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Application of capillary electrophoresis to the separation of structurally diverse *N*-(substituted)-glycine–peptoid combinatorial mixtures

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Abstract

The capillary electrophoresis (CE)-based separation of five *N*-(substituted)-glycine (NSG)–peptoid mixtures with a wide range of physical and chemical properties was studied. A CE separation, initially developed using a single representative peptoid mixture, with a backround electrolyte (BGE) modified by the addition of both methyl-β-cyclodextrin and heptane sulfonic acid was found to provide good separations of most of the combinatorial mixtures investigated. For those mixtures not separated well by this procedure, the use of SDS micelles in conjunction with methyl-β-cyclodextrin resulted in dramatic improvements in the separation. While no single set of separation conditions proved sufficient for all of the NSG–peptoid combinatorial mixtures, the two methods were able to provide separation sufficient for characterization of a set of mixtures with a wide range of physical and chemical properties. The efficiency of the CE-based separation of the combinatorial mixtures studied was compared to a reversed-phase liquid chromatographic method using gradient elution.

Keywords: *N*-(substituted)-glycine; Peptoids

1. Introduction

A capillary electrophoretic separation was developed to characterize peptoid-based combinatorial mixtures [1]. The development of this method was based on the use of a particular *N*-(substituted)-glycine (NSG)–peptoid mixture, CHIR 4580, as a representative of mixtures which may be encountered during drug discovery. In order to provide the highest separation efficiency for the widest range of peptoid species, a CE background electrolyte (BGE) containing multiple additives was developed [2]. The use of additives to the BGE to enhance the CE separations of hydrophobic and neutral molecules was introduced by Terabe et al. [3,4]. An ion-pairing agent was incorporated to diminish self-association interactions of the peptoids and interactions with the capillary wall. This role for ion-pairing agents in CE was first proposed by McLaughlin et al. [5]. A cyclodextrin was also incorporated into the BGE to add hydrophobic partitioning properties to the separation [6–8].

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In this report, this separation method was applied to five structurally-diverse peptoid combinatorial mixtures. Although the five mixtures were composed of the same twenty-four side chains (Fig. 1), each mixture contained a physicochemically unique set of compounds in terms of shape, molecular weight, hydrogen bonding capabilities, lipophilicity, and acid/base properties as shown in Table 1 [9].

The aim in developing the CE separation was to provide a method capable of rapidly characterizing a wide range of NSG–peptoid combinatorial mixtures. Using the diverse mixtures in this study, the general applicability of the CE method was evaluated. The flexibility of the method for modification to characterize mixtures not well resolved using the original separation conditions was evaluated. This CE method was compared to a reversed-phase liquid chromatography method using gradient elution.

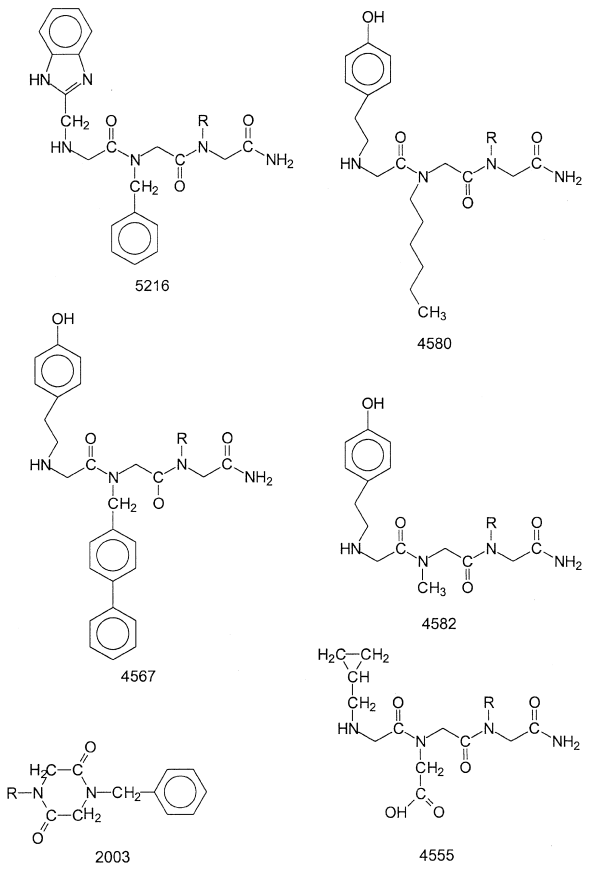

Fig. 1. Common structural elements in the NSG–peptoid mixtures investigated. (CHIR 4580, CHIR 4582, CHIR 4555, CHIR 4567, CHIR 5216 and CHIR 2003). The position where the side chains varied are denoted R.

2. Experimental

2.1. Chemicals

The NSG–peptoid mixtures were synthesized as previously described by Chiron (Emeryville, CA, USA) and were provided as 1 m*M* solutions in DMSO with the exception of CHIR 4580 which was in ethanol. All mixtures were diluted 1:1 with NANOpure water prior to use except CHIR 4580 which was diluted tenfold. Sodium dodecyl sulphate was obtained from Fisher Scientific (Fairlawn, NJ, USA). Methyl-β-cyclodextrin (CD) was obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were reagent grade or better and used as received.

