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LETTERS

Determination of Enantiomeric Composition by Fluorescence Anisotropy

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A new method is developed whereby the enantiomeric composition of a chiral analyte is determined by steady-state fluorescence anisotropy. A theoretical model is presented showing that the measured anisotropy of an enantiomeric mixture in the presence of a chiral selector is dependent on the selectivity, the concentration of free selector, and the enantiomeric composition. Furthermore, for a given system the relationship between the measured anisotropy and the enantiomeric composition is predicted to be linