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Enantiomeric separation of small chiral peptides by capillary electrophoresis

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Abstract

Different approaches to the enantiomeric separation of sixteen derivatized di- and tripeptides, having one or two chiral centers, by means of capillary electrophoresis have been examined. The approaches were: direct separation using cyclodextrin (CD), vancomycin or teicoplanin and indirect separation. The highest plate numbers, 10^6 plates/m, were obtained with CD and the indirect method. The maximum resolution was 3 with CD and 11 when using the indirect method. The antibiotics gave somewhat lower plate numbers, $0.5 \cdot 10^6$ plates/m, but the resolution was high, up to 38. All examined peptides, except Leu–Ala, Leu–Leu and Arg–Gly, were fully resolved with the antibiotics as chiral selectors. The three remaining peptides were fully resolved when using the indirect method. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Chiral selectors; Diastereomer separation; Peptides; Teicoplanin; Vancomycin

1. Introduction

During recent years, it has been demonstrated that capillary electrophoresis (CE) is a potentially powerful technique for the rapid separation of enantiomers. The main virtue of CE, in this context, is the high number of theoretical plates which makes it possible to fully utilize the separation power of chiral selectors. Traditionally, chiral separations have been performed with the high-performance liquid chromatography (HPLC) technique where, in practice, a different separation column is required for each type of chiral selector. In CE, the selector is part of the buffer, and that makes the system very flexible. Selectors can be added to the mobile phase in HPLC; however, since most selectors are expensive, this is, in most cases, not a practical approach. Finally, HPLC provides much lower plate numbers than CE, but as a consequence of gradient elution, selectivity may still be quite powerful in HPLC. At the present state of the art the two techniques can be considered as complementary.

Chiral separation of small peptides is an important issue because of the different biological activity of the enantiomers. Such separations have been performed on gas chromatography [1], HPLC [2–9] and CE [10–15]. At our laboratory, the performance of some different chiral selectors for direct chiral separation, by means of CE, of a group of di- and tripeptides has been evaluated. The selectors were cyclodextrin (CD) [15], vancomycin [16,17] and teicoplanin [18]. Further, the application of the indirect approach was evaluated [15]. In the present work, a comparison of different approaches to chiral separation of derivatized di- and tripeptides is pre-

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sented. Further, the influence of buffer pH on the performance of teicoplanin and vancomycin as selectors for chiral separation of small peptides by CE is investigated.

2. Experimental

2.1. Materials

The chiral selectors β-CD and γ-CD were provided by Sigma (St. Louis, MO, USA). Teicoplanin was from Resolution Systems (Wilmette, IL, USA), and vancomycin was obtained from ICN Biomedicals (Aurora, OH, USA). The peptides (D-form and DL-peptides) examined in this work included: Ala–Ala, Ala–Gly, Ala–Gly–Gly, Ala–Leu, Gly–Ala, Gly–Asn, Gly–Asp, Gly–Leu, Gly–Met, Gly–Phe, Gly–Val, Leu–Ala, Leu–Gly, Leu–Gly–Gly, Leu–Leu, all these were obtained from Sigma. The Arg–Gly was from Ferring (Malmö, Sweden). Sodium dodecyl sulfate (SDS) was obtained from Fluka (Buchs, Switzerland). The derivatization reagent, 9-fluorenylmethyl chloroformate (FMOC) was obtained from Fluka, and (+)- and (−)-1-(9-fluorenyl)-ethyl chloroformate (FLEC) were provided by Eka Nobel (Bohus, Sweden). Other chemicals used in this work were of analytical grade.

2.2. CE separation

All CE separations were performed on a Prince (Emmen, Netherlands) CE autosampler with a high voltage supply (0–30 kV). On-column detection was done with a UV detector (254 nm), CV[4] (Isco, Lincoln, NE, USA).

The temperature of the capillary was, if not stated otherwise, maintained at 25°C. Data were collected by means of an ELDS 900 laboratory data system and ELDS Win v1.0 (Chromatography Data System, Kungshög, Sweden). Non-coated fused-silica capillaries (25 μm I.D. or 50 μm I.D.) were obtained from Polymicro Technologies (Phoenix, AZ, USA). New uncoated capillaries were first pre-treated by flushing with 0.2 *M* NaOH for 1 h and then with water for 10 min. Between runs, the capillary was flushed with 0.2 *M* NaOH for 5 min, followed by water rinsing for 5 min, then with running buffer for 5 min. Samples were introduced by pressure (50 mbar×0.2 min for 50 μm I.D. capillaries and 50 mbar×0.35 min for 25 μm I.D. capillaries). All buffer pH values are given as they were before the addition of organic modifiers.

2.3. Optimization

Statistical experimental design for the optimization experiments was done in Codex (Sum IT System, Sollentuna, Sweden). The procedures of optimization has been described in earlier papers [19–21].

2.4. Resolution and electroosmotic flow (EOF)

Calculation of resolution (R_s) was same as in Ref. [19]. The EOF was measured by means of a neutral marker, mesityl oxide where the UV absorbance was measured at 204 nm.

2.5. Derivatization

The derivatization of dipeptides with FMOC and FLEC were the same as for amino acid derivatization [19]. A newly developed derivatization reagent, (+)- and (−)-1-(9-anthryl)-2-propyl chloroformate (APOC) [22] was also used. Derivatization with APOC was, in principle, as with FLEC [22]. The concentrations of the samples were in the range of 100–200 μ*M*.

3. Results and discussion

3.1. Comparison of chiral separation of peptides by means of different approaches

The separation can be done according to two principally different approaches, the indirect and the direct. In the indirect method, the analytes are first derivatized to form diastereomers and these are subsequently separated on a non-chiral system. In the direct method, chiral separation is performed on a chiral system. Here, derivatization is, in principle, not necessary, but it has been applied in our work to improve chiral recognition and detectability.

A comparison of the general performance of the

Table 1
Comparison of different separation approaches

Type	Migr. time (min)	R_s	$N\times10^5$ (plates/m)	Organic modifier	MEKC	Ref.
CD	~12	1–3	4–10	IPA	+	[15]
Vancomycin	5–8	2–23	2–6	IPA	−	[16,17]
Teicoplanin	6–7	2–38	3–7	ACN	−	[18]
Indirect						
(a) no IPA	5–6	1–4	~8		+	[15]
(b) With IPA	9–13	3–11	~3–11	IPA	+	[15]

IPA=2-propanol; MEKC=micellar electrokinetic chromatography.

different methods is presented in Table 1. The table is a compilation of data from published experiments.

3.2. Indirect chiral separation of small peptides

In the indirect method, separation is achieved by means of different analyte interactions with anionic micelles (SDS) that are present in the buffer. The separation is according to hydrophobicity, and optimal micellar concentration depends on the analyte hydrophobicity. Separation of hydrophilic analytes (early eluting analytes in the present system) is thus achieved at relatively high SDS concentrations, while hydrophobic analytes (late eluting) require low or no SDS concentration. If a mixture of analytes covering a wide range of hydrophobicity is to be separated, the SDS concentration has to be adopted to suit the needs regarding early eluting peaks, which leads to quite long retention times for the most hydrophobic analytes. Moreover, the presence of 2-propanol in the buffer leads to increased retention times, Fig. 1. However, 2-propanol also leads to improved plate numbers, and that is often necessary for the separation, Table 1.

The hydrophobicity of the derivatization reagent is of great importance for the optimal SDS concentration. Most reagents give relatively polar derivatives which require high SDS concentrations for their separation. The necessary SDS concentration for 2, 3, 4, 6 - tetra-O-acetyl-β-D-glucopyranosylisothiocyanate (GITC) is 200 m*M* [23], for *o*-phthalaldehyde, 150 m*M* [24] and for Marfey's reagent, about 100 m*M* [10]. Derivatives obtained with FLEC are more hydrophobic than above mentioned derivatives and only 10–20 m*M* SDS is required here. This is a great advantage since high SDS concentrations may result in excessive Joule heating.

Finally, it should be mentioned that the indirect method suffers from an inherent limitation. Thus, the intermolecular distance between the chiral centers of the derivatization reagent and of the analyte must not be too long [25,26]. For example, peptides beginning with Gly could not be resolved with the indirect method [15].

3.3. Direct chiral separation of small peptides

3.3.1. CD

Systems containing CD, in general, show somewhat lower plate numbers than systems without chiral selector, the indirect method, Table 1. Obviously, the interaction with a chiral selector gives a contribution to band broadening. However, addition of 2-propanol to the buffer led to increased plate numbers, Table 1. The importance of organic modi-

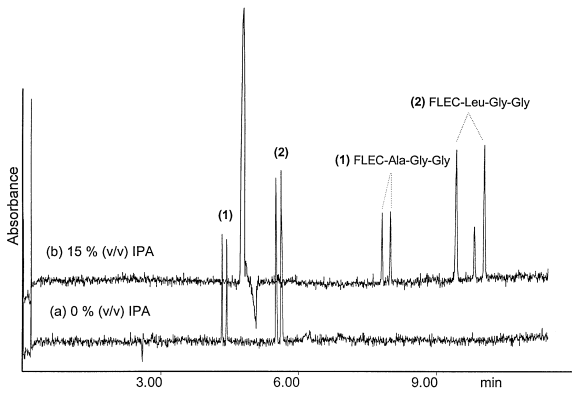


Fig. 1. Diastereomeric separation of FLEC-tripeptides. Conditions: buffer, 40 m*M* borate; pH, 9.2; 15 m*M* SDS; capillary, 67 cm (45 cm to detector)×25 μm I.D.; 30 kV. (a) 7.0 μA, (b) 4.8 μA.

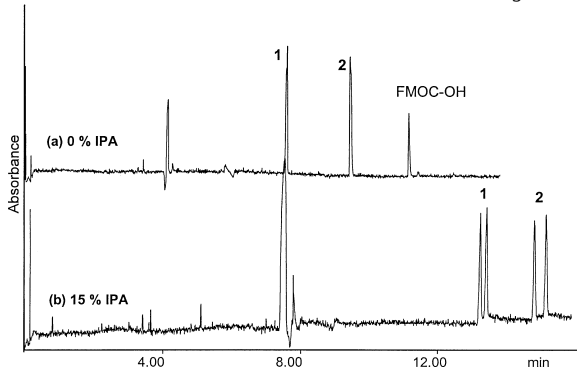


Fig. 2. Enantioseparation of FMOC-dipeptides, using cyclodextrin as chiral selector. Conditions: buffer, 40 m*M* phosphate, pH, 7.5; 40 m*M* SDS; 12 m*M* γ-CD; in (b) 15% (v/v) 2-propanol was added to the buffer; capillary, 67 cm (45 cm to detector)×25 μm I.D.; 25 kV; (a) 16 μA, (b) 10 μA. Analytes: (1) FMOC–Gly–Met; (2) FMOC–Gly–Phe.

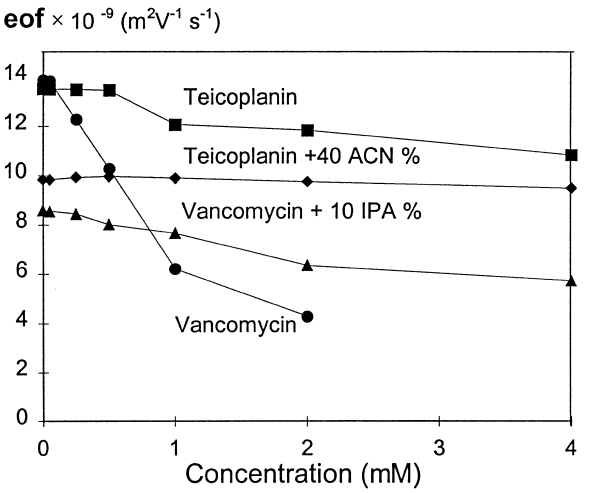


Fig. 3. Electroosmotic flow dependence of selector concentration. Conditions: buffer, 25 m*M* acetate–Tris; pH, 5.5; capillary, 67 cm (47.5 cm to detector)×50 μm I.D.; 25 kV.

fiers for chiral separation with CD as selector is illustrated in Fig. 2 where the presence of 2-propanol was a presupposition for the enantioseparation of FMOC–Gly–Met and FMOC–Gly–Phe. In summary, although the selectivities obtained with CD were lower than those obtained with vancomycin and teicoplanin, Table 1, the overall performance of CD systems was, by virtue of the relatively high plate numbers, excellent in many cases.

