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Separation of neuropeptide Y diastereomers by high-performance liquid chromatography and capillary zone electrophoresis

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ABSTRACT

Separation of analogues of neuropeptide Y (NPY) in which a single D-amino acid replaced the corresponding naturally occurring residue was performed by chromatographic techniques to ensure the quality of the synthetic peptides to be used for structural and biological studies. Of the 35 compounds, 28 were easily separated ($\alpha = 1.02–2.76$) from native NPY by standard reversed-phase high-performance chromatography (RP-HPLC) methods using a Vydac C_{18} column and a gradient buffer system developed in our laboratory comprised of triethylammonium phosphate (TEAP) at pH 2.25 and acetonitrile at 40°C. The identical diastereomers could be separated on the same solid support and by using 0.1% trifluoroacetic acid (TFA) as the mobile phase modifier, however separation factors were smaller and retention times were longer. Three of the remaining seven unresolved analogues were separated ($\alpha = 1.02–1.96$) by changing the solid-phase support to Vydac diphenyl derivatized silica and a buffer system consisting of 0.1% TFA and acetonitrile. Of the four remaining unresolved analogues, only two could be separated by capillary zone electrophoresis (CZE) in 0.1 *M* sodium phosphate at pH 2.5, but all four were finally resolved by changing the electrophoretic buffer to 0.1 *M* TEAP buffer at pH 2.5. Migration times of the diastereomers differed by 0.2–2.0 min from that of the natural NPY. In addition to confirming the uniqueness of each isomer, this investigation demonstrated the expansive utility and high efficiency of the TEAP buffer system for both RP-HPLC and CZE as well as the difference in selectivity produced by the TEAP and TFA buffers in RP-HPLC. The conditions described here have broad applications for the analysis and preparative separation of synthetic and native peptides.

INTRODUCTION

Neuropeptide Y (I) is a 36-residue, C-terminally amidated polypeptide. It is widely distributed in the central and peripheral nervous systems of many species of mammals and fish. It has been implicated in the pathology of many disease states including hypertension and obesity [1,2]. As part of a structure-activity relationships investigation, we synthesized the entire series of D-isomer substitutions to explore the role of backbone modifications at each position in the sequence [3].

1 5

Tyr–Pro–Ser–Lys–Pro–Asp–Asn–Pro–Gly–

10 15

Glu–Asp–Ala–Pro–Ala–Glu–Asp–Leu–Ala–

20 25

Arg–Tyr–Tyr–Ser–Ala–Leu–Arg–His–Tyr–

30 35

Ile–Asn–Leu–Ile–Thr–Arg–Gln–Arg–Tyr–NH_2

I

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The use of racemic mixtures of amino acids in peptide synthesis or epimerization at any one of the many amino acid α carbons during synthesis could lead to the undesired incorporation of D-isomer impurities. Such impurities represent one of the greatest challenges to chromatographic detection systems, since they differ by only a single inversion of a peptide backbone α carbon. Analogues for our studies were assembled by standard solid-phase peptide synthesis (SPPS) methodology, which virtually eliminates the occurrence of epimerization, and were subsequently purified by preparative reversed-phase high performance liquid chromatography (RP-HPLC). Standard characterization of synthetic peptides includes assessment of purity by RP-HPLC, amino acid analysis and determination of molecular mass by mass spectroscopy. Although these three methods allow (in one way or another) the detection of the most common side products in peptide chemistry (adducts and truncations), the latter two techniques will not distinguish diastereomers and thus could not detect racemic contamination that could lead to ambiguous biological results. In this study of a series of NPY analogues we have confirmed both the uniqueness of each derivative differing only by the inversion of a single chiral center and the resolution power of RP-HPLC and that of capillary zone electrophoresis (CZE), especially when combined with the use of the trietylammonium phosphate (TEAP) separation buffer [4].

EXPERIMENTAL

All reagents and solvents were of analytical grade (Aldrich, Milwaukee, WI, USA; Fisher Scientific, Springfield, NJ, USA) and were used without further purification except triofluoroacetic acid (TFA) (Halocarbon, Hackensack, NJ, USA) and triethylamine (TEA) (Aldrich), which were reagent grade and used without further purification for syntheses but were distilled to constant boiling point for use in the chromatographic buffers.

Peptide synthesis

All peptides were assembled using standard SPPS techniques on *p*-methylbenzhydrylamine (MBHA) resin, as previously described, [5,6]. Briefly, *tert.*-butyloxycarbonyl (BOC) was used for N-terminal protection and deblocked with TFA in the presence of ethanedithiol (EDT). Most couplings employed 1,3-diisopropylcarbodiimide (DIC) as primary coupling reagent; more difficult cycles required the use of benzotriazol-1-yl-oxytris (dimethylamino)phosphonium hexafluorophosphate (BOP) in N-methylpyrrolidone (NMP) or dimethylformamide (DMF) in the presence of an excess of diisopropylethylamine (DIPEA). Couplings were generally carried out for 1 h, completion of the couplings was monitored by qualitative Kaiser test [7]. Coupling steps were repeated as necessary. After TFA deprotection, resin washes included 2-propanol (containing 1% EDT), triethylamine [10% in dichloromethane (DCM)] for neutralization, MeOH and DCM. The protected peptide was cleaved from the resin with anhydrous HF in the presence of 10% anisole at 0°C for 90 min. Volatiles were removed *in vacuo*. The crude peptides were precipitated with anhydrous ethyl ether, filtered to remove ether soluble non-peptide materials and extracted in water. After lyophilization, crude peptides were purified by preparative RP-HPLC [8,9] on a Waters DeltaPrep LC 3000 system equipped with a Waters 1000 Prep Pak Module and a Shimadzu SPD-6A variable-wavelength UV detector. The cartridges used were hand packed, in Waters polyethylene sleeves and frits with Vydac C_{18} packing material (15–20 μm particle size, 30 nm pore size). The material was eluted first using a linear TEAP–acetonitrile (40:60) (pH 2.25 or 5.20) buffer system gradient; acceptable fractions were pooled, reloaded onto the preparative cartridge and desalted in 0.1% TFA. Final products were >95% pure by RP-HPLC analysis.

Peptide characterization

Purified peptides were subjected to hydrolysis in 4 *M* methanesulfonic acid at 110°C for 24 h, and analyzed by ion-exchange chromatography with post column derivatization with *o*-phthalal-

dehyde. Liquid secondary ion (LSI)-MS analysis was performed on a Jeol JMS-HX110 double focusing mass spectrometry (Jeol, Tokyo, Japan) fitted with a Cs^+ gun. Samples were added directly to a glycerol–3-nitrobenzyl alcohol (1:1) matrix.

RP-HPLC

Samples for coinjection experiments contained native NPY (0.3 μg/10 μl) and a single diastereomer (1.0 μg/10 μl) dissolved in 0.1% TFA. In addition, each sample contained an analogue of ovine corticotropin releasing factor, [NIe^{21},Tyr^{27}]-oCRF (a 41-peptide) as an internal standard. Coinjection experiments were performed on a Hewlett-Packard Series II 1090 liquid chromatograph with diode array detector and HPLC ChemStation. The Vydac C_{18} and diphenyl columns were from the Separations Group and were 15 × 0.21 cm. Buffer A was 0.1% TFA or TEAP. TEAP was 0.1% phosphoric acid adjusted to the desired pH by addition of TEA. Buffer B (TEAP) was composed of acetonitrile–TEAP A buffer (60:40) while the B buffer (TFA) was 0.1% TFDA in acetonitrile–water (60:40). Analyses were performed at 40°C at a flow-rate of 0.2 ml/min. and detected at 210 nm. Linear gradient conditions were 45–75% B in 30 min. Uniform column characteristics were confirmed by reanalysis of several closely eluting diastereomers following the lenghty C_{18} series.

CZE

CZE was performed on a Beckman P/ACE System 2050 with Spectra-Physics ChromJet SP4400 integrator. Fused silica capillaries from Beckman were 50 cm × 75 μm. The buffer was 100 m*M* phosphoric acid adjusted to pH 2.50 by addition of 2 *M* sodium hydroxide or 100 m*M* phosphoric acid adjusted to pH 2.50 by the addition of TEA. Voltage ranged from 12 to 20 kV and produced from 66 to 100 μA. Detection was at 214 nm and the temperature was maintained at 30°C. Native NPY and the diastereomers were used as prepared for RP-HPLC and applied by pressure injection. After each separation, a 1-min rinse with 0.5 *M* TEAP or sodium phosphate pH 2.50 was performed.

RESULTS AND DISCUSSION

Separation of NPY diastereomers by RP-HPLC

Neuropeptide Y is a relatively large polypeptide (M_r 4252) with 38 chiral centers and a member of the pancreatic polypeptide (PP) family. The tertiary structure of avian PP (aPP) has been elucidated in detail (<1 Å) by X-ray diffraction analysis revealing an intramolecularly stabilized helical structure referred to as the PP-fold [10]. Recent spectral studies as well as molecular dynamics simulation suggest that NPY may have a similar intramolecularly stabilized three-dimensional structure as illustrated in Fig. 1; specifically, a polyproline-type II helix for residues 1–8, a β-turn through positions 9–14, an amphipathic α-helical segment extending from residues 15–32 and a C-terminal turn structure from residues 33–36 [11]. While the α-helical component of the structure of either NPY or NPY C-terminal fragment 18–36 could be demonstrated by NMR [12–14] and circular dichroism (CD) spectroscopy [11,15] other features such as the N-terminal poly-proline helix remain hypothetical or are not substantiated by NMR. Supposing therefore that NPY has a high

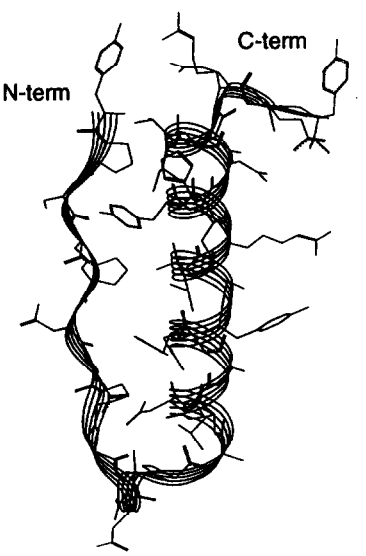

Fig. 1. Ribbon illustration of NPY obtained by modeling of NPY by homology to X-ray crystallographic structure of avian pancreatic polypeptide and tethered minimization to relieve side-chain stretch.

degree of conformational integrity, any modification of the backbone by the introduction of a single D-amino acid was expected to change the solution conformation and thus the chromatographic properties of the resulting diastereomers. Previous attempts at resolving diastereomers of NPY or peptides of similar size by chromatographic techniques have met with only limited success, and have therefore relied heavily on difference in optical rotation or biological activities for complete demonstration of uniqueness such as was the case for bombesin [16], neurotensin [17], somatostatin [18] and CRF [19]. A thorough investigation of retention behavior of the D-series of NPY_{18-36} was recently published, although demonstration of uniqueness of each analogue was not intended [20].

Initially, we obtained retention data for the 35 isomers of NPY in the standard analysis system developed in our laboratory, using a Vydac C_{18} column and mobile-phase composed of TEAP at pH 2.25 at 25°C (Table I). This chromatographic system, which favours basic peptides and amines, was first described by Rivier [4] and has proven to be very useful in the separation of closely associated impurities generated in peptide synthesis (typically fragments or adducts differing from the major component by size and/or charge). As indicated, resolution was achieved for 26 of the 35 analogues yielding separation factors (α) that were greater than 1.03. Centrally modified residues produced the clearest separation with poor resolution of N-terminally modified analogues under the same conditions. These results suggest that a more flexible conformation at the termini may produce few side chain interactions between the peptide and the stationary phase. Since any difference in retention would reflect a difference in the solute's binding affinity to the stationary phase, it is also possible that the N-terminus under these conditions is not exposed to the surface and therefore is prevented from interacting with the stationary ligand. Under the same gradient conditions at 40°C efficiency increased, allowing the separation of three additional N- and C-terminal analogues (Fig. 2 and Table I). Using other supports than the Vydac material reported here, in this case Baker-bond silica, we found that the D-isomer series of NPY_{18-36} displayed maximum retention to a C_8 support at 25°C [20]. Since the magnitude of log k' is a measure of the free energy associated with the binding of the solute to the

TABLE I

COMPARISON OF SEPARATION FACTORS FOR 35 STEREOISOMERS OF NPY UNDER GIVEN RP-HPLC CONDITIONS

A = Vydac C_{18} using TEAP pH 2.25 mobile phase modifier at 40°C; B = Vydac C_{18} using TEAP pH 2.25 mobile phase modifier at 25°C; C = Vydac C_{18} using 0.1% TFA mobile phase modifier at 40°C; D = Vydac diphenyl using 0.1% TFA mobile phase modifier at 40°C.

Isomer	α			
	A	B	C	D
D-Tyr[1]	1.00	1.00	1.00	1.00
D-Pro[2]	1.02	1.00	1.01	1.02
D-Ser[3]	1.00	1.00	1.00	1.00
D-Lys[4]	1.00	1.00	1.00	1.00
D-Pro[5]	1.00	1.00	1.00	1.03
D-Asp[6]	1.03	1.00	1.01	1.02
D-Asn[7]	1.00	1.00	1.00	1.03
D-Pro[8]	1.05	1.03	1.03	1.04
D-Glu[10]	1.07	1.06	1.05	1.09
D-Asp[11]	1.12	1.11	1.08	1.08
D-Ala[12]	1.07	1.06	1.05	1.07
D-Pro[13]	1.05	1.03	1.04	1.06
D-Ala[14]	1.05	1.04	1.03	1.06
D-Glu[15]	1.24	1.18	1.16	1.20
D-Asp[16]	1.06	1.05	1.04	1.05
D-Leu[17]	1.26	1.18	1.16	1.22
D-Ala[18]	1.16	1.09	1.10	1.16
D-Arg[19]	1.34	1.20	1.20	1.32
D-Tyr[20]	1.07	1.14	1.06	1.00
D-Tyr[21]	1.20	1.10	1.13	1.10
D-Ser[22]	1.18	1.07	1.09	1.16
D-Ala[23]	1.39	1.34	1.20	1.27
D-Leu[24]	1.35	1.17	1.19	1.31
D-Arg[25]	2.05	1.56	1.44	1.57
D-His[26]	1.24	1.13	1.12	1.20
D-Tyr[27]	1.41	1.25	1.22	1.32
D-Ile[28]	2.76	1.90	1.62	1.96
D-Asn[29]	1.07	1.04	1.04	1.04
D-Leu[30]	1.46	1.28	1.23	1.32
D-Ile[31]	1.62	1.40	1.31	1.45
D-Thr[32]	1.33	1.19	1.19	1.26
D-Arg[33]	1.15	1.15	1.12	1.10
D-Gln[34]	1.07	1.12	1.06	1.06
D-Arg[35]	1.00	1.00	1.00	1.02
D-Tyr[36]	1.00	1.00	1.00	1.00

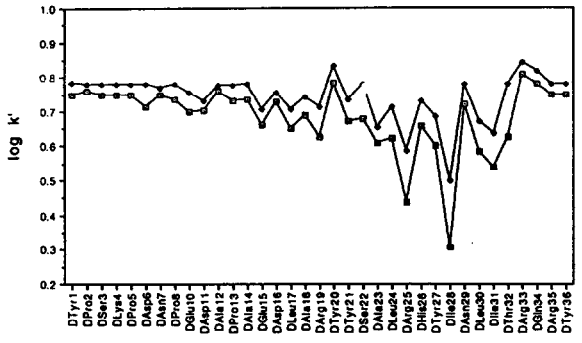

Fig. 2. Plot of log k' *versus* position of D-amino acid substitution in NPY: TEAP pH 2.25–acetonitrile buffer system on Vydac C_{18} at (□) 40°C, and (◆) 25°C. See Experimental section for additional chromatographic details.

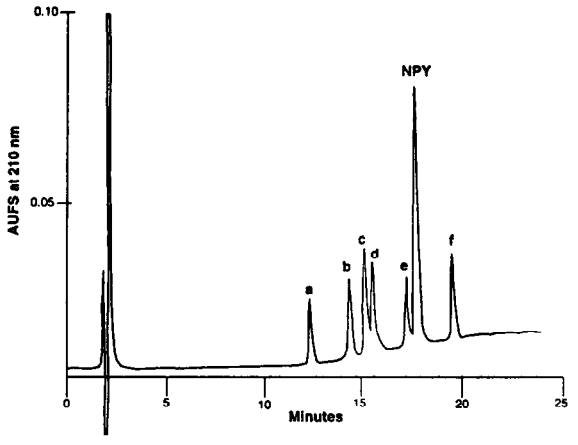

Fig. 3. Coelution of diastereomers of NPY by RP-HPLC using TEAP pH 2.25 buffer at 40°C on Vydac C_{18}. Peaks: a = [D-Ile^{28}]-NPY; b = [D-Ile^{31}]-NPY; c = [D-Leu^{30}]-NPY; d = [D-Thr^{32}]-NPY; e = [D-Asn^{29}]-NPY; f = [D-Arg^{33}]-NPY. See Experimental section for additional chromatographic details.

stationary phase, it is inferred that the interactive behavior of NPY and analogues with the octadecylsilane (Vydac) is stabilized by the presence of N-terminal residues at 25°C. At 40°C, the free energy contribution by each residue was magnified (Fig. 2). Hence, the higher temperature permitted the additional discrimination between D-isomers of [Pro^2]-, [Asp^6]-, and [Arg^{35}]-NPY from the native peptide. At both temperatures, greatest selectivity was exhibited by analogues modified in the region proposed to exist as an alpha helix, as expected since these residues are held in close proximity by hydrogen bonding and hydrophobic interactions and critically depend on the orientation of the side chains to maintain conformational stability. Coinjection of six such centrally modified analogues with NPY is shown in Fig. 3.

