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# Analysis of amino acid enantiomers derived from antitumor antibiotics using chiral capillary electrophoresis

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**Abstract**

The chiral separation of enantiomeric forms of derivatized amino acids have been achieved based on a metal–chelate chiral capillary electrophoretic method and a cyclodextrin mediated host–guest interaction approach in micellar electrokinetic chromatography (MEKC) mode with laser-induced fluorescence detection. This approach has been applied to the determination of enantiomeric forms of amino acids derived from novel depsipeptide antitumor antibiotics, BMY-45012 and its analogs. Amino acids were analyzed by complete hydrolysis and the hydrolysate was derivatized with either dansyl chloride for UV absorbance detection or fluorescein isothiocyanate for laser based fluorescence detection. The presence of several amino acids, serine and $\beta$-hydroxyl-*N*-methy-valine in the proposed structure have been confirmed as D-serine and L-$\beta$-hydroxyl-*N*-methy-valine enantiomeric forms by both chiral capillary electrophoresis (chiral CE) and MEKC approaches. A non-chiral amino acid, sarcosine, was also confirmed. These methodologies provide a quick and sensitive approach for the determination of amino acids racemization of pharmaceutical natural products and have proven to be useful for structural elucidation refinement. © 1997 Elsevier Science B.V.

*Keywords:* Capillary electrophoresis; Chiral separation; Amino acids; Antibiotics

## 1. Introduction

In the past decade, the importance of chirality in biological phenomena, chemical synthesis and drug design has considerably accelerated the demands in areas dealing with enantiomeric separation and identification of racemic mixtures. The resolution of amino acid enantiomers is of interest to drug discovery and pharmaceutical development fields, especially, in peptide synthesis and structure determination and elucidation. Chromatographic methods such as high-performance liquid chromatography (HPLC) and gas

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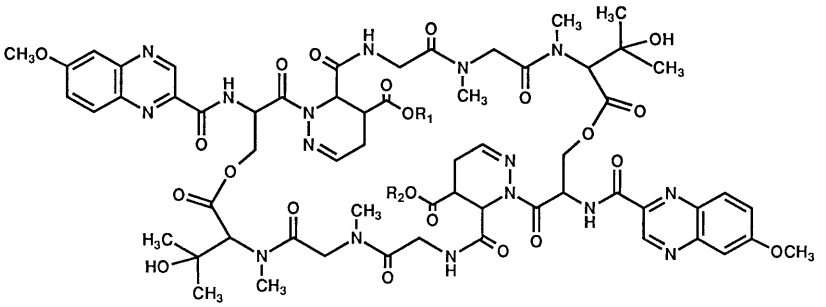

| Compound | $R_1$ | $R_2$ |
|---|---|---|
| BMY45012 | $CH(CH_3)_2$ | $CH(CH_3)_2$ |
| Analog-G439B | $CH_2CH_3$ | $CH_2CH_3$ |
| Analog-G451 | $C(CH_3)_3$ | $C(CH_3)_3$ |
| Analog-G435 | $C(CH_3)_3$ | $CH(CH_3)_2$ |
| Analog-G439A | $CH(CH_3)_2$ | $CH_3$ |

Fig. 1. Proposed structure of BMY-45012 and analogs.

chromatography (GC) have been the most commonly used analytical tools to resolve these enantiomers [1–5]. Various approaches have been introduced to facilitate the resolution of chiral compounds (such as chiral reagents either immobilized in the stationary phase or added to the mobile phase to assist the separation process). Generally, the design of proper chiral stationary phases to meet the needs of sophisticated chiral separations of enantiomeric mixtures has been a complex and expensive procedure. Furthermore, consumption of large amounts of chiral reagents in the mobile phase has drawbacks. Precolumn derivatization using appropriate agents leading to the formation of the more readily separated diasteromeric derivatives is a very useful approach and has recently been incorporated into other separation techniques.

