Tripeptide Discriminations Using Circular Dichroism Detection

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Abstract □ A general spectroscopic method is described that might be applied to validating amino acid sequences in peptides and protein fragments with a view to it becoming a routine procedure with which to characterize biotechnology drug products. The tripeptides are the L-enantiomers of GGA, GGH, GGI, GGL, GGF, GHG, LGG, and YGG. The simple procedure calls for their complexation with Cu(II) ion in strong aqueous base. Binding the first three residues in the sequence, beginning at the amine terminus, completes the coordination sphere of the Cu(II) ion, so duplication of the initial sequence from peptide to peptide could be an important limiting factor in determining the extent of differentiation that is possible. The analytical focus is the selectivity associated with the chirality properties of the peptides. Detection is by circular dichroism operating in the visible range. The eight analytes were chosen as representative of a series where the sequences are most similar and therefore potentially the most difficult to discriminate spectroscopically. All have just one chiral center. Using ellipticity data at all ($n = 1500$) wavelengths in the measured spectra, and two novel data reduction procedures, total discrimination among all eight analytes is achieved. The method has considerable potential for use in quality control of peptide and protein biotechnological drug forms, especially their enantiomeric purities.

Introduction

Modern pharmaceutical and biotechnology conglomerates are committed to the production of chiral drug substances.[1,2] Manufacturers have the option to prepare chiral drugs either as pure single substances (enantiomers) or as racemic mixtures. While racemates are easier to make, many good reasons exist for choosing to manufacture enantiomerically pure forms, not the least of which are considerations of the relative therapeutic values and relative toxicity levels of each enantiomer either by itself or as half of a racemate. All of this means that there is a need for simple routine analytical methods that are adaptable to all chiral drug forms which can be used for regulatory control of their chemical and enantiomeric purities (EP), whether it is done by the manufacturer or by a federal agency.

Analytical options currently applied to these tests generally involve simultaneous derivatization of both enantiomers in a partial racemic mixture to their corresponding diastereoisomers by selective reactions with a third chiral species. Unlike enantiomers, diastereoisomers can be differentiated by physical properties other than just the direction of rotation of linearly polarized light.[3] Chiral chromatography is a major player in the development of these methods. The chiral third party is introduced either in the mobile phase or immobilized on the stationary phase.[4,5] Since diastereomers elute after different retention times, achiral detectors, e.g., absorbance, electrochemical, and mass spectrometry, are sufficient to effect quantitative distinctions, within the limit of the detection capabilities of the chosen methods.

If the experimental preference is to determine chemical purities without a prior separation step, spectroscopic procedures generally call for the use of two detectors, one of which is a chiral detector such as polarimetry or circular dichroism (CD).[6−8] By combining CD with absorbance detection, measuring spectral differences or spectral ratios are different strategies that can be applied to handling the data. Generally speaking most of these methods are based on single wavelength detection data.

By combining multiple wavelength detection with modern chemometric methods for data analysis, a third alternative procedure was described.[9,10] The procedure exploits the best characteristics of both of the other methods, namely a single chiral detector, bulk in situ derivatizations, and no separations. Results obtained for the determinations of chemical and enantiomeric impurities using visible CD detection for binary mixtures of the four ephedrine stereoisomers complexed to Cu(II) ion were an improvement over what was capable at that time by either the chiral chromatographic or two-detector methods.

A major new frontier in the pharmaceutical industry is the focus on the therapeutic properties of peptide and protein drug forms. Because the number of chiral centers has virtually no limit, the magnitude of the chirality regulatory control problem is increased almost exponentially. Since derivatizations will not produce a single diastereoisomer, even the very best chiral chromatographic methods face what are probably insurmountable challenges unless the peptides are first cleaved enzymatically.

Problems that are associated with chirality detection also increase. The total CD signal for a metal−peptide complex is not determined by just the number and sequence of chiral centers in the primary peptide structure. It also includes contributions from longer range chiral interactions between side-chain substituents that modify the ternary structure when peptides are coordinated to metal ions. Experimental conditions must be very carefully controlled, otherwise these very pH-sensitive structural modifications would give false information about the analyte to the detector. On the other hand the simple accumulation of these additive chiral properties could conceivably produce a level of analytical selectivity that is unmatched by other detectors and might even approach specificity. Enzymatic cleavage followed by CD detection is also an option. What chirality detection contributes that the others do not is a direct look at the enantiomeric form. This ability will increase in value as long as manufacturers continue to use D- for L-enantiomeric substitutions as a strategy in peptide drug design.

Eight tripeptides were chosen for the study. Common to all eight are two glycine residues which occupy positions 1,2-, 1,3-, and 2,3- in the sequence. The remaining residues are L-enantiomers of aliphatic and aromatic amino acids. The tripeptides have no stable ternary structure to speak of, so variability in the sequence is really the only parameter affecting the chiral response of the CD detector. The order of residues in short peptides is crucial to the extension of the study to peptides and proteins as a whole because coordination of the latter to Cu(II) involves the first

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three amino acids from the amine terminus. If there is no CD selectivity associated with changes in the initial sequence, the method has no value in the study of oligopeptides and proteins where too frequently the same initial sequence is common to several potential analytes.

The experimental procedure is a combination of the methods that were used to discriminate among related dipeptides[11] and insulins[12] and to measure EP's for glycyl-L-alanine and ephedrine mixtures.[9−11] Data reduction and spectral differentiations are done using variations on standardized mathematical algorithms and principal component analysis (PCA).