When changing the mobile phase modifier to 0.1% TFA, a more hydrophobic counterion, the same 28 isomers were again resolved at 40°C, although α values, in general, were smaller than those obtained with TEAP, and the elution order of two isomers, [D-Pro^2]- and [D-Asp^6]-NPY, was reversed. These observations are not unique to NPY and have been also described for opiate peptides by Zhu *et al.* [21] who used both TFA and phosphate buffers. The decrease in ionic strength of the TFA buffer is implied from the increase in k' values under identical gradient conditions, as graphically illustrated in Fig. 4. The magnitude of the log k' value is a measure of the affinity of the peptide surface to the stationary phase in the absence of the organic modifier. Ion-pairing reagents such as H_3PO_4 and TFA effect changes in peptide retention solely through interaction with positively charged groups on the peptide, with the greater strength of the phosphate counterions reducing the affinity of the peptide to the stationary phase.

Under the acidic conditions of the previous buffer systems, it was expected that the peptide would be partially denatured and all basic residues would be fully protonated. CD and NMR spectroscopy data have indicated that the secondary structural features of NPY, especially α helical content increased as the solution ap-

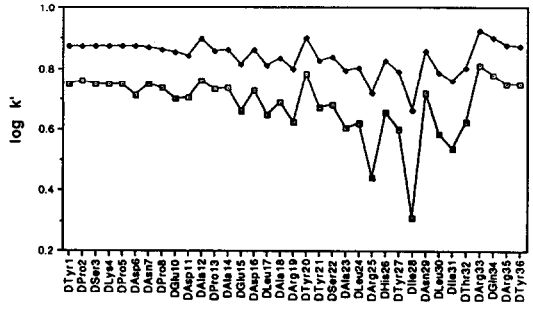

Fig. 4. Plot of log k' *versus* position of D-amino acid substitution in NPY: Vydac C_{18} at 40°C using (□) TEAP pH 2.25 and (◆) 0.1% TFA mobile phase modifiers. See Experimental section for additional chromatographic details.

proached neutral pH [13,14]. Expecting the same effect on the RP-HPLC column, we therefore attempted to separate the four remaining isomers using a Vydac C_{18} stationary phase in a buffer system comprised of TEAP at pH 6.6 and 4.3 [9]. Interestingly, severe band broadening and significantly increased k' of NPY was observed, although the similarly sized and charged corticotropin releasing factor analogue ([Nle^{21},Tyr^{27}]-oCRF), used as an internal standard, eluted as a narrow band indicating no change in H_{eff} (Fig. 5). Since the apparent column efficiency was unchanged, this phenomena alludes to the possibility that NPY may adopt multiple similar transitions or conformations on the C_{18} stationary phase at neutral pH producing a variety of similar surface interactions with the stationary phase. In support of this theory, recently reported structural determination of NPY by NMR spectroscopy using water as the solvent at pH 3.20 and 37°C suggested the exclusive presence of an NPY dimer, though at higher concentrations than those that were used in our studies [14]. Similar effects have been reported for β-endorphin and other related opioid peptides [22] and for NPY and related analogues at elevated temperatures [20].

Of the four unresolved derivatives from the previous stage, we expected that the two terminal D-Tyr analogues (positions 1 and 36) would present the greatest challenge since the physicochemical properties of these derivatives would be expected to be nearly identical to that of the native peptide and the inversion of chirality would assert little effect upon hydrophobicity. Additionally, molecular modeling and other structural investigations [11–13] indicated that the termini display little interaction with neighboring residues. Because of the high content of aromatic residues present in NPY, it was hypothesized that the selectivity of the diphenyl-derivatized column might be more sensitive to the modified orientation at one of these tyrosine residues. Analysis in 0.1% TFA–acetonitrile permitted the resolution of three additional diastereomers, [D-Pro^5]-, [D-Asn^7]- and [D-Arg^{35}]-NPY, but failed to demonstrate increased discrimination of D-tyrosine containing molecules (Table I and Fig. 6). Co-elution of native NPY with the corresponding D-isomers of each of the tyrosines at positions 1, 27 and 36 indicated that the inversion of chirality at these positions was without significant effect on the peptide–diphenyl interaction under the buffer conditions used in these experiments.

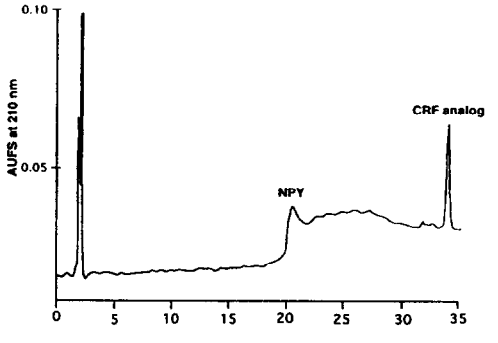

Fig. 5. Chromatogram of NPY and [Nle^{21},Tyr^{27}]-oCRF using TEAP pH 6.50–acetonitrile gradient system on Vydac C_{18} at 40°C. See Experimental section for additional chromatographic details.

The evaluation of data generated by this study of 35 peptide isomers under a variety of separation conditions provides a unique opportunity to discuss trends in chromatographic behavior. First, we have seen that the TEAP buffer system under identical linear gradient conditions produces faster elution of peptide solutes with equal efficiency as the 0.1% TFA buffer system (Fig. 4), long regarded as standard in peptide chemistry. Although the same isomers were resolved from NPY by TEAP, separation factors were greater. Additionally, we have demonstrated improved separation by analysis at 40°C in TEAP (Fig. 2). Second, while the comparison of peptide retention on C_{18} and diphenyl derivatized silica has not proven the advantage of one ligand over the other, concurrent exploitation of both sorbents was shown to be complementary in some cases, with the combination of data producing unequivocal separation of all but four stereoisomers (Table I). Finally, we have recognized the influence of conformation in the variation of peptide retention to a stationary phase. Substitution of NPY by a D-amino acid at positions 22–31 produced analogues with the largest deviations in chromatographic behavior, suggesting that such substitutions were the most disrup-

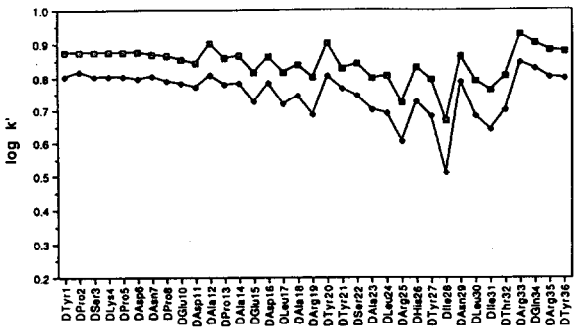

Fig. 6. Plot of log k' *versus* position of D-amino acid substitution in NPY: 0.1% TFA–acetonitrile mobile phase at 40°C using (□) Vydac C_{18} and (◆) Vydac diphenyl. See Experimental section for additional chromatographic details.

tive of NPY's conformation in the buffers used. In all four RP-HPLC analysis conditions, we have seen that the D-amino acid isomer of NPY was overwhelmingly (>80%) the first to elute from the column. This phenomena was independent of particular functional group or position in the sequences. It was also shown that the retention times could vary by as many as 6 min for diastereomers using the identical linear (30 min) gradient conditions, making prediction of peptide retention highly speculative.

Separation of isomers by CZE

As a final approach to separating the remaining isomers, CZE was employed. CZE has been proved to be a very useful analytical tool in our laboratory and that of others [23–25]. The large number of theoretical plates in CZE lends this system the ability to discriminate between small differences in charge densities of peptide molecules and has thus allowed the resolution of species with very subtle differences in structure. The separation mechanism in free solution electrophoresis is based on the differential electrophoretic mobilities of the solutes, which in turn is related to their charge densities [25]. It was expected that, although the global charge of each NPY isomer would be identical, the altered local orientation of a charged group might be exposed, and thus could be expressed by differing migration times. Under the first set of conditions, [D-Ser^3]- and [D-Tyr^{36}]-NPY were resolved from NPY (Fig. 7a) when subjected to the electric field of 12 kV (62 μA) in 0.1 *M* sodium