2.2. Capillary electrophoresis system

An ISCO Model 3850 capillary electropherograph (ISCO, Lincoln NE, USA) connected to a Spectra Physics Model 5P4400 Integrator for data acquisition was used for all analyses. Data was collected in ASCII format and any processing of data was performed using Origin 3.5 (Microcal Software, Northhampton, MA, USA). Fused-silica capillaries from Polymicro Technologies (Tucson AZ, USA) of 50 μm I.D. with a length of 45 cm to the detection window and a total length of 80 cm were used for the separation. Capillary columns were rinsed with 0.1 *M* sodium hydroxide for 20 min followed by conditioning with the BGE for 30–45 min prior to use. In all experiments a constant electrophoretic voltage of 15 kV was applied. Vacuum injection for 15 s at 3.5 kPa was used for all experiments. UV absorbance detection at a wavelength of 210 nm was used for all mixtures.

2.3. Preparation of buffer solutions

Sodium phosphate buffer, pH 2.0, was prepared by diluting the appropriate volume of concentrated phosphoric acid to the desired concentration and

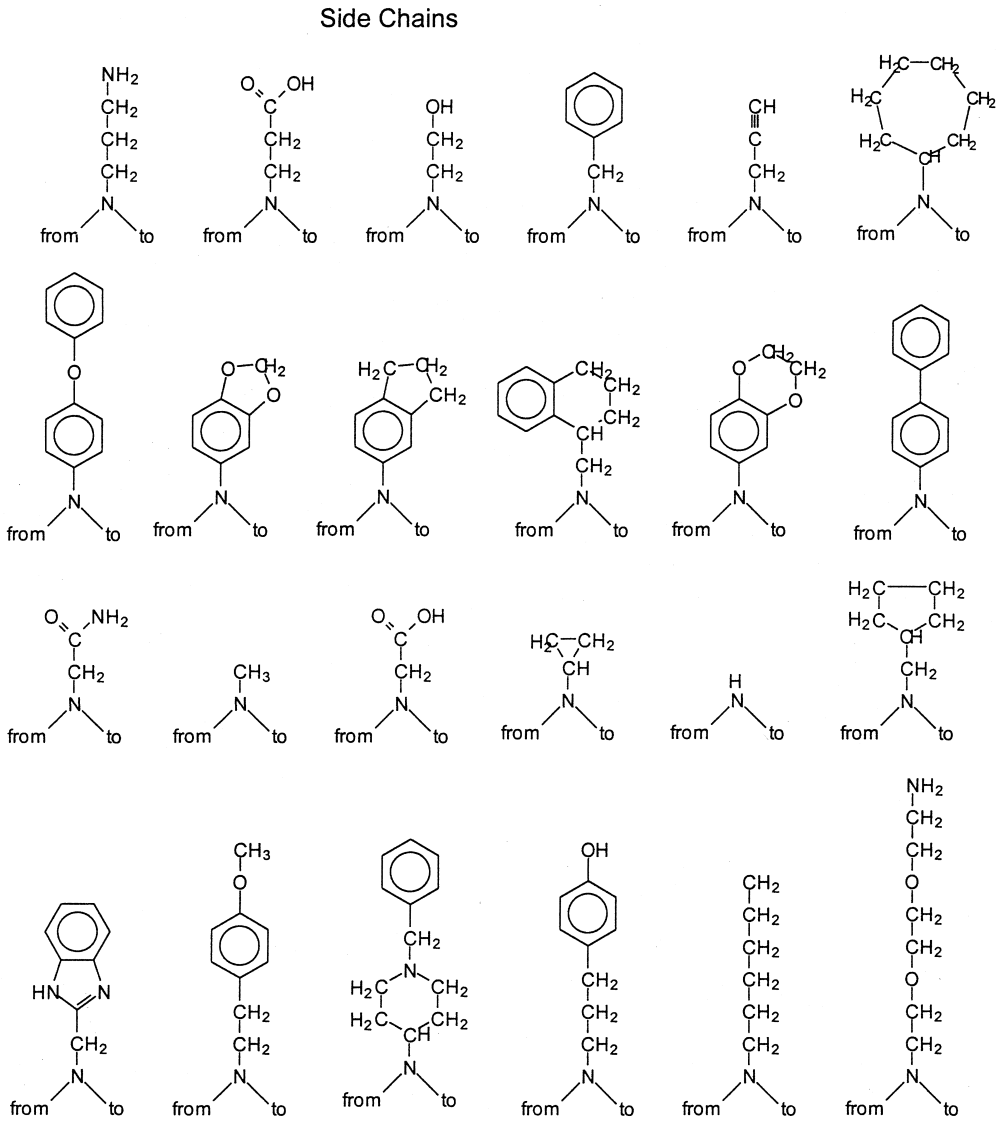

Fig. 1. (*continued*)

Table 1
Physicochemical characteristics of the NSG–peptoid mixtures investigated

Peptoid mixture	pK_a	pK_b	Log $D^a_{o/b}$	M_w	Structure class
CHIR 4580	3.4–10.1	9.8–7.6	−6.1–1.2	392–581	Linear
CHIR 4582	3.4–10.1	9.8–7.6	−8.7–1.0	322–495	Linear
CHIR 5216	3.4–6.8	9.8–7.6	−7.7–0.1	408–581	Linear
CHIR 4555	3.1–3.3	9.8–7.6	−8.7–1.5	300–473	Linear
CHIR 4567	3.4–10.1	9.8–7.6	−5.0–2.3	460–633	Linear
CHIR 2003	3.4–>14	9.8–<1	−4.7–3.1	204–377	Cyclic

[a] Distribution coefficient between octanol and a pH 2.0 phosphate buffer containing 50 m*M* NaCl.

adjusting the pH with 0.1 *M* sodium hydroxide. The appropriate amounts of HSA and CD were then dissolved in the sodium phosphate buffer. The solution was sonnicated for 30 min to assure complete dissolution of the HSA and the pH was then adjusted to 2.0 with 0.1 *M* sodium hydroxide. Phosphate–borate buffer was prepared by mixing 100 m*M* sodium tetraborate and 250 m*M* sodium dihydrogen phosphate to give pH 8. The SDS and CD buffer solutions were prepared by dissolving the SDS and/or CD in phosphate–borate buffer followed by sonication for 30 min. The pH was readjusted to 8.0 as required.

2.4. Liquid chromatography

Stock solutions of the NSG–peptoid mixtures at 1 m*M* per compound in DMSO were diluted 1:100 in 0.2% heptane sulfonic acid/0.1% trifluoroacetic acid. Ten microliters of this solution was injected onto an Ultrafast Microprotein Analyzer microbore HPLC system equipped with a 150×1.0 mm I.D. monitor C_{18} column containing 5-μm diameter 100X pore highly base-deactivated silica and protected with a guard cartridge (Michrom BioResources, Aubum, CA, USA). The chromatographic conditions were 5% acetonitrile in 0.2% heptane sulfonic acid–0.08% trifluoroacetic acid as buffer A and 75% acetonitrile in 0.2% heptane sulfonic acid–0.08% trifluoroacetic acid as buffer B. Analytes were eluted using a linear gradient from 0% to 100% buffer B over 35 min at a flow-rate of 50 μl/min. The elution of NSG–peptoids was detected at 214 nm.

2.5. Physicochemical characterization of the NSG peptoids

Predictions of apparent 1-octanol/buffer distribution coefficient (log $D_{o/b}$) and pK_a were performed using PrologD v2.0 and pK_{alc} v3.1 software, respectively, both from CompuDrug Chemistry (Budapest, Hungary).

3. Results and discussion

In the previous report that described the development of a CE-based separation of an NSG–peptoid mixture, CHIR 4580, the BGE consisted of 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* heptane sulphonic acid (HSA) and 0.04 g/ml methyl-β-cyclodextrin [1]. A low pH was used to ensure that all of the peptoids were fully protonated. The HSA, an ion-pairing reagent, was added to reduce the intramolecular interactions of the peptoids and electrostatic interactions with the capillary wall. The methyl-β-cyclodextrin was added to provide greater resolution of the mixture by adding a partitioning component to the electrophoretic separation. In this report, this method was applied to five new, structurally-diverse peptoid mixtures. The structures and the physicochemical characteristics of these peptoid mixtures are shown in Fig. 1 and listed in Table 1, respectively. CHIR 4582 and CHIR 4567 only differ from CHIR 4580 the mixture initially used for the development of the CE method, in that the nonpolar side chain at the second position is different in size, shape and hydrophobicity. In CHIR 5216, although the side chain at the second position is nonpolar, the *N*-terminal 4-hydroxyphenethyl side chain is replaced by 2-methylbenzimidazole. CHIR 4555 differs significantly from the previously described mixtures in that, the side chain at the second position is acidic (a carboxylic acid) and the *N*-terminal group is methylcyclopropyl, which is nonpolar but not aromatic. Unlike the linear NSG–peptoids which all contain a 2° amine at the N-terminus, CHIR 2003 contains a dimeric cyclic, diketopiperazine backbone thereby eliminating the positive charge on the backbone and making this structure more compact and less flexible. The 24 side chains used to constitute each mixture were identical for all of the mixtures studied. The modifications in the basic structure of the various mixtures impart different physicochemical characteristics to each mixture. The mixtures selected for this study were chosen to represent a wide range of shapes, molecular masses, hydrogen bonding capabilities, lipophilicities (log $D_{o/b}$), and acid/base characteristics (pK_as and pK_bs) [9].

3.1. Initial modification to the previous method

The development of the initial optimized method was performed using a laboratory-built apparatus which did not have cooling capabilities. For this

reason, the electrophoretic current had to be kept below 80 μA to avoid loss of efficiency due to Joule heating. The current was minimized by using slightly lower than optimal concentrations of the ion-pairing agent, HSA. For the current experiments a commercially-available ISCO Model 3850 system was used which contained a cooling device to protect the capillary from Joule-heating effects. To enhance the separation, the concentration of the HSA was therefore increased to 75 m*M* from the previously used 25 m*M*. The higher HSA concentration decreased the cyclodextrin concentration required for optimal separation. A lower cyclodextrin concentration results in faster migration times of the peptoids and higher separation efficiencies without decreasing resolution. The modified BGE consisted of 200 m*M* sodium phosphate buffer, pH 2.0, containing 75 m*M* HSA and 0.03 g/ml methyl-β-CD. While the electrophoretic currents were high, ca. 80 μA, degradation of the separation by Joule heating was avoided by the column cooling capabilities of the commercial CE system.

