trans	0.10	0.34	0.44
cis–cis	0.05	0.19	0.27
trans–trans	1.05	0.41	0.56

Results expressed as % of total isomer content.

electropherogram and therefore indicates that CE would not be capable of detecting to such low levels as HPLC. The relative standard deviations were calculated for the CE and carbon column methods and indeed confirm this observation: for CE, the R.S.D. is 20%, yet for HPLC, it is 6%. This observation is indeed typical of CE and represents one analytical aspect where HPLC has an advantage over CE.

The results shown in Table 1 reveal that there is reasonable agreement between the CE and carbon column methods, but poor agreement between these two methods and the Vydac method, a probable consequence of the poorer resolution achieved by the Vydac procedure. The agreement between CE and carbon column method is corroborated by NMR data (Lilly Research, internal report). NMR gives a combined value for *cis–cis* and *cis–trans* of 0.5% for this batch which agrees very well with the carbon column method. The CE data is not in such good agreement, but can still be regarded as reasonable considering the low levels being assayed and the greater variability of the CE data.

4. Conclusion

The use of cyclodextrins as run buffer additives in CE can be used to separate and monitor geometric isomers as well as enantiomers. This extends the range of isomeric species that may be controlled in new pharmaceuticals and so help to ensure that a single-component product is produced. It has been demonstrated that CE can markedly improve the resolution of isomer separations compared to HPLC and to decrease analysis times. One disadvantage of CE is its lack of sensitivity.

References

[1] S.J. Fanali, J. Chromatogr., 474 (1989) 441.
[2] S.J. Fanali and P. Bocek, Electrophoresis, 11 (1990) 757.
[3] S.J. Fanali, J. Chromatogr., 545 (1991) 437.
[4] P.J. Gilbert, M.J. Dethy and M.L. Lesne, in 2nd International Symposium on Chiral Discrimination, Rome, July, 1991.
[5] M. Tanaka, S. Asano, M. Yoshinago, Y. Kawaguchi, T. Tetsumi and T. Shono, J. Fresenius, Anal. Chem., 339 (1991) 63.
[6] M. Yoshinaga, S. Asano, M. Tanaka and S. Toshiyuki, Anal. Sci., 7 (1991) 257.
[7] R. Porra, M.G. Quaglia and S.J. Fanali, Cromatographia, 41 (1995) 383.
[8] A. Werner, T. Nassauer, P. Kiechle and F. Erni, J. Chromatogr. A, 666 (1994) 375.
[9] B.J. Clark, P. Barker and T. Large, J. Pharm. Biomed. Anal., 10 (1992) 723.
[10] D.K. Bempong and I.L. Honiberg, J. Pharm. Biomed. Anal., 11 (1993) 829.
[11] G.N. Okafo, K.K. Rana and P. Camilleri, Chromatographia, 39 (1994) 627.
[12] K. Wetterlin, J. Med. Chem., 15 (1972) 1182.
[13] A.J. Nichols, A.C. Sulpizio, D.J. Ashton, J.P. Hieble and R.R. Ruffolo, Chirality, 1 (1989) 265.
[14] D.B. Windhorst and T. Nigra, J. Am. Acad. Dermatol., 6 (1982) 675.
[15] D.M. Kochler, J.D. Penner and C. Tellone, Terrat. Carcinog. Mutagen., 4 (1984) 377.
[16] C.E. Orfanos, R. Ehlert and H. Gollnick, Drugs, 34 (1987) 459.
[17] United States Pharmacopoeia XX111, United States Pharmacopoeial Convention, Rockville, MD, 1995.
[18] British Pharmacopoeia, HMSO, London, 1993.
[19] B.J. Fish, J. Pharm. Biomed. Anal., 11 (1993) 517.
[20] J.H. Knox, B. Kaur and G.R. Millward, J. Chromatogr., 352 (1986) 3.