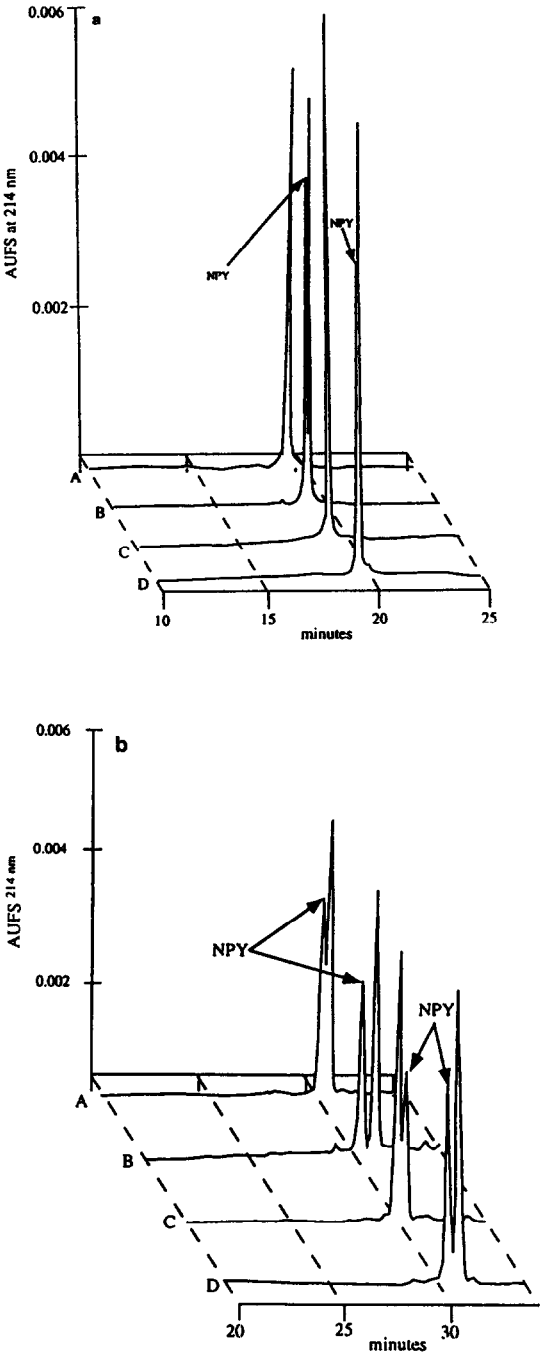

Fig. 7. Coelution of diastereomer pairs of NPY by CZE. (a) Electrophoresis performed at 12 kV, 62 μA using 0.1 *M* sodium phosphate at pH 2.50. (b) Electrophoresis performed at 20 kV, 100 μA using 0.1 *M* TEAP at pH 2.50. For each electropherogram, native NPY was coinjected with one of the following stereoisomers: A = [D-Tyr^1]-NPY; B = [D-Ser^3]-NPY; C = [D-Lys^4]-NPY; D = [D-Tyr^{36}]-NPY.

phosphate at pH 2.5. Migration times differed from that of native NPY by 0.1 min. The other isomers, [D-Tyr[1]]- and [D-Lys[4]]-NPY, were not resolved under the same conditions.

Replacing the sodium cations of the electrolyte with TEA, proven effective in RP-HPLC separations, once again demonstrated the utility of such an acidic TEAP buffer system in another separation system (Fig. 7b). Acidic pH was beneficial in the electrophoretic system as electroosmosis is greatly reduced and solute-silica wall interactions are minimal. As triethylamine replaced sodium as the electrolytic cation the electrophoretic mobility was greatly decreased. This may be due to the increased radius of the TEA cation resulting in an increase in viscosity of the buffer. In order to accelerate analysis times the applied voltage was increased to 20 kV (migration time of the isomers was approx. 30 min) and a current of approximately 100 μA was produced. Under these conditions the entire series of 35 isomers was evaluated and resulted in the separation of 32 out of 35 isomers.