3.3.2. Vancomycin

Although the mechanisms of the chiral selectivity are not exactly known, it is clear that, for the separation of anions, electrostatic attraction between analytes and cationic sites at the chiral selector are highly important for the selectivity [27–31]. The pH of the buffer must therefore be selected so that the selector maintains a sufficiently high cationic character. Thus, the p*I* of the selector has to be considered when selecting the buffer pH. The p*I* of vancomycin is about 7.2 [32,16] and the p*I* of teicoplanin is about 3.8 [28]. It has been demonstrated that selectivity is improved with decreasing pH [27,28,32]. Thus, when highest selectivity is a primary concern, the pH should be low. However, when using non-coated fused-silica capillaries, the positively charged selectors will be adsorbed at the capillary wall. Such adsorption leads to broader peaks and it can be observed that the EOF is decreasing with increasing selector concentration [27,32]. Fig. 3 shows EOF as a function of selector concentration at pH 5.5. It can be seen that vancomycin is more strongly adsorbed than teicoplanin at pH 5.5. Addition of 10% 2-propanol to the buffer leads to decreased adsorption, but the adsorptive activity that still remains leads to excessive band broadening. Another approach to decrease surface adsorption of vancomycin at lower pH is the addition of SDS to the buffer. The concentration of vancomycin at the surface is thereby decreased; at equilibrium, a major part of the positively charged vancomycin is associated with the negatively charged micelles [33]. However, the best method to avoid adsorption of vancomycin at pHs below its p*I* is the application of coated capillaries [29,34,35].

In earlier work [16,17], we applied a pH just above the p*I* of vancomycin. In this way, the number of cationic sites on the vancomycin was traded to advantage. The cationic character was thus reduced so that surface adsorption of the selector could be virtually eliminated by the addition of 10% (v/v) 2-propanol to the buffer. On the other hand, the number of vancomycin cationic sites remaining was sufficient for the chiral recognition. Chiral resolutions of dipeptides as high as 31 were obtained [17]. Nevertheless, application of lower pH would result in improved selectivities. Therefore, separation was

attempted at pH 6.25. However, at this pH adsorption of vancomycin at the surface of non-coated fused-silica capillaries was extensive. Operation at pH 6.25 on such capillaries resulted in very broad peaks (not shown) and enantioresolution was not achieved.

3.3.3. Teicoplanin

In an earlier paper, we applied a pH of 6.25 (after addition of 40% acetonitrile (ACN), pH* was 6.6) for separation of derivatized small chiral peptides [18]. Rundlett et al. [28] examined the separation of some different types of chiral compounds at three different pHs, 5.0, 6.0 and 7.0. The best resolutions were obtained for all compounds at pH 5.0 but the improved separations were gained at the expense of the analysis time.

In spite of its low p*I*, teicoplanin may be adsorbed at the surface of a non-coated capillary also at a relatively high pH. Fig. 3 demonstrates that the EOF is strongly influenced by selector concentration at pH 5.5. However, in the presence of 40% ACN, the EOF was only slightly dependent on teicoplanin concentration. This indicates that teicoplanin can be used at pH 5.5 without serious adsorption problems. An additional function of the ACN is to suppress the formation of teicoplanin micelles.

The influence of pH, from 5.0 to 7.0, on resolution was examined for four dipeptides. Performance at lower pH was not evaluated since the teicoplanin was barely soluble in the buffer at pH 4.5 and lower. In addition, teicoplanin is considered to be unstable at pHs below 4 [27]. Maximum resolutions were obtained in the pH range 5.0 to 6.0 and maximum plate number was in the pH range 5.5 to 6.0. Optimal pH was dependent of the nature of the analyte, Fig. 4. In order to assure sufficient buffer capacity and still keep the buffer composition constant, a mixed acetate–phosphate buffer was used.

Peptides having two chiral centers are, in general, more difficult to resolve than one chiral center peptides. When using teicoplanin as chiral selector at pH 6.25 [18] the FMOC-derivatives of the peptide Ala–Ala gave three fully separated peaks and FMOC–Ala–Leu gave three fully separated peaks and a fourth peak that was partially separated. FMOC–Leu–Leu gave two fully separated peaks and a third peak was partially separated, FMOC–Leu–