Experimental Section

Chemicals—Tripeptides used in the study were glycylglycyl-L-alanine (GGA), glycylglycyl-L-histidine (GGH), glycylglycyl-L-isoleucine (GGI), glycylglycyl-L-leucine (GGL), glycylglycyl-L-phenylalanine (GGF), glycyl-L-histidylglycine (GHG) and its D-enantiomer (GhG), l-leucyl-glycylglycine (LGG) and its D-enantiomer (lGG), and L-tyrosylglycylglycine (YGG) and its D-enantiomer (yGG). All eight L-enantiomers were supplied by Sigma Chemical Co. which reported an EP in excess of 99.8%. The D-enantiomers GhG, lGG, and yGG of GHG, LGG, and YGG were prepared by Multiple Peptide Systems (MPS), San Diego, CA. Certificates of Analysis described them as unpurified off white powders. Percent purities as determined by RP-HPLC analyses were reported as 86.57, 99.10, and 97.86, respectively. The low value for GhG is explained as being due to two elution peaks that correspond to the same compound. The percentage is based on the relative area of the first peak which corresponds with most of the material eluting with the void volume peak. The second peak is related to the hydrophobicity of the molecule that causes it to stick to the column to be eluted later. D-Histidine was also a Sigma Chemical Co. product with an EP reported at better than 99.8%. Reagent grade $CuSO_4 \cdot 5H_2O$ was obtained from Fisher Scientific.

Solution Preparations—The chemistry of the derivatization reaction is a simple chiral variation of the classical biuret "color reaction" for the determination of total serum proteins in which the reagent is a solution of [Cu(II)] = 2.0 mM and [tartrate] = 8.0 mM in 0.1 M NaOH. Racemic NaK-tartrate is the solubilizing ligand for Cu(II) and is completely exchanged by protein in the test. The chemistry for the reaction is well understood and relatively uncomplicated.[13] Determinations were done based upon absorbance spectrophotometric detection. Being relatively insensitive and not sufficiently selective, the biuret reaction is no longer the method of choice for serum proteins.

In this instance, aqueous stock solutions at pH 13 were prepared for Cu(II)−D-histidine and each of the Cu(II)−L-tripeptide complexes in which the Cu^{2+} concentration was always 0.020 M. Ligands were present at 0.080 M concentrations, a 4:1 excess over the Cu(II) ion. KI at a concentration of 0.03 M was added as a stabilizer.[9,10] Spectra were measured for working solutions prepared by diluting stocks by a factor of 10 with 0.10 M NaOH. Spectra for the working solutions are the bases for testing the extent of the qualitative analytical selectivity accessible to CD detection.

Quantitation tests were done on two kinds of mixtures. For the first kind, GGA was arbitrarily selected as an enantiomerically pure "reference" material. Aliquots from the stock were spiked with "chemical impurities", i.e., smaller volume aliquots of the other L-tripeptide stocks to cover the impurity range from 1 to 10%, prior to dilution with NaOH. For the second, "enantiomeric purity" tests, aliquots of YGG, LGG, and GHG stocks were spiked with smaller volume aliquots of the corresponding D-enantiomer stocks, yGG, lGG, and GhG, over the same 1−10% impurity range.

Measurements—CD spectra were measured using a Jasco 500-A automatic recording spectropolarimeter coupled to an IBM-compatible PC through a Jasco IF-500 II serial interface and data processing software. Experimental parameters: wavelength range 400−700 nm; sensitivity 100 mdeg/cm; time constant 0.25 s; scan rate 200 nm/min; path length 5.0 cm; ambient temperature.

Calibration of the day to day reproduciblity of the system was done by measuring the CD spectrum for the Cu(II)−D-histidine complex. Statistical data for reproducibilities of the maximum ellipticities measured at wavelengths 487 nm and 682 nm were 7.42 ± 0.07 mdeg and −214 ± 0.60 mdeg, respectively.

Results and Discussion

Cu(II)−Peptide and D-Histidine Complexes—The local microsymmetry of the Cu(II) ion in aqueous solution is essentially square-planar due to axial elongation of the typical octahedral symmetry, assumed by most first row transition metal ions, by Jahn−Teller distortion.[14] Complexation serves to keep the Cu(II) ion in solution at high pH conditions. At pH 13, D-histidine and the amide-nitrogen protons are fully ionized,[15] which essentially eliminates competitive complex formation equilibria when partially protonated anions are present in solution at lower pH.

D-Histidine, the ligand used for instrument calibration, binds via the amine N-atom, the carboxylate functional group, and a pyrimidine N-atom in an equatorial three-coordinate arrangement. Stoichiometry for the complex is 1:1.[15] Complexing a peptide to Cu(II) at pH > 12 involves first attachment through the N-atom of the terminal amine followed by ring closure(s) through bonding with the N-atoms of successive amide bonds until maximum thermodynamic stability is achieved.[15] Side chain substituents on the amino acid residues lie out of the coordinate plane and are factors only in inter- and intramolecular interactions within the inner coordination sphere, unless a potential Lewis base is present, e.g., a histidine residue. Axial positions might be occupied by hydroxide ions which is the only feature that might complicate the stoichiometry of the generic metal−peptide, $(MP)_n$, equilibrium.[16]

By analogy with the Cu(II)−D-histidine equilibrium reaction, the stoichiometry of the Cu(II)−tripeptide complexes is also believed to be 1:1. If the only purpose of the study were to develop analytical selectivity, the question of the stoichiometry of the metal−tripeptide complexes is not relevant. If the stoichiometry were to change from one ligand to another, the analytical selectivity might very well be enhanced. It is only when making an analytical determination by conventional mathematical procedures that knowledge of the stoichiometry is a prerequisite.

CD activity in the visible range for chiral Cu(II) complexes is a result of disymmetric perturbations of ground and excited state ligand field orbitals by the chiral ligands. Bands in the UV range, attributable to only the chirality in the ligands, bound and unbound, are typically very intense but quite insensitive to the environment of the coordinating metal ion. The lack of selectivity is the major reason for not exploiting the obvious analytical sensitivity that is inherent in the intense UV bands.