Capillary electrophoresis (CE), as a complementary separation technique to HPLC, has made remarkable progress as a highly efficient and relatively fast method providing high resolution in various application areas. Several different separation modes, such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), and capillary gel electrophoresis (CGE), have been developed to facilitate chiral separation of racemic compounds. The versatility of various chiral selectors, such as cyclodextrins, proteins, and metal-chelate complexation, or pseudophases, micelle and chiral additives, has made these electrophoretic methods very attractive for the separation of various types of chiral compounds [6–12]. The previous approaches using diastereomeric interaction adapted from HPLC methods have proven to be very successful for enantiomeric separation of DL-amino acids [8,9]. Current studies focus on the achievement of chiral separation of amino acids by chiral CE based on both metal–chelate complexation in the normal CE mode and host–guest inclusion involved with cyclodextrins in the MEKC mode. The established methodologies were subsequently applied to the chirality analysis of pharmaceutical drug derived compositions in structural determination and elucidation.

Racemization of amino acids in many microbial products, such as polypeptide antibiotics, is a commonly occurring natural phenomena, which often needs to be elucidated in the determination of structure with regard to synthesis, regulatory and patent issues. BMY-45012 and its analogs are

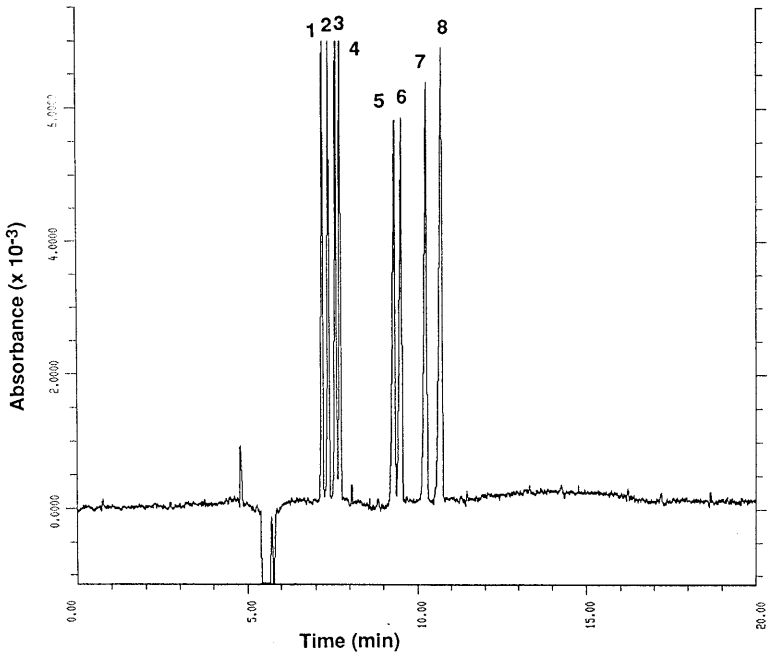


Fig. 2. Electrophoretic separation of enantiomeric forms of standard dansylated amino acids. Peak identification: 1, D-Trp; 2, L-Trp; 3, D-Val; 4, L-Val; 5, D-Asp; 6, L-Asp; 7, D-Glu; 8, L-Glu. Background electrolyte, 10 mM ammonium acetate–5.0 mM aspartame–2.5 mM copper(II) sulfate pentahydrate (pH 7.55). Capillary, 360 μm o.d., 75 μm i.d. × 43 cm (36.2 cm effective separation length). Pressure injection, 5 s. Separation voltage, 10 kV (20 μA).

a class of compounds isolated from a fermentation product and have been identified as novel depsipeptide antitumor antibiotic compounds. Structural studies indicate that these compounds are closely related to the cyclic depsipeptide sandramycin [13], which was found strongly inhibitory to the Gam-positive organisms *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus faecalis* and also moderately active against leukemia P388 in mice. The proposed structure of BMY-45012 and representative analogs are shown in Fig. 1. The chiral identification of the amino acids provided valuable information for refining structural data and subsequent chemical synthesis of analogs. Our approach required the establishment of analytical methods to facilitate the analysis of amino acids and chiral recognition of the corresponding enantiomers. In this communication, the metal–chelate based CE approach with UV detection and cyclodextrin modified MEKC method with laser based detection have been used for the chiral separation of several amino acids derived from novel depsipeptide antitumor antibiotic BMY-45012, which have been derivatized with either dansyl chloride or fluorescein isothiocyanate (FITC) to facilitate the appropriate detection method. As will be demonstrated in this paper the goal of the current chiral CE studies is to develop a practical CE based analytical approach for the identification of chirality of natural product derived amino acids in drug discovery and pharmaceutical development processes.