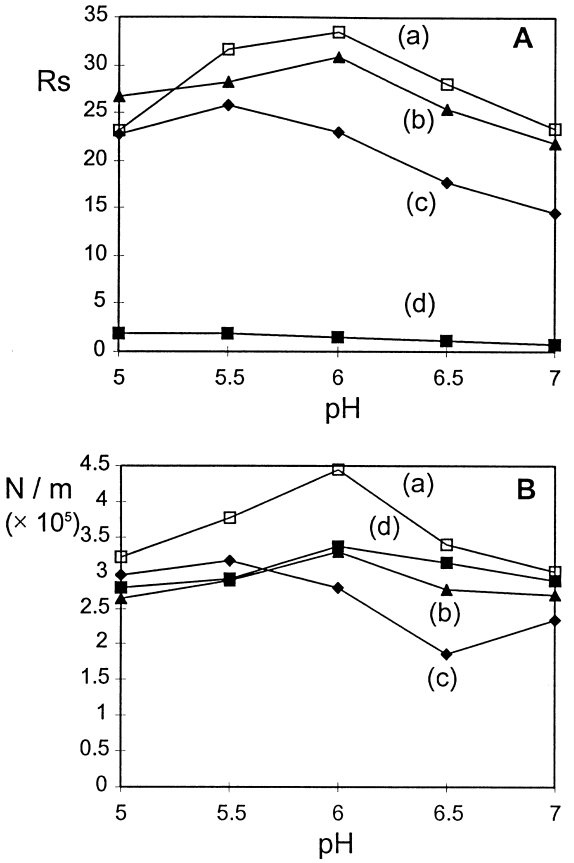


Fig. 4. Enantioseparation of FMOC derivatized dipeptides as a function of pH using teicoplanin as chiral selector. Conditions: detection, UV at 254 nm; buffer, 12.5 m*M* phosphate+12.5 m*M* acetate–Tris, 1 m*M* teicoplanin, 40% (v/v) ACN; capillary, 67 cm (47 cm to detector)×25 μm I.D.; 30 kV. A=resolution; B=number of theoretical plates. Analytes: (a) Ala–Gly; (b) Gly–Ala; (c) Gly–Leu; (d) Leu–Gly.

Ala gave two separated peaks. Using the same selector at pH 5.5, the FMOC-derivatives of Ala–Ala and Ala–Leu were fully resolved, Leu–Leu gave three separated peaks and a fourth peak was partially separated, and Leu–Ala gave three fully separated peaks, Table 2. The resolution of FMOC–Ala–Ala at pH 5.0 and 6.0 is shown in Fig. 5. The migration time of the last peak was 12 min at pH 5.0, 9.5 min at pH 6.0 and in an earlier investigation performed at pH 6.25 it was 7.9 min (in a phosphate buffer) [18]. The prolonged analysis time is a drawback when operating at the lower pH. However, in cases when

Table 2
Number of resolved peaks for dipeptides having two chiral centers

Selector	Ala–Ala	Ala–Leu	Leu–Ala	Leu–Leu	Ref.
CD	2	4[a]	3	3	[15]
Vancomycin	3	4[a]	3	4[a]	[16,17]
Teicoplanin	4[b]	4[b]	3[b]	4[c]	[18][e]
	4[c,d]	4[c,d]	2	3	
Indirect	2	2	4[a]	4[a]	[15]

[a] and [b] Baseline separation or better of all four peaks.
[b] Under the condition, pH=5.00.
[c] Partial separation of peaks 3 and 4.
[d] Under the condition, pH=6.25.
[e] Present work.

CE at the higher pH does not result in the desired separation, application of a low buffer pH should be considered.

The selectivities of the two antibiotics are often different. For example, vancomycin gave a quite high resolution, 20.6, of FMOC–Leu–Gly (Fig. 6), as compared to teicoplanin which gave a resolution of 1.68 [36,18].

In order to examine the influence of temperature on the chiral separation, performance at two temperatures, 15°C and 25°C, was investigated. Resolutions were found to be similar at the two temperatures.

3.4. Comparison of methods for the resolution of peptides having two chiral centers

Table 2 shows a comparison of the performance of different methods for chiral separation of peptides having two chiral centers. In the table, the number of fully or partially resolved peaks are given. Some separations were readily performed with the direct method, while the indirect method failed and vice versa. At this state of the art, it is not possible to predict whether a method will be successful or not. In our opinion, knowledge that would permit such predictions is highly desirable.

3.5. Separation mechanisms

In order to study interaction mechanisms, chiral separation of three quite similar amino acid derivatives was attempted with different types of chiral selectors. The structures of the derivatives and separation data are given in Table 3. The chiral separation of the derivatives, using different chiral

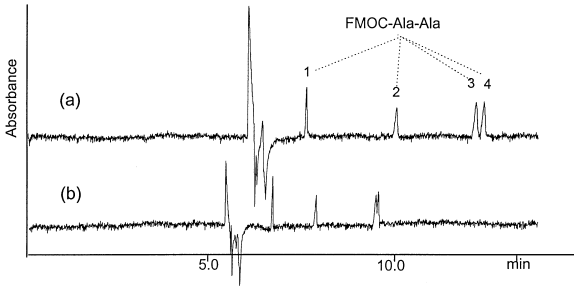


Fig. 5. Enantioseparation of a dipeptide, FMOC–Ala–Ala, using teicoplanin as selector. Conditions: buffer 25 m*M* acetate–Tris; 40% (v/v) ACN; capillary, 65 cm (45 cm to detector)×25 μm I.D.; 30 kV; current, 4.0 μA in (a), 4.8 μA in (b). (a) pH 5.0; (b) pH 6.0.