Visible CD Spectra for Cu(II)−Tripeptide Complexes—Spectra for all eight copper−L-tripeptide complexes, in which [Cu(II)] = 2.0 mM and ligand concentrations are 8.0 mM, are shown in Figure 1. Only GGH, GHG, LGG, and YGG are uniquely differentiable by their zero order CD spectra. Spectra for the histidyl-containing ligand complexes, GGH and GHG, are blue shifted compared with the Cu(II)-D-histidine complex itself which has an intense negative band with a maximum at 689 nm and a weaker positive maximum at 570 nm. The magnitude of the shift is greatly dependent upon the position occupied by the histidyl residue. The sensitivity of the CD spectral response to the histidine position is a significant first result in the context of possibly sequencing short peptides by this spectroscopic method.

Of the five GGX peptides, only the spectrum for GGH is unique, which might be attributable to a special involvement of the pyrimidine N-atom in binding to Cu(II). The remaining four have but one broad negative band that

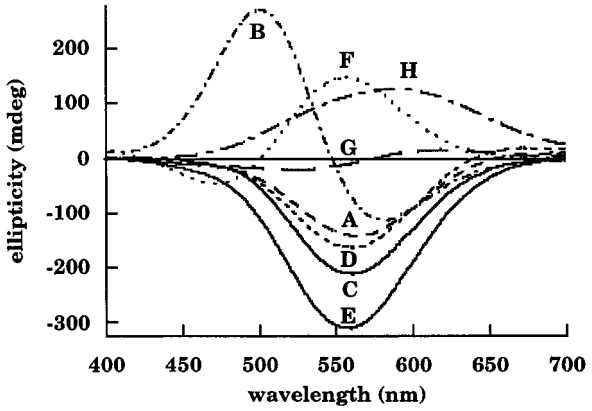

Figure 1—Visible CD spectra for the Cu(II) complexes of (A) GGA, (B) GGH, (C) GGI, (D) GGL, (E) GGF, (F) GHG, (G) LGG, and (H) YGG. Similarities are greatest for the GGA, GGI, GGL, and GGF complexes over the entire wavelength range.

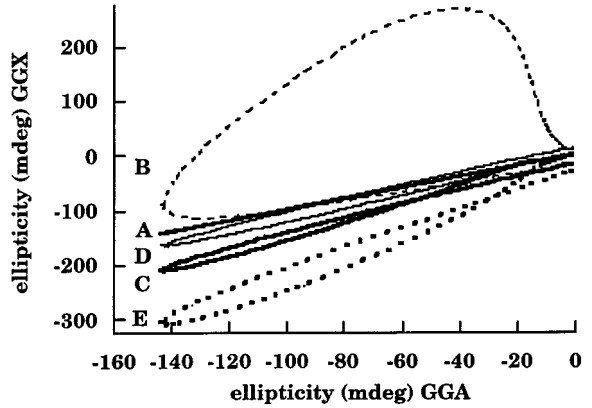

Figure 2—Correlation plots of ellipticity for the Cu(II)GGA complex versus the ellipticities for the analogous complexes with equimolar amounts of (A) GGA, (B) GGH, (C) GGI, (D) GGL, and (E) GGF.

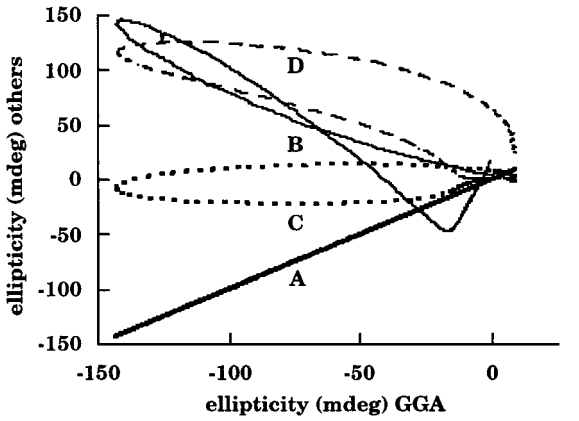

Figure 3—Correlation plots of ellipticity for the Cu(II)GGA complex versus the ellipticities for the analogous complexes with equimolar amounts of (A) GGA, (B) GHG, (C) LGG, and (D) YGG.

maximizes around 550 nm. Aromaticity in the side chain may (YGG) or may not (GGF) induce a spectral change, which with further developments, might be exploited for short range sequencing. There is ambiguity in differentiating among GGA, GGI, GGL, and GGF unless the solution concentrations are carefully controlled.

The L-leucine structural isomers can ostensibly be differentiated in a quality control context. The lack of band intensity for the LGG is a potential problem in quantitation. Although the glycyl residue is achiral, the relative positions that it occupies affect the CD spectra quite

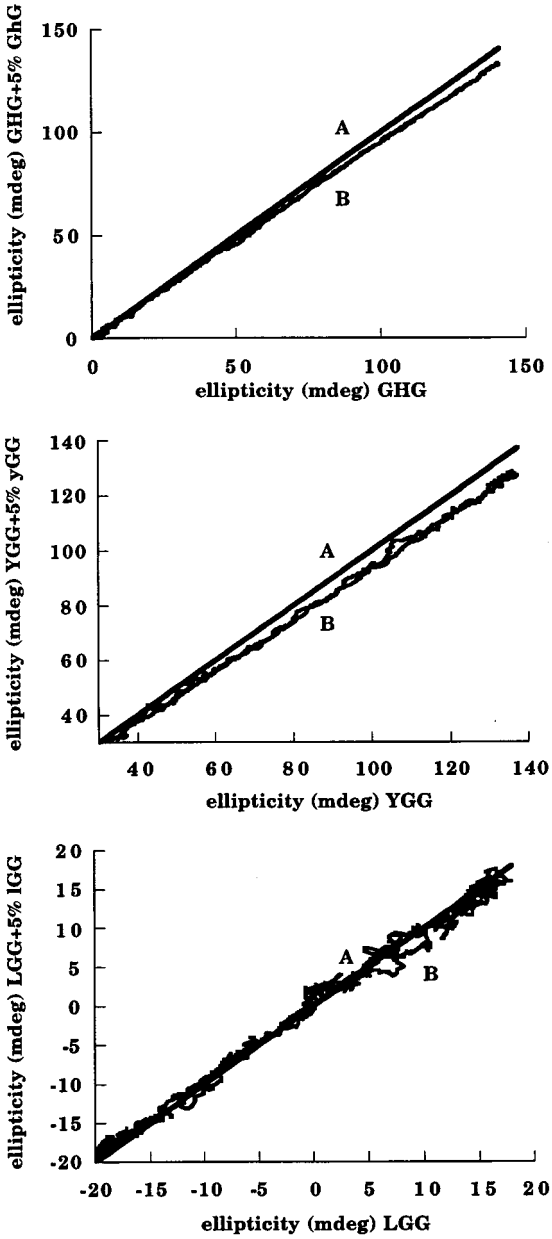

Figure 4—Correlation plots of ellipticities for the Cu complexes of L-GHG, L-YGG, and L-LGG versus ellipticities for 5% racemic mixtures with GhG, yGG, and lGG. In each case line A is for the L-enantiomer against itself and line B is for the L-enantiomer against the mixture.

dramatically, which is good reason to believe that specific interligand interactions occur within the first coordination sphere of the complex.

It is quite clear at this point that total differentiation among all eight analytes is not possible.

Alternative Algorithms for Data Reduction and Enhancing Selectivity—Conventional algorithms typically deal with data measured at just the wavelength of the maximum signal; unless a chemometrics approach is employed.[17] The intent of the algorithms described here was to start with ellipticity data measured at all 1500 wavelengths and, using novel mathematical procedures, reduce the data to a single variable (or factor) upon which selectivity decisions are made. Having a simple numerical means for making selectivity judgments is superior to relying upon subjective graphical superpositions of the CD spectra. Furthermore, if that same numerical factor were to correlate linearly with ligand concentration, then quantitative differentiations might also be accomplished. As a

Table 1—Determination of Enantiomeric Purities for Prepared Binary Mixtures of GHG/GhG, LGG/lGG, and YGG/yGG

% L-form in prepared solution	regression slope (enantiomeric excess)	regression coefficient
	GHG/GhG	
99	0.9919	0.9998
97	0.9685	0.9998
95	0.9503	0.9999
90	0.8973	0.9999
	LGG/lGG	
99	0.9974	0.9951
97	0.9723	0.9957
95	0.9455	0.9946
90	0.8819	0.9932
	YGG/yGG	
99	0.9924	0.9996
97	0.9675	0.9996
95	0.9359	0.9996
90	0.8854	0.9995

final consequence, resultant analytical determinations will be more accurate since experimental uncertainties are significantly reduced when 1500 data points are used rather than only one.

A 2-D Data Reduction Algorithm for Enhancing Selectivity—To illustrate this data reduction procedure, GGA is arbitrarily assigned the status of an enantiomerically pure standard reference material. In a pharmaceutical context, GGA might represent a commercial drug product. The others fill the roles of potential "chemical" and "enantiomeric" impurities.

The simple concept is to plot the 1500 data points for the 8.0 mM GGA spectrum (on the *x*-axis) against analogous data for 8.0 mM solutions for each of the others (on the *y*-axis). To get a baseline reference check for the absolute enantiomeric purity of GGA, its CD spectrum is plotted on both axes. The correlation is a straight line of unit slope and zero intercept. Spectra for the remaining seven tripeptide complexes are plotted against GGA in Figure 2 for the GGX subseries and in Figure 3 for the other sequences.

Plots are decidedly nonlinear and individually distinct from one another. The ellipsoidal shapes for GGI and GGL might appear similar but the best-fit lines do have different slopes. On enlargement, however, the ellipse for GGI is seen to "fold over" on itself in a partial figure 8, implying a latent three-dimensional property in these plots. The same phenomenon can be seen more clearly for the plot of the GGH analogue vs GGA in Figure 3. Differentiation among enantiomerically pure forms of the tripeptides has apparently been achieved at least when the number of possibilities is limited to a small closed set, as they are here. It should be emphasized that in order to reproduce these curves exactly the concentrations must be carefully controlled.

The only other possible correlation line of unit slope (but opposite in sign) and zero intercept is the plot of GGA vs the D-enantiomer, GGa, if their purities are equivalent. This is a consequence of their being chemically identical. The feature that is common to all cases where spectra for chemically dissimilar compounds are correlated is splitting of the correlation line relative to the ideal reference line. Some splittings are extreme, Figures 2 and 3. Conversely, if the correlation plot of the CD spectrum for a newly manufactured lot of GGA vs the reference is linear with a slope less than one, and shows no evidence of splitting, this

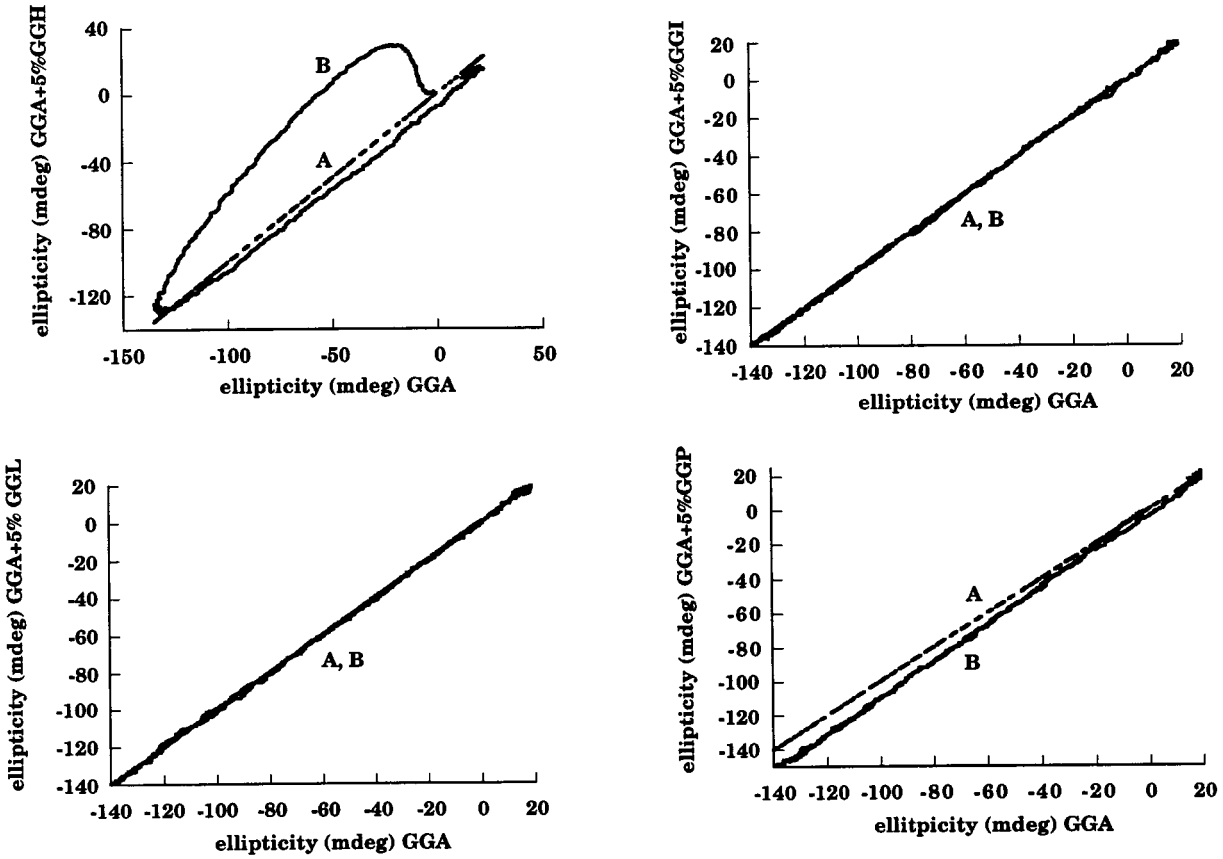

Figure 5—Correlation plots of ellipticity for the Cu(II)–(GGA) complex versus ellipticities for 5% chemical mixtures with GGH, GGI, GGL, and GGF. In each case line A is for GGA against itself and line B is for GGA against the mixture.

is evidence for the presence of the enantiomer. Splitting is instant evidence for the presence of a chemical impurity.

Nonlinear plots, typical of Figures 2 and 3 do not yield easily to quantitation of the "chemical impurities".

(a) Quantitation of Enantiomeric Mixtures—Enantiomeric purity tests were made on three analyte pairs, GHG/GhG, LGG/lGG, and YGG/yGG. As the D-enantiomers were added in increasing amounts, over the range 1, 3, 5, 10% of the L-enantiomer concentration, the slopes of the correlation lines decreased. Data are shown for 5% "impurity" levels only, Figure 4.

Judging by the regression coefficient of 0.9998 for the GHG vs GhG plot, there is no significant loss of linearity compared to the reference baseline, meaning that the EP of GhG is equivalent to that of GHG. The explanation given in the Experimental Section for the low percent purity for GhG, as described in the MPS Certificate of Analysis, is apparently vindicated by the results of this spectroscopic method. Splitting of the YGG/yGG correlation line is consistent with the MPS reported purity level of 97.86% or total impurity of 2.14%. Noise on the LGG/lGG correlation line conceals whether there is splitting of the line or not. A poor S/N ratio is expected since the CD spectral intensity for LGG is the weakest, Figure 1,being approximately one-tenth of the band intensities for the other tripeptides.

Enantiomeric excess, defined for example as:

$$\{[Cu(II)(GHG)] - [Cu(II)(GhG)_x]\}/\{[Cu(II)(GHG)] + [Cu(II)(GhG)_x]\}$$

is given by the correlation slope for each mixture. Calculated values for spiked GHG solutions are in excellent agreement with the measured values for prepared mixtures, Table 1. Imprecisions based on data from three to five repeat measurements are an improvement by almost a factor of 10 over results obtained from the analyses of binary ephedrine mixtures in which a chemometric analysis method was applied to data at five wavelengths.[9] Despite the splitting of the YGG/yGG and the noise in the LGG/lGG plots, by using best-fit correlation lines, the agreements between calculated and measured EP's are still very good. The method is quantitatively valid over the full range of enantiomeric ratios from 100% L to 100% D.

(b) GGA +"Chemical Impurity" Levels for All Other Tripeptides—The question with respect to "chemical impurities" that needs to be addressed is not how great the differences are between the curve for an 8.0 mM solution of GGA and curves for the other tripeptides at equimolar concentrations, Figures 2 and 3, but rather, are the differences sufficient enough to identify and quantitate anonymous chiral "chemical impurities" when these amount to only a few percent of the total composition of a binary mixture? The answer to the question lies in how sensitive the CD detector is in discovering splitting of the correlation line when spectra for "impure" samples are plotted against the spectrum for the primary reference standard.