Thus, even those stereoisomeric pairs which could not be resolved by RP-HPLC were clearly separated in this electrophoretic system at an applied voltage of 20 kV. Other contributing factors to a better separation in TEAP as compared to sodium phosphate buffer at the same molar concentration may include such parameters as viscosity, interaction of the triethylammonium ion with the silanols of the capillary walls and/or differences in ionic strength. Migration times, band width and amperage, were highly reproducible in the TEAP system (relative standard deviation = 4%) (Table II). Migration times of each diastereomer did not vary as much as they did under RP-HPLC from that of NPY and in some cases co-elution profiles did not allow unequivocal identification of NPY *versus* its diastereomer. Since the actual charge-to-mass ratio is identical for all diastereomers ($q/M_r^{2/3}$, where q is the calculated charge and M_r is the molecular mass), it was concluded that any separation would be the direct result of intramolecular interactions created by inverting the chirality at a single residue. Because we were able to separate most diastereomers using CZE (see Table II), such an inversion clearly affected the expression of the local charge density. Our conclusion disputes the premise of Rickard *et al.* [24] who proposed that any minor irregularities in solute shape (such as that resulting from inversion of chirality at one center) was likely to

TABLE II

ELECTROPHORETIC MIGRATION TIME DATA FOR 35 STEREOISOMERS OF NPY USING 0.1 *M* TEAP pH 2.50 BUFFER MATRIX

Average migration time (t_m) for NPY was 29.03 min with standard deviation of the mean = 1.31 ($n = 35$). See Experimental section for additional chromatographic details.

Isomer	First elutant	NPY t_m (min)	D isomer t_m (min)	Δt_m (min)
D-Tyr[1]	L	30.32	30.64	0.32
D-Pro[2]	L	29.94	31.17	1.23
D-Ser[3]	L	30.50	31.12	0.62
D-Lys[4]	D	30.11	29.69	0.42
D-Pro[5]	–	28.19	28.19	0
D-Asp[6]	L	28.86	29.15	0.29
D-Asn[7]	L	26.19	27.33	1.14
D-Pro[8]	L	27.42	27.79	0.37
D-Glu[10]	–	28.39	28.39	0
D-Asp[11]	D	29.04	28.74	0.30
D-Ala[12]	D	27.67	26.30	1.37
D-Pro[13]	D	28.28	28.04	0.24
D-Ala[14]	D	28.03	27.63	0.40
D-Glu[15]	–	28.46	28.46	0
D-Asp[16]	D	28.14	27.68	0.46
D-Leu[17]	L	27.91	28.52	0.61
D-Ala[18]	L	27.87	28.56	0.69
D-Arg[19]	L	27.93	28.66	0.73
D-Tyr[20]	L	28.10	29.58	1.48
D-Tyr[21]	L	28.12	28.66	0.54
D-Ser[22]	L	28.27	28.85	0.58
D-Ala[23]	L	28.17	28.91	0.74
D-Leu[24]	L	28.21	28.59	0.38
D-Arg[25]	D	30.28	29.01	1.27
D-His[26]	D	30.56	28.57	1.99
D-Tyr[27]	D	30.71	30.41	0.30
D-Ile[28]	D	28.61	28.37	0.24
D-Asn[29]	D	30.41	29.96	0.45
D-Leu[30]	L	32.72	33.78	1.06
D-Ile[31]	L	30.84	31.37	0.53
D-Thr[32]	D	30.43	29.03	1.40
D-Arg[33]	D	28.65	28.01	0.64
D-Gln[34]	D	29.28	28.62	0.66
D-Arg[35]	D	29.17	28.71	0.46
D-Tyr[36]	L	30.25	30.72	0.47

be smoothed out by the solvation sphere and ionic atmosphere that surrounds peptide ions in solution.

While 80% of the D-amino acid containing analogues of NPY eluted before NPY under RP-HPLC conditions, we did not discover any obvious trends displayed by the 35 isomers using capillary electrophoresis. Migration times were so close in most cases, that any attempt to draw from such results would likely lead to over-interpretation. There was no pattern to the isomers which failed to be resolved, nor was there any pattern as to which diastereomers displayed the highest electrophoretic mobilities (Table II).

CONCLUSIONS

This study has demonstrated the rapid and effective employment of modern chromatographic techniques for the resolution of diastereomers of NPY. For complete resolution of diastereomers or closely associated impurities in peptides of this size, the challenge presented will likely necessitate the use of several overlapping detection systems to eliminate ambiguity, as in the present study. In this way, many physicochemical properties may be exploited leading to an optimized separation profile. Additionally, we have proven the utility of the TEAP separation buffer system for both RP-HPLC and CZE applications. Although it was proposed that retention times of peptides could be predicted [26] the data presented here clearly indicate that a conformational parameter should be introduced in any such methods. Similarly, although a correlation between q/M_r was suggested by Rickard *et al.* [24] for peptide and proteins using CZE, a conformational component also needs to be introduced as a further refinement of such studies.

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