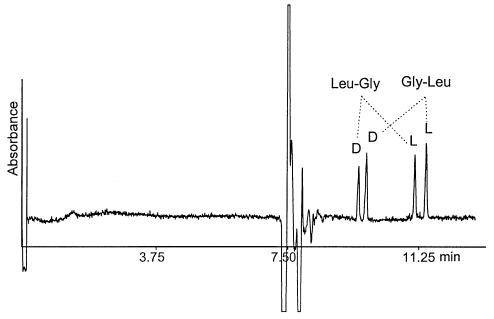
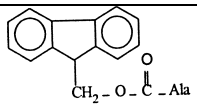
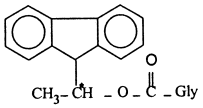
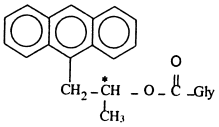


Fig. 6. Enantioseparation of FMOC-dipeptides, using vancomycin as chiral selector. Conditions: buffer, 50 m*M* phosphate, pH, 7.5; 1 m*M* vancomycin; 10% (v/v) 2-propanol; capillary, 67 cm (45 cm to detector)×25 μm I.D.; 25 kV; 13.6 μA.

Table 3
Comparison of chiral resolution, obtained with different types of chiral selectors, of some derivatives having similar structures

Eanatiomer	Structure	Molecular dimensions	Selectivity (R_s)		
			Teicoplanin[a]	Vancomycin[b]	CD
FMOC–Ala (a)		8.58 Å×13.0 Å	2.42	8.60	1.85[c] 0.89[d]
FLEC–Gly (b)		9.10 Å×11.8 Å	0	0	2.20[e] 5.85[f]
APOC–Gly (c)		9.58 Å×13.5 Å	–	<0.50	3.93[e] 2.10[f]

Separation conditions:
[a] Buffer, 25 m*M* phosphate–Tris, pH, 6.25; 1.2 m*M* teicoplanin, 40% (v/v) ACN; capillary, 67 cm (45 to detector)×25 μm I.D.; 30 kV; 4.8 μA.
[b] 40 m*M* Phosphate, pH, 7.50; 2 m*M* vancomycin, 25 kV; 21 μA.
[c] 50 m*M* Phosphate, pH, 7.50; 50 m*M* SDS, 12 m*M* β-CD, 15% IPA; 25 kV; 8.8 μA.
[d] 12 m*M* γ-CD; other conditions as in (c).
[e] 40 m*M* HEPES–Tris, pH, 7.50; 40 m*M* SDS, 25 m*M* β-CD, 15% (v/v) IPA; 25 kV; 5.5 μA.
[f] 8 m*M* γ-CD; other conditions as in (e).

selectors, is shown in Fig. 7. FMOC–Ala could be resolved with teicoplanin, vancomycin and CD, but FLEC–Gly was resolved with CD only. The main difference between the two derivatives is that FLEC–Gly lacks the possibility of polar interactions in the vicinity of the chiral center. In addition, interactions with the chiral center of FLEC–Gly may be sterically hindered. A third derivative, APOC–Gly, could be partially resolved with vancomycin, Table 3 and Fig. 7b. It seems that the sterical hinderance is less prominent for this derivative than for the FLEC–Gly.

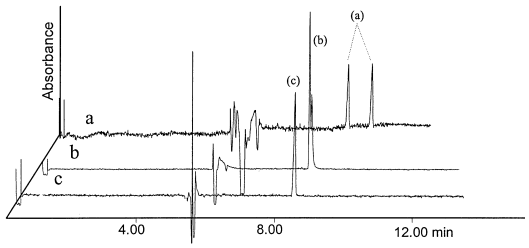


Fig. 7. Comparison of chiral separation of amino acid derivatives using vancomycin as chiral selector. Conditions: buffer, 40 m*M* phosphate; pH, 7.5; 2 m*M* vancomycin; capillary, 67 cm (45 cm to detector)×25 μm I.D.; 25 kV; 21 μA. (a) FMOC–Ala; (b) APOC–Gly; (c) FLEC–Gly.

Acknowledgements

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