Spectra were measured for mixtures in which GGA solutions were spiked with small volumes of the other L-tripeptides at levels of 1, 3, 5, and 10%. Data for only the 5% mixtures are plotted in Figure 5 for the GGX subseries and in Figure 6 for GHG, LGG, and YGG. Splittings range from being very small, where they are barely discernible, e.g., for GGI, GGL, and GGF, to extreme, for GGH, GHG, and LGG. Where they are small, the best-fit lines, determined by simple linear regression, are seen to deviate from the unit slope of the reference line. Because of the "absence" of splitting at the lowest concentrations, the plots fail to confirm the presence of GGI, GGL, or GGF at a level of 5% or less, Figure 5. In general the extreme nonlinearity of the split correlations associated with chemical impurities makes it very difficult to determine the amount of impurity.

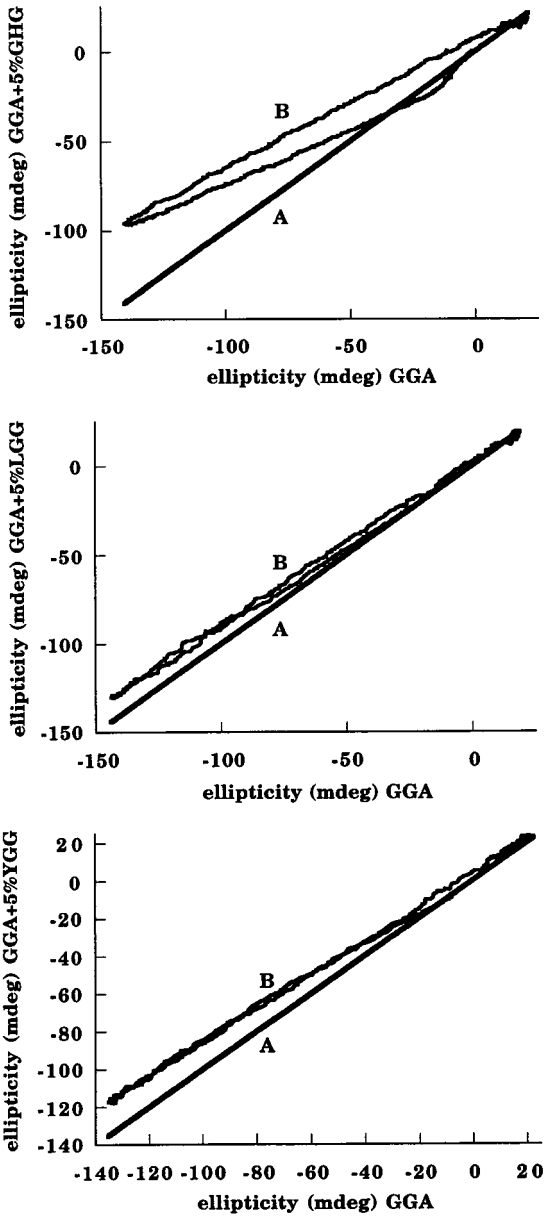

Figure 6—Correlation plots of ellipticity for the Cu(II)–(GGA) complex versus ellipticities for 5% chemical mixtures with GHG, LGG, and YGG. In each case line A is for GGA against itself and line B is for GGA against the mixture.

Briefly recapping the results, the 2-D algorithm has effectively reduced the 1500 spectral data points to one number (the correlation slope), from which EP's can be determined with excellent accuracy over the complete range. Recognition that a potential "chemical impurity" is present is elementary for a limited number of cases, but its analytical determination is not easily done.

A 3-D Data Reduction Algorithm for Enhancing Selectivity—The objectives that relate to this second data reduction algorithm were to discover if the GGA, GGI, GGL, and GGF series can be completely differentiated both qualitatively and quantitatively. The same objectives were achieved when the 3-D algorithm was applied to a series of dipeptides all of which had just one chiral center.[11]

In the 2-D presentations of Figures 2, 3, 5, and 6, wavelength is an implied variable. For the evolution of the 3-D algorithm, wavelength is the third dimension. Since

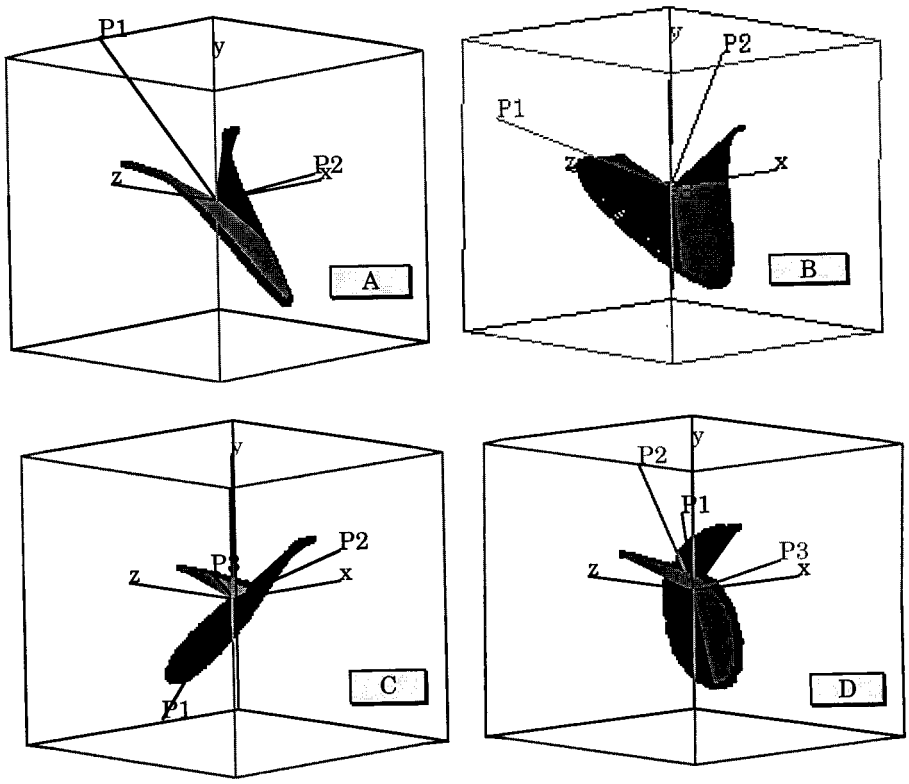

Figure 7—Spinning Plots for the presentation of wavelength (*x*-coordinate), spectral data for the GGA complex (*y*-coordinate), and spectral data for (A) GGA, (B) GGH, (C) GHG, and (D) LGG complexes. The lines P1, P2, and P3 are the principal component axes from the PCA solutions. Dark and light areas distinguish the front four quandrants of the cube from the rear four quadrants.

the experimental parameter measured in CD detection is an absorbance difference, observed signals are positive, negative, and zero. When two CD spectra are plotted against each other, four sign combinations are possible at any wavelength. Repeats of coordinate points, e.g., zero crossover points, can occur at wavelength values that are not adjacent to one another in the spectra. When that occurs, 2-D plots "wrap around" and become three-dimensional. In retrospect what are observed as 2-D plots are simple projections of the 3-D plots on to the $x-y$ coordinate plane, which explains why some of the plots in Figures 2 and 3 appear to have a 3-D character. The added value of the third dimension is that there should be an increase in the overall analytical selectivity.

The algorithm used for the visual presentation of the three-parameter plot was Spinning Plot[18] which is an integral part of a number of commercially available statistical analyses software packages. The software used for these calculations was JMP 3.1 produced by SAS Institute Inc. Four 3-D plots of wavelength (nm) vs ellipticity data for GGA vs ellipticity data for GGA, GGH, GHG, and LGG are shown in Figure 7. By analogy with the 2-D algorithm procedure, GGA plotted against itself is included to provide a baseline for comparison. Front and back quadrants are distinguished by dark and light shading to enhance the 3-D presentation. Discriminations are clearly more evident than they were in Figures 5 and 6.

Factor Analyses of Spinning Plot Data—To derive a quantitative mathematical algorithm, data reduction was done using a Principal Component Analysis (PCA) procedure on the Spinning Plot data.[18] Eigenvalues and eigenvectors for the three principal components, P1, P2, and P3, calculated for the GGA/GGH combination plot are given in Table 2. Spatial projections of these same principal components are superimposed on the coordinate axes of Figure 7.

Table 2—Principal Components Calculated for the GGA versus GGH System

		P1	P2	P3
eigenvalues		+1.9603	+0.9798	+0.0599
eigenvectors	nm	+0.19154	+0.97273	–0.13080
	GGA	–0.68522	**+0.22794**	+0.69175
	GHG	+0.70270	–0.04287	+0.71019

Of the twelve resultant eigenfactors, the one that is most sensitive to variations in the identity of the analyte is P22, highlighted in bold type in Table 2. The 22 tag indicates the entry is in the second row of the second column of the eigenvector matrix. Comparative P22 values for all combinations with GGA are as follows: 0.04519 (vs GGA); 0.88194 (vs GGH); 0.13171 (vs GGI); − 0.01932 (vs GGL); 0.10312 (vs GGF); 0.22794 (vs GHG); 0.89248 (vs LGG); 0.57491 (vs YGG). Standard deviations in P22 determined for data from three to five independent repeat measurements are ±0.002, meaning that total analytical selectivity is accomplished. The 3-D algorithm effectively reduced the 1500 original spectral data points to a single discretionary number, P22. The test sets up well in a quality control environment for proving that a chiral substance is or is not a single chemical.

The remaining question is whether the test has the potential to be quantitative. If P22 values were to correlate linearly with the amount of "chemical impurity", then EP's can be determined by difference. Representative plots of P22 vs percent impurity for solutions of GGA spiked with GGH, GGF, LGG, and YGG are shown in Figure 8. The plots cease to be linear when the impurity concentration approaches 2.0 mM, the concentration of the Cu(II) ion. Differences in the slopes of these lines assist in the identification of the chiral impurity. With the exceptions of GGI and GGL, correlation slopes are greater than two

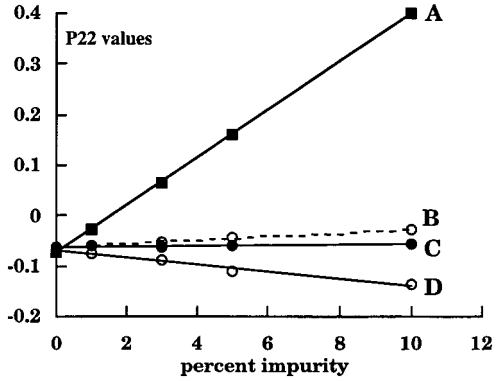

Figure 8—Linear plots of the percent chemical impurity versus the eigenvector P22 for the Cu complex of GGA at a concentration of 8.0 mM spiked with increasing amounts of (A) GGH, (B) GGF, (C) LGG, and (D) YGG.

times the ±0.002 SD in the mean for P22 values, which means that impurity levels as little as 1−3% can be measured with confidence provided the impurity is a single chiral substance. Analytical sensitivities are at least 10 times more accurate than analogous plots in which maximum ellipticity values measured at a single wavelength are plotted against concentration. Why this is so is easily understood when one sees changes in maximum ellipticity values at a single wavelength over the 1−10% impurity range that are less than the best resolution of ± 2.0 mdeg for the CD instrumentation used in this study. The additional accuracy comes from the ability to conveniently include data at 1500 wavelengths.