## 2. Experimental

### 2.1. Apparatus

Chiral capillary electrophoresis was performed using untreated fused silica capillaries (polymicro Technology, Phoenix, AZ) with 75 μm i.d. and 360 μm o.d. and an effective separation length of

37–50 cm. The detection window was made by burning the polyimide coating and placed at about 7 cm from the outlet. A Beckman P/ACE model 2100 CE system (Beckman Instruments, Fullerton, CA) equipped with a UV absorbance detector was used for all separations. An alternative 5.0 mW argon-ion laser emitting at 488 nm was also incorporated into the system. The laser beam is transmitted via a fiber-optic cable to the detector and illuminates the capillary window. The fluorescence signal was collected through a narrow-band 520 nm emission filter. The temperature controlled capillary chamber was maintained at 25°C by employing a liquid coolant. Pressure injection mode (0.5 p.s.i. low pressure) was employed throughout all experiments. The components derivatized with dansyl chloride were monitored with the UV detector at a wavelength of 214 nm and the FITC derivatives were monitored by laser-induced fluorescence (LIF) detection. Data were collected and analyzed with Beckman System Gold software.

### 2.2. Materials and reagents

Standard native and dansylated amino acids, dansyl chloride, FITC, aspartame, $\gamma$-cyclodextrin were purchased from Sigma (St. Louis, MO). BMY-45012 and analogs are natural antitumor antibiotic products isolated from a fermentation product and purified in-house. Background electrolytes consisted of 10 mM ammonium acetate, 5 mM aspartame, and 2.5 mM copper(II) sulfate pentahydrate with pH adjusted to 7.55 in metal–chelate chiral CE separations. The buffer consisted of 20 mM borate, 20 mM phosphate, 10 mM $\gamma$-cyclodextrin, and 50 mM SDS (pH 9.50) was employed in chiral MEKC experiments. The standard dansylated amino acids were dissolved in 50% methanol–50% water solvent at a concentration of $5.0 \times 10^{-4}$ M. The fused silica capillary was pre-rinsed sequentially with 1 M sodium hydroxide and deionized water prior to use. The column was then equilibrated with running buffer before sample injection. The capillary was washed and refilled with fresh buffer between electrophoretic runs.

### 2.3. Hydrolysis and derivatization

The total hydrolysis of BMY-45012 and analogs was carried in a vacuum hydrolysis tube with 6 M HCl at 110°C for 24 h. The hydrolyzed residue (about 7.8 mg) was redissolved in water–acetonitrile solvent. For subsequent derivatization, the solution was further diluted to about 1.7 mg/ml by water. Dansyl chloride dissolved in acetonitrile (3.0 mg/ml) was utilized for the derivatization of standard native amino acids and those present in hydrolysate. A 50 μl sample solution was mixed with 50 μl dansyl chloride solution and an aliquot of borate buffer at pH 9.08. The mixture was held at room temperature for 2 h and used directly for injection. For fluorescence detection, the fluorogenic reagent FITC was dissolved in acetone (0.01 M) as stock solution. Amino acids were derivatized with FITC derivatizing solution ($5.0 \times 10^{-4}$ M) under basic conditions (borate buffer, pH = 9.08). The reaction was allowed to proceed for 2–4 h in the dark at room temperature and then stored at −20°C prior to use.

## 3. Results and discussion

Several different approaches have been utilized to achieve chiral separation of DL-amino acids. Diasteromer formation through metal-chelation and other derivatization and cyclodextrins based host–guest interaction are among the most commonly used methods. Using of Cu(II)–aspartame complex as chiral selector in the mobile phase in HPLC separation [14] has recently been extended to the electrophoretic separation format ([8,9]) where the metal–chelate complex was utilized in the background electrolyte for chiral recognition. The formation of a ternary complex of Cu(II) in the presence of aspartame appears to be the essential step responsible for the chiral separation of enantiomeric forms [9]. In order to establish a practical method for general applications in pharmaceutical development, a similar approach with UV absorbance detection was employed in the present studies.

The electropherogram shown in Fig. 2 was obtained with a commercial instrument capable of accommodating both laser and UV detection approaches. The electrophoretic buffer contained 10 mM ammonium acetate, 5.0 mM aspartame and 2.5 mM copper(II) sulfate with pH adjusted to 7.55, i.e. essentially the same support electrolyte as reported. The fused silica capillary was directly used for the separation without any further treatment. Standard amino acids are derivatives of dansyl chloride which facilitate both UV and fluorescent detection. Since our argon-ion laser based detector does not match the maximum UV excitation wavelength of 360 nm for the dansylated amino acids, we chose UV absorbance detection in our preliminary studies. As shown in Fig. 2, several dansylated DL-amino acids were resolved into their enantiomeric forms. The separation is comparable with those obtained using laser detection although larger amounts of sample are required for UV detection which can result in a loss of resolution in CE separation as it may overload the column capacity. These studies indicate the present method is suitable for subsequent determination of amino acid racemization in the antitumor antibiotic BMY-45012 and analogs.

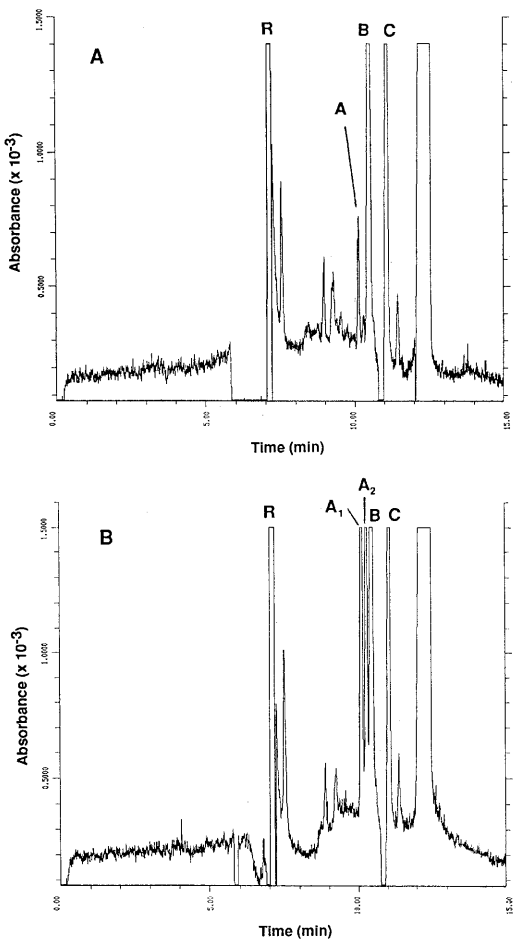


Fig. 3. Electrophoretic separation of dansyl chloride derivatized hydrolysate from original BMY-45012 analog (A) and spiked electropherogram with one standard amino acid (B). A: R, reference peak (Dan–L–Arginine); corrected migration time: peak A, 1.43; peak B, 1.48; peak C, 1.57. B: R, reference peak; corrected migration time: peak $A_1$ (Dan–D-Serine), 1.43; peak $A_2$ (Dan–L-Serine), 1.46; peak B, 1.48; peak C, 1.57. Pressure injection, 2 s. Other conditions are the same as in Fig. 2.

The unique cyclic depsipeptide structure of BMY-45012 and analogs has been proposed as shown in Fig. 1. Complete amino acid analysis revealed the three major amino acids contained in these structures (serine, a valine derivative, and sarcosine) although they have different substituent functional groups, $R_1$ and $R_2$. Two of these amino acids possess chirality and are separable into their enantiomeric forms. The chiral forms were well resolved and served as a reference for the subsequent identification of amino acid racemization of BMY-45012 and analogs. Since standard DL-$\beta$-hydroxyl-*N*-methyl-valine is not readily available, we have substituted it with DL-valine. Little effect on charge status would be expected with this substitution at neutral pH conditions. The substituted functional groups do not participate in the formation of ternary complex with Cu(II) and aspartame based on the described mechanism of action [9] and thus will not affect chiral recognition.

To determine the enantiomeric forms of amino acids in BMY-45012 and analogs, the compound was subjected to complete hydrolysis under vacuum in 6 M HCl and 110°C for 24 h. The hydrolysate was subsequently derivatized with dansyl chloride for UV detection. Fig. 3 illustrates the identification of chirality of serine derived from hydrolysis of a BMY-45012 analog. A dansyl chloride derivatized L-arginine was used as an

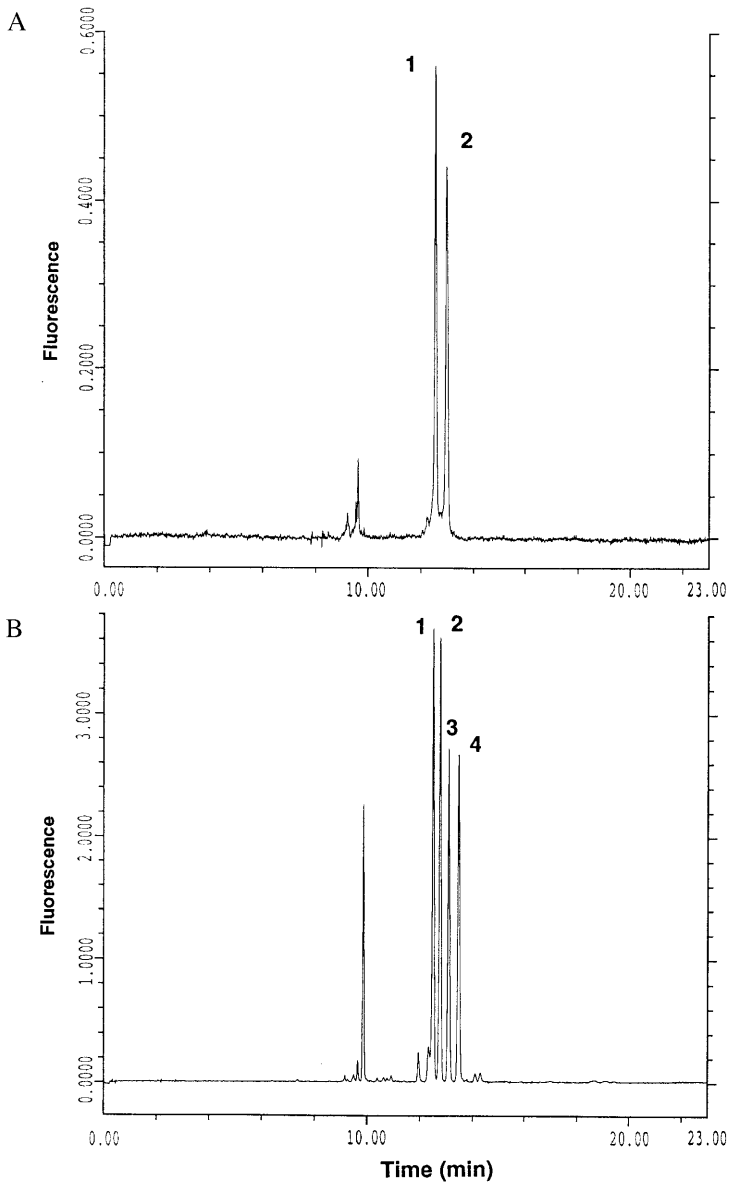


Fig. 4. Separations of FITC derivatized amino acids by micellar electrokinetic chromatography with laser-induced fluorescence detection. (A): 1, FITC–DL-Val; 2, FITC–DL-Ser; buffer, 10 mM ammonium acetate–5.0 mM aspartame–2.5 mM copper(II) sulfate pentahydrate (pH 7.55). (B): 1, FITC–D-Val; 2, FITC–L-Val; 3, FITC–D-Ser; 4, FITC–L-Ser; buffer, 20 mM borate–20 mM phosphate–10 mM $\gamma$-cyclodextrin–50 mM SDS (pH 9.50). Capillary, 75 μm i.d. × 57 cm (50 cm effective separation length). Applied voltage, 10 kV (87 μA).

internal reference for the calculation of corrected migration times of other amino acid enantiomeric derivatives [9,15]. The enantiomeric forms have been identified by adding either derivatized DL-amino acid or L enantiomer to the hydrolyzed mixture. The electropherogram in Fig. 3A was obtained from original hydrolyzed sample. Peak R is the reference and peaks A, B, and C have

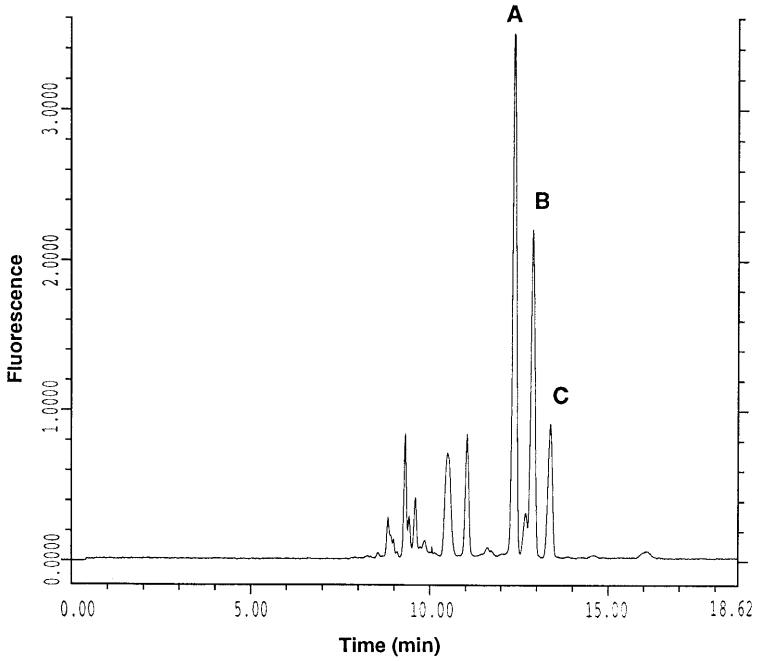


Fig. 5. Separations of FITC derivatized amino acids derived from a BMY-45012 analog by micellar electrokinetic chromatography with laser-induced fluorescence detection. Peaks identified: A, FITC–L-$\beta$-hydroxyl-*N*-methyl-valine; B, FITC–D-Ser; C, sarcosine. Buffer, 20 mM borate–20 mM phosphate–10 mM $\gamma$-cyclodextrin–50 mM SDS (pH 9.50). Capillary, 75 μm i.d. × 57 cm (50 cm effective separation length). Applied voltage, 10 kV (87 μA).

corrected migration times, 1.43, 1.48 and 1.57, respectively. Addition of dansylated DL-serine to the sample resulted in two extra peaks designated as $A_1$ and $A_2$ due to the resolved enantiomeric forms as shown in Fig. 3B. The corrected migration time was found to be 1.43 for D-serine (peak $A_1$) which correlates with peak A found in Fig. 3A and 1.46 for L-serine (peak $A_2$) that appeared between the original peaks A and B in Fig. 3A. The corrected migration time remains unchanged for peaks B and C. These results suggest that the amino acid serine originated from BMY-45012 analog is a D enantiomer. Similar experiments were also performed for enantiomeric identification of the valine derivative. As discussed above, dansylated DL-valine was used to add to the derivatized hydrolysate. It was found that the L enantiomer co-migrated with peak B found in Fig. 3A with same corrected migration time of 1.48, indicating a L enantiomeric form of valine derivative is present in BMY-45012. The presence of a non-chiral amino acid, sarcosine was also confirmed as peak C with corrected migration time of 1.57. The unidentified peaks are most likely related to the other parts of the studied structure, or from impurities and reagent, which are not the subject of interest in the current studies.

To further explore the enantiomeric separation and chirality identification of amino acids in drug substances, we have also investigated the feasibility of MEKC with chiral additives, one of the chiral CE versions. A laser-induced fluorescence detection approach has been incorporated into the system to enhance the sensitivity and selectivity. A common fluorogenic reagent, FITC, was used to derivatize amino acids, which yielded fluorescence at 550 nm while excited at 495 nm. The argon-ion laser with 488 nm blue line equipped with our CE system is well suited for this purpose. Fig. 4 shows the electrophoretic separations of two FITC derivatized amino acids, which were expected to be present in antibiotic agents as previously dis-

cussed, FITC–DL-serine and FITC–DL-valine. It was interesting to note that the two FITC derivatives were well separated from each other (Fig. 4A), but their enantiomeric forms were not resolved under the same conditions as previously described for dansylated amino acids with UV detection. By employing a buffer system consisting of 20 mM borate, 20 mM phosphate and 50 mM SDS (pH 9.50) with 10 mM $\gamma$-cyclodextrin, the enantiomeric forms of these derivatives were well resolved as seen in Fig. 4B. The different chiral separation mechanism between the metal chelation, where a ternary complex is formed, and the host–guest interaction involved with cyclodextrins and derivatives may play a key role in this phenomenon. The structural alteration of FITC derivatives may prevent the molecules from forming the ternary complex as proposed. Instead, this perhaps is a favorable situation for cyclodextrin inclusion.

This approach has been applied to the analysis of BMY-45012 and analogs for the chiral identification of derivatized amino acids. As described previously, the sample was hydrolyzed prior to FITC derivatization and a electropherograms of the derivatives are shown in Fig. 5. Standard addition methods have been utilized to identify the separated peaks. Both the L enantiomeric form of valine derivative and the D form of serine have been identified as peak A and B, respectively, as indicated in Fig. 5. Peak C was identified as sarcrosine. It was also interesting to note that the last major peak found in Fig. 3 disappeared in this case, most likely due to the lack of fluorophore attachment. The results obtained by the MEKC approach in conjunction with chiral cyclodextrin inclusion and laser based fluorescence detection appear to correlate well with those found by using chelating complex and UV detection.

CE based chiral separation has been proven to be a useful approach for the identification of enantiomeric forms of amino acids present in natural compounds. The methodology combines the resolving power of electrophoretic separation, speed and sensitivity with the unique chiral selectivity inherited from other chromatographic techniques. The information obtained in these studies provided important details for refining the structure of the depsipeptide antitumor antibiotic agents. The approach is not limited to the examples presented in this study. It demonstrates examples for developing practical methods for various applications in drug discovery and pharmaceutical development research.

## References

[1] V.A. Davankov, A.A. Kurganov and A.S. Bochkov, in J.C. Giddings, E. Grushka and P.R. Brown (Eds.), Advances in Chromatography, Vol. 22, Marcel Dekker, New York, 1983, p. 71.
[2] B. Koppenhoefer, E. Bayer, Chromatographia 19 (1984) 123.
[3] D.W. Armstrong, Anal. Chem. 59 (1987) 84A.
[4] C. Pertersson, Trends Anal. Chem. 7 (1988) 209.
[5] W.H. Pirkle, T.C. Pochapsky, Chem. Rev. 89 (1989) 347.
[6] S. Terabe, Trends Anal. Chem. 8 (1989) 129.
[7] T. Arai, M. Ichinose, H. Kuroda, N. Nimura, T. Kinoshita, Anal. Biochem. 217 (1994) 7.
[8] E. Gassmann, J.E. Kuo, R.N. Zare, Science 230 (1985) 813.
[9] P. Gozel, E. Gassmann, H. Michelsen, R.N. Zare, Anal. Chem. 59 (1987) 44.
[10] K. Otsuka, S. Terabe, J. Chromatogr. 515 (1990) 221.
[11] T.J. Ward, Anal. Chem. 66 (1994) 633A.
[12] M.V. Novotny, H. Soini, M. Stefansson, Anal. Chem. 66 (1994) 646A.
[13] J.A. Matson, J.A. Bush, J. Antibiotics. 42 (1989) 1763.
[14] C. Gilson, R. Leshem, Y. Tapuhi, E. Grushka, J. Am. Chem. Soc. 101 (1979) 7612.
[15] A. Guttman, N. Cooke, J. Chromatogr. 685 (1994) 155.

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