3.1.1. NSG–peptoid mixture: CHIR 4582

The previous separation conditions provided adequate separation of this particular mixture with approximately 19 of the 24 compounds separated (Fig. 2A). With the modified separation, 23 peaks were observed (Fig. 2B). While the overall number of individual peptoid peaks did not increase significantly, the resolution of the later eluting peaks was improved. In addition, the separation time was somewhat decreased. Overall, the two methods yielded similar separation of this combinatorial mixture.

3.1.2. NSG–peptoid mixture: CHIR 5216

Using the previous conditions, 24 peaks were detected with a separation time of 60 min (Fig. 3A). With the modified method, 25 peaks were still resolved, however, the analysis time was decreased to 45 min (Fig. 3B).

3.1.3. NSG–peptoid mixture: CHIR 4555

This mixture differs from the previous mixtures investigated in that the second position side chain was a carboxylic acid yielding a mixture where all of the components have a pK_a at pH 3. However, carboxylic acid should be protonated at the CE

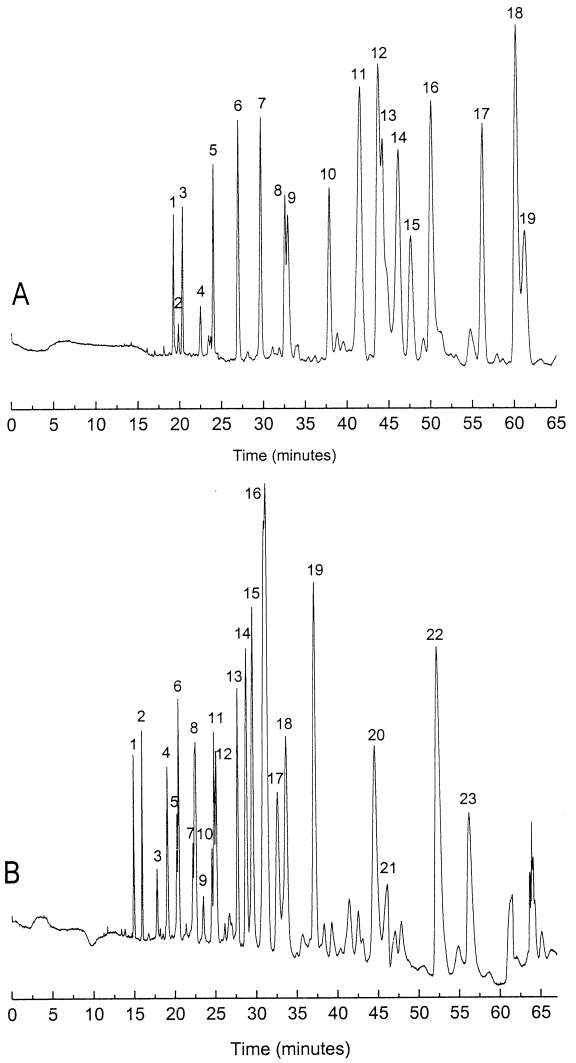

Fig. 2. CE separation of the linear trimeric NSG–peptoid mixture; CHIR 4582. Running buffer conditions were (A) 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* HSA and 40 mg/ml methyl-β-CD; (B) 200 m*M* sodium phosphate buffer, pH 2.0, with 75 m*M* HSA and 30 mg/ml methyl-β-CD.

separation condition of pH 2. Using the previous conditions, only 14 peaks were detected, many of which were not baseline resolved (Fig. 4A). The separation efficiency for this mixture was considerably lower than for the other mixtures as can be seen in the broad peaks throughout the electropherogram. The lack of an aromatic group on the peptoid backbone possibly limits the interactions with the

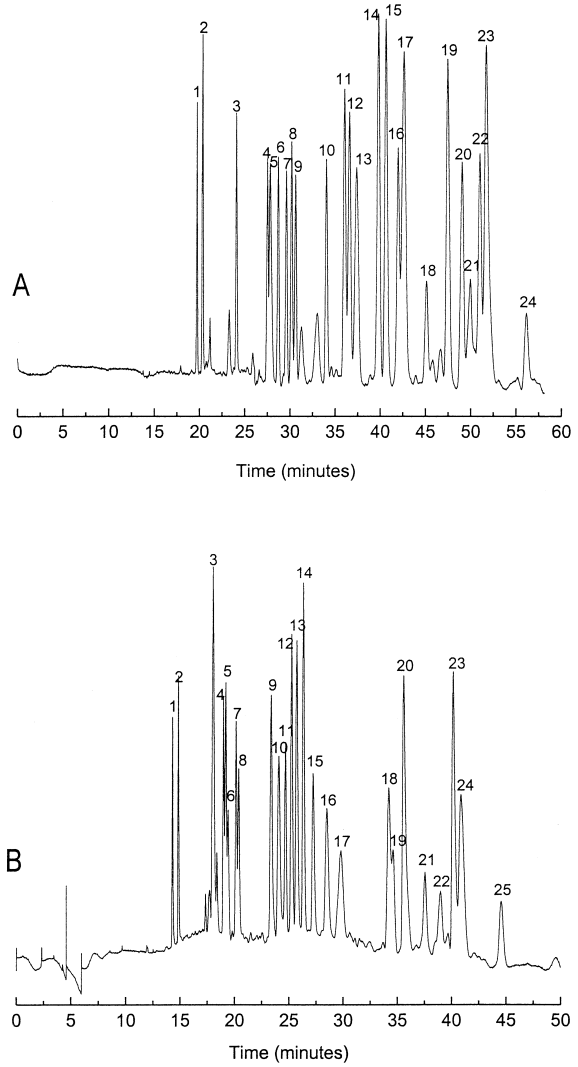

Fig. 3. CE separation of the linear trimeric NSG–peptoid mixture CHIR 5216. Running buffer conditions were (A) 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* HSA and 40 mg/ml methyl-β-CD; (B) 200 m*M* sodium phosphate buffer, pH 2.0, with 75 m*M* HSA and 30 mg/ml methyl-β-CD.

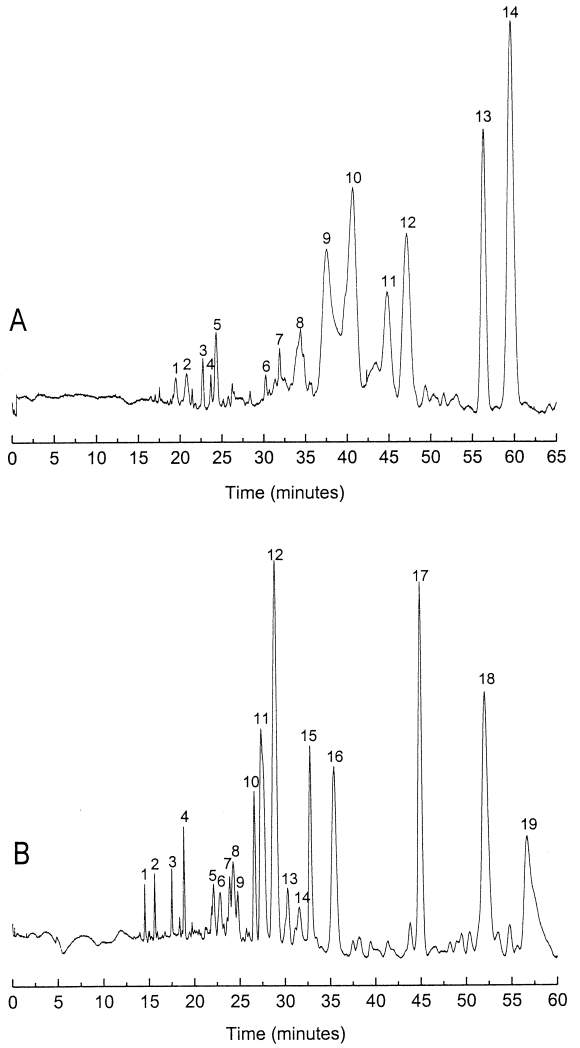

Fig. 4. CE separation of the linear trimeric NSG–peptoid mixture CHIR 4555. Running buffer conditions were (A) 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* HSA and 40 mg/ml methyl-β-CD; (B) 200 m*M* sodium phosphate buffer, pH 2.0, with 75 m*M* HSA and 30 mg/ml methyl-β-CD.

cyclodextrin leading to band broadening through intramolecular interactions between the hydrophobic peptoids. The development of the previous separation demonstrated a synergistic effect between the ion-pairing agent and the cyclodextrin. Therefore, in this case where interaction with the cyclodextrin appeared to be minimal, the effectiveness of the HSA may be less effective for this peptoid mixture. Upon increasing the HSA concentration to 75 m*M* the separation efficiency was improved and approximately 19 peaks were detected (Fig. 4B). The peak widths were considerably narrower and comparable to the separation of the previous combinatorial mixtures. This was likely because the increased HSA concentration more effectively disrupted intermolecular interactions between the peptoids and

interactions with the silica wall even in the absence of interactions with the cyclodextrin.

3.1.4. Comparison to a reversed-phase HPLC separation

Separations of these three NSG–peptoid mixtures by reversed-phase HPLC with gradient elution are shown in Fig. 5. The CE separation resulted in 24 peaks for CHIR 4582, 25 peaks for CHIR 5216.a, and 20 peaks for CHIR 4555 while the HPLC separation resulted in 16 peaks, 23 peaks and 16 peaks, respectively. The CE separation time was approximately 60 min for all three libraries while the HPLC method required 50 min. For these NSG–peptoid mixtures the CE method exhibited slightly greater resolution than the HPLC method but with a somewhat longer analysis time.

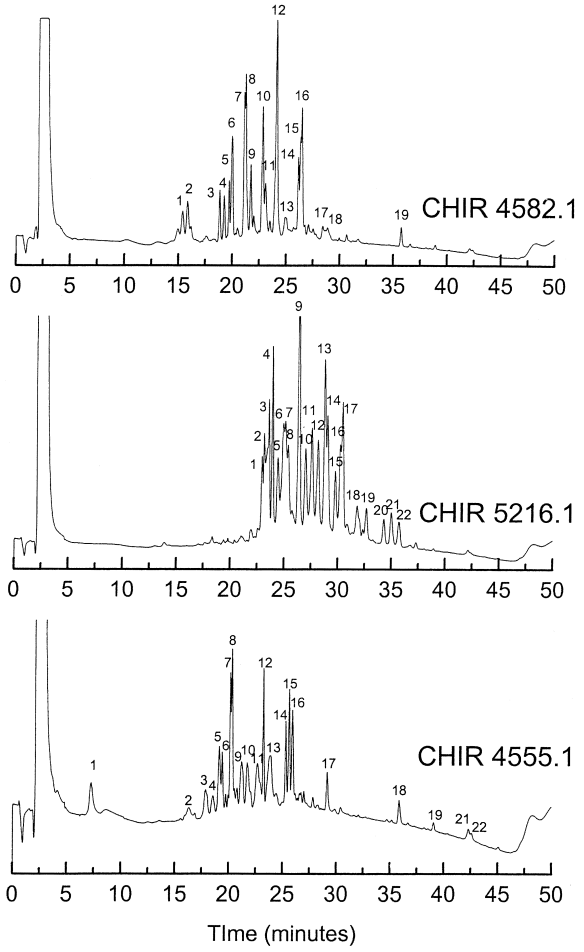

Fig. 5. Reversed-phase HPLC chromatograms of CHIR 4582, CHIR 5216 and CHIR 4555.

3.2. Modification to the separation for problematic mixtures

3.2.1. NSG–peptoid mixture: CHIR 2003

Using the previously-developed separation conditions, only 12 peaks from this mixture were resolved (Fig. 6A). Because the backbone of this particular mixture did not contain an ionizable group, the use of a micellar phase was investigated. Charged micelles can provide a pseudophase in CE by which neutral molecules are separated through their varying affinities for the micellar pseudophase [3]. Addition of ionic micelles to a CE BGE has been termed micellar electrokinetic chromatography (MEKC) and was introduced by Terabe et al. [3,4]. An ionic detergent such as sodium dodecylsulfate (SDS) is typically used to form the ionic micelles. As the SDS micelles are negatively charged, they migrate at a rate considerably less than the EOF. Partitioning of neutral molecules between the buffer solution and the interior of the micelle result in the neutral species exhibiting a net migration between that of the electroosmotic flow (EOF) and the mobility of the micelles. Hydrophobic molecules tend to remain in the hydrophobic core of the micelle and elute with the micelle. This is illustrated in Fig. 6B where MEKC was implemented using a BGE of 75 m*M* phosphate, 25 m*M* borate buffer, pH 8.0, and 75 m*M* SDS. A large band of poorly resolved peaks is seen to elute between 40 and 45 min which is near the elution time of SDS. These poorly resolved peaks were likely the more hydrophobic molecules in the mixture which had a high affinity for the SDS micelles and migrated at nearly the same rate as the micelles.

Terabe et al. [6] modified MEKC by addition of cyclodextrin in order to separate highly hydrophobic neutral compounds. Due to the hydrophilic nature of the CD surface, they will not interact with the micelles. Therefore the CD will behave as a separate phase relative to the micelles. The neutral cyclodextrin migrates at the EOF and the migration of hydrophobic molecules will be dependent upon the relative affinities for the CD and SDS micelles.

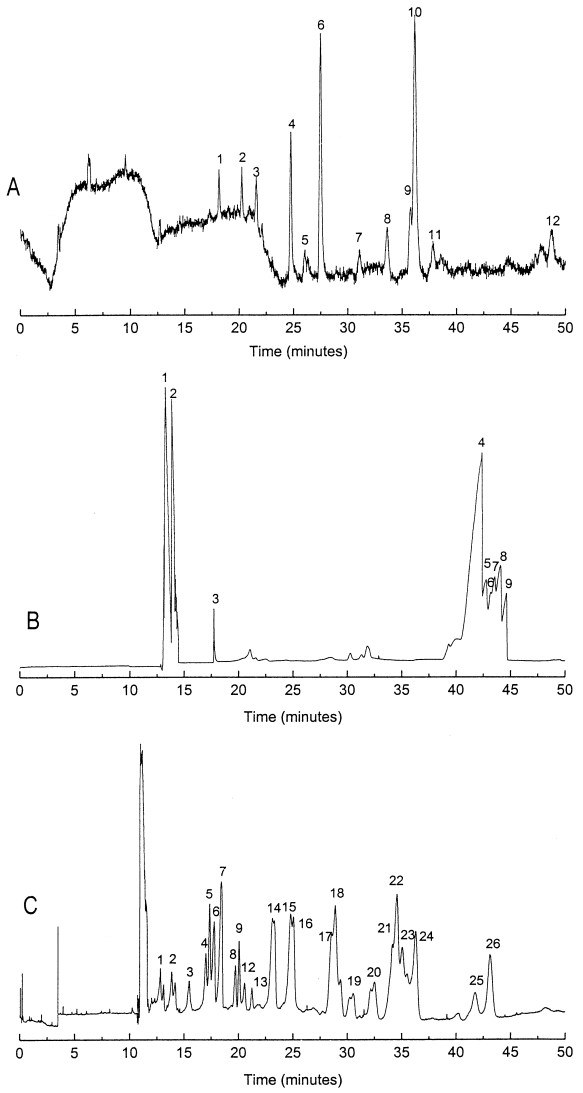

Fig. 6. CE separation of the cyclic dimeric NSG–peptoid mixture CHIR 2003. Running buffer conditions were (A) 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* HSA and 40 mg/ml methyl-β-CD; (B) 75 m*M* sodium phosphate–25 m*M* sodium borate buffer, pH 8.0, with 40 mg/ml methyl-β-CD; (C) 75 m*M* sodium phosphate–25 m*M* sodium borate buffer, pH 8.0, with 40 mg/ml methyl-β-CD and 75 m*M* SDS.

When large hydrophobic molecules such as the NSG–peptoids in this mixture are injected into the CD-modified MEKC system, the molecules distribute between three phases, the aqueous phase of the BGE, the micellar phase, and the CD cavity. The affect of these phases on the separation of this mixture is evident in Fig. 6C in which a BGE of 75 m*M* sodium phosphate, 25 m*M* sodium borate buffer, pH 8.0, containing 75 m*M* SDS and 0.04 g/ml methyl-(3-CD was used. As can be seen, the large late-eluting band occurring when using only SDS micelles was spread over a wider elution window in the presence of CD. The CD migrated with the EOF which was much faster than the SDS micelles, therefore the migration times of the NSG–peptoids were shorter in the CD-modified MEKC system than with SDS micelles only. More than the expected 24 peaks were identified using this method. As the purity of the sample was unknown, it was assumed that these extraneous peaks were impurities from the synthesis.

The separation of CHIR 2003 using the reversed-phase gradient HPLC method is shown in Fig. 7. The HPLC method resulted in more than 24 peaks being resolved with an analysis time of 50 min. The separation efficiency of the HPLC method is higher than the CE method as evidenced by the sharper peaks in the chromatogram of Fig. 7 relative to the electropherogram of Fig. 6C.

3.2.2. NSG–peptoid mixture: CHIR 4567

The final mixture which was studied (CHIR 4567) consisted of a highly aromatic linear trimeric backbone. Using the previous conditions, only 11 peaks were observed (Fig. 8A). This mixture, along with CHIR 2003, was the most hydrophobic mixture

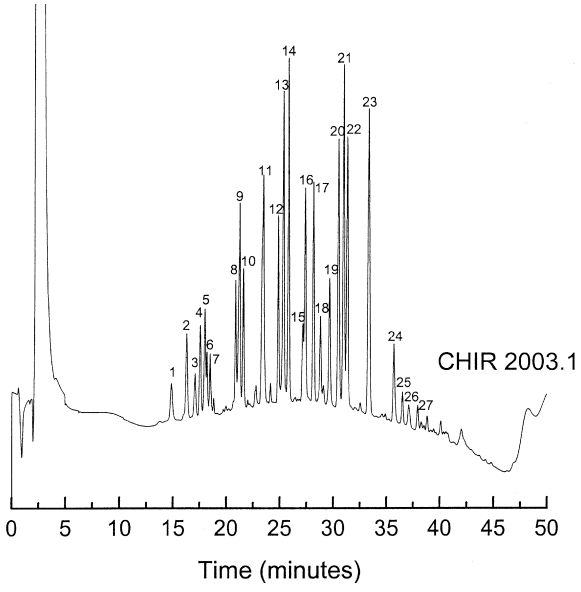

Fig. 7. Reversed-phase HPLC chromatogram of CHIR 2003.

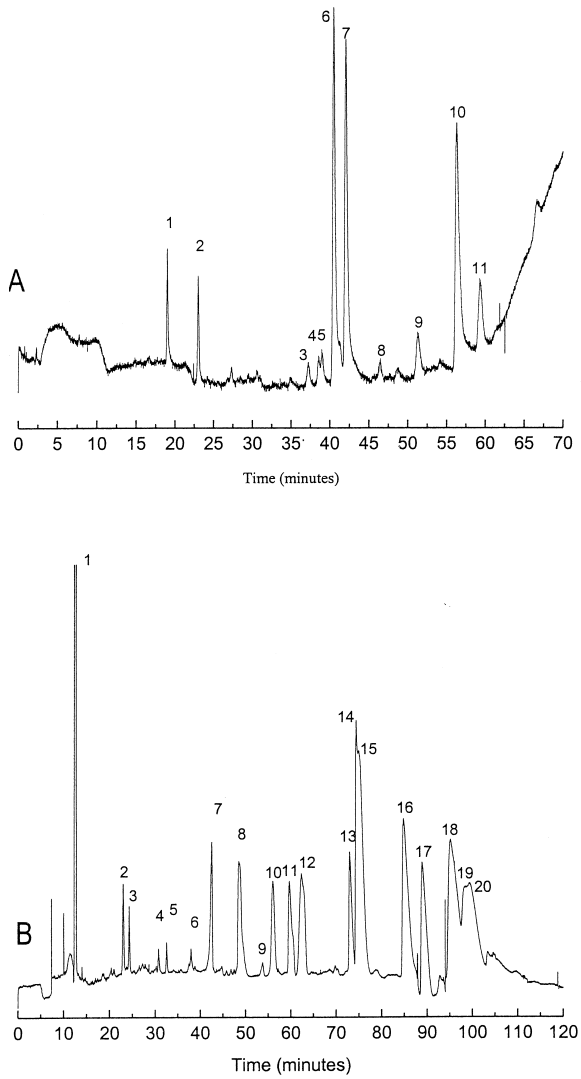

Fig. 8. CE separation of the linear trimeric NSG–peptoid mixture CHIR 4567. Running buffer conditions (A) 250 m*M* sodium phosphate buffer, pH 2.0, with 25 m*M* HSA and 40 mg/ml methyl-β-CD; (B) 75 m*M* sodium phosphate–25 m*M* sodium borate buffer, pH 8.0, with 40 mg/ml methyl-β-CD and 75 m*M* SDS.

studied as determined from the log $D_{o/b}$ values (Table 1). The template for CHIR 4567 contained aromatic side chains at the first and second positions. While the N-terminal primary amine indicates that this mixture was charged at all pH values used, the greater aromaticity may lead to significant intermolecular interactions as a result of the hydrophobicity and pi-bonding capabilities of the aromatic

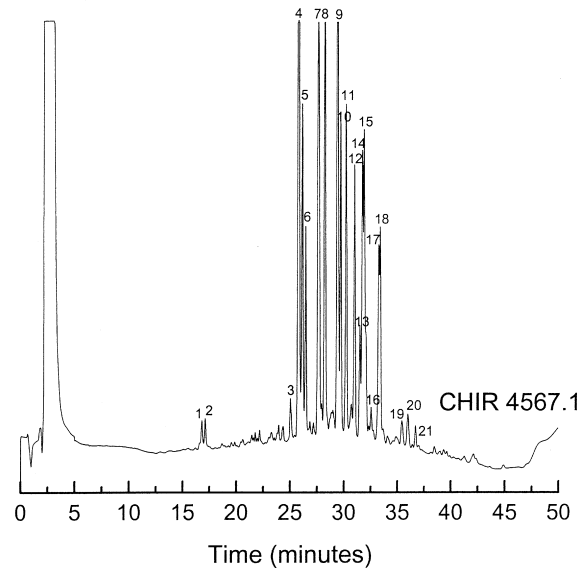

Fig. 9. Reversed-phase HPLC chromatogram of CHIR 4567.

groups. Both CD and SDS were evaluated as buffer additives to decrease the intermolecular interactions between these peptoids. Neither CD or SDS alone was sufficient to achieve the efficient separation of this peptoid mixture. However, when combined as for the separation of CHIR 2003, a reasonable separation was achieved (Fig. 8B). The BGE of 75 m*M* phosphate, 25 m*M* sodium borate, pH 8.0, with 75 m*M* SDS and 0.04 g/ml methyl-β-cyclodextrin resulted in 20 peaks for this mixture.

The reversed-phase HPLC method produced 21 peaks for the CHIR 4567 NSG–peptoid mixture (Fig. 9). As for CHIR 2003, the HPLC method exhibited higher separation efficiencies than the CE method, particularly for the late-eluting components in the electropherogram. This is likely a reflection of the different separation mechanisms being exploited. The elution of early peaks in the electropherogram was dominated by electrophoresis while the elution of the later peaks was dominated by partitioning into the micelles.

4. Conclusion

The separation power and versatility of CE makes it an interesting tool for many of the analytical challenges in the bio-pharmaceutical industry. A

particular attraction of the use of CE for analyses is the small mass of sample necessary for analysis. A second attractive feature is the range of separation mechanisms which can be achieved, including electrophoresis and partitioning. Selectivity can be readily modified by the addition of additives such as cyclodextrins, ion-pair reagents, or micelles to the electrophoretic BGE. These buffer additives provide alternate separation mechanisms in addition to the native electrophoretic mobility of the analytes.

In this report the separation of a variety of NSG–peptoid mixtures, synthesized by combinatorial methods, using modified CE BGEs was demonstrated. A BGE containing CD and an ion-pairing agent was found to provide good separations of most of the mixtures investigated. Unfortunately, this method was not sufficient to characterize peptoid mixtures which were composed of predominantly very hydrophobic and/or neutral compounds. In this case, addition of an anionic micellar phase greatly enhanced the resolving power of the separation. Using SDS micelles in conjunction with CD resulted in the separation of hydrophobic and neutral compounds as a function of their relative affinity for the micelle relative to the CD. While no single set of separation conditions proved sufficient for all of the NSG–peptoid mixtures, two methods provided sufficient separation of mixtures of compounds which exhibited a wide range of physical and chemical properties and for physicochemically different mixtures. In addition, if further resolution is required for a particular mixture, the methods are readily modified in a relatively predictable manner to achieve the desired separation. CE with modified BGEs provides an extremely powerful separation technique for combinatorial mixtures.

Although the reversed-phase HPLC separations described in this report yielded separation efficiencies similar to or worse than those of CE, all of the NSG–peptoid mixtures studied were separated using the same linear gradient conditions. The gradient elution conditions could be tailored for any given mixture to increase resolution and/or reduce total run time. One advantage of reversed-phase HPLC is the higher compatibility with and availability of mass spectrometric detectors. The ability of MS to identify specific components of mixtures as well as to verify the content of mixtures, makes compatibility with MS essential for characterization of synthetic products. As the development of new high-resolution separation techniques continues to advance for the characterization of physicochemically diverse compounds, compatibility with MS remains a challenge.

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