Where the 2-D method succeeded in providing a means to get accurate values for EP's, the 3-D method provides a way to get a quantitative measure of nonenantiomeric chiral impurities.

Summary and Application of the Method

By the simple chiral modification of the biuret reagent, combined with two novel data reduction algorithms for the handling of visible CD data, a potentially useful QC regulatory procedure for peptides, oligopeptides, and proteins has been developed.

A typical procedure begins with the measurement of the visible CD spectrum for the Cu(II) complex of the chromatographically purest available form of the substance being regulated. Data are archived in a computer file onboard the spectrometer and are updated each time the reference material is measured. Spectra for aliquots taken from each newly manufactured product lot are plotted against the standard and successive on-screen visual comparisons are made.

Deviation from a slope of 1.0 in the 2-D test is an instant indication that the purity is less than that of the reference standard. If the value of the regression coefficient indicates an enantiomeric "impurity", the EP is readily calculated from the regression slope. Splitting of the correlation line for the standard reference material gives instant recognition that a "chemical impurity" is present whose identity may be confirmed by the P22 value calculated from the Spinning Plot algorithm. The percent impurity is calculated from the correlation slope of the P22 vs impurity line.

The two algorithms are complementary in the sense that whereas the 2-D model is capable of measuring EP's with excellent accuracy but only capable of differentiating qualitatively among the eight tripeptides, the 3-D model was capable of quantitatively measuring the compositions of binary mixtures of dissimilar compounds, but incapable of measuring EP's. The latter was not discussed in detail, but is a consequence of the fact the P22 values for an enantiomeric pair are invariant with concentration.

The method is quick, rugged, uses stable inexpensive reagents, requires no specific precautions, and a minimum of technical expertise for a potential operator. Derivatization reactions are instantaneous and data collection is done in a matter of minutes. Spectral data are stored on an onboard computer that is programmed to perform all the mathematical comparisons and quantitative analyses in situ.

All of these advantages point to a very satisfactory and very competitive routine alternative to chromatographic and mass spectrometry methods for the quality control of small peptides. Its successful applications to oligopeptides and proteins[12] are the subjects of subsequent articles.

References and Notes

1. Collins, A. N.; Sheldrake, G. N.; Crosby, J., Eds. *Chirality in Industry, The Commercial Manufacture and Applications of Optically Active Compounds*; Wiley: New York, 1994.
2. DeCamp, W. H. The FDA Perspective on the Development of Stereoisomers. *Chirality* **1989**, *1*, 2−6.
3. Charney, E. *The Molecular Basis of Optical Activity: Optical Rotatory Dispersion and Circular Dichroism*; Wiley: New York, 1960.
4. Zief, M., Crane, L. J., Eds. *Chromatographic Chiral Separations*, *Chromatographic Science Series*; Marcel Dekker: New York, 1988; Vol. 40.
5. Souter, R. W. *Chromatographic Separations of Stereoisomers*; CRC Press: Boca Raton, FL, 1985.
6. Purdie, N. Analytical applications of CD to the forensic, pharmaceutical, clinical, and food sciences. In *Analytical Applications of Circular Dichroism*, *Techniques and Instrumentation in Analytical Chemistry*; Purdie, N., Brittain, H. G., Eds.; Elsevier Scientific: Amsterdam, 1994; Vol. 14, pp 241−278.
7. Gergely, A. The use of circular dichroism as a liquid chromatographic detector. In *Analytical Applications of Circular Dichroism*, *Techniques and Instrumentation in Analytical Chemistry*; Purdie, N., Brittain, H. G., Eds.; Elsevier Scientific: Amsterdam, 1994; Vol. 14, pp 279−292.
8. Horvath, P.; Gergely, A.; Noszal, B. Determination of enantiomeric purity by simultaneous dual circular dichroism and ultraviolet spectroscopy. *Talanta* **1997**, *44*, 1479−85.
9. Engle, A. R.; Purdie, N. Determination of enantiomeric purities using CD/CD detection. *Anal. Chim. Acta* **1994**, *298*, 175−182.
10. Engle, A. R.; Lucas, E. A.; Purdie, N. Determination of enantiomers in ephedrine mixtures by polarimetry. *J. Pharm. Sci.* **1994**, *83*, 1310−14.
11. Purdie, N.; Province, D. W. Algorithms for the quantitative validation of chiral properties of peptides. *Chirality*, in press.
12. Purdie, N.; Province, D. W.; Layloff, T. P.; Nasr, M. M. Algorithms for validating chiral properties of insulins. *Anal. Chem.*, submitted.
13. Tietz, N. W. *Fundamentals of Clinical Chemistry*, 3rd ed.; Saunders: Philadelphia, 1987.
14. Cotton, F. A.; Wilkinson, G. *Advanced Inorganic Chemistry. A Comprehensive Text*, 4th ed.; Wiley-Interscience, New York, 1980.
15. Sigel, H.; Martin, R. B. Coordinating properties of the amide bond. Stability and structure of metal ion complexes of peptides and related ligands. *Chem. Rev.* **1982**, *82*, 384−426.
16. Martell, A. E.; Sillen, L. G. *Stability Constants*; Special Publication No. 17, The Chemical Society: London, 1964.
17. Martens, H.; Naes, T. *Multivariate Calibration*; Wiley: New York, 1989.
18. Kaiser, H. F. The varimax criterion for analytic rotation in factor analysis. *Psychometrika* **1985**, *23*, 187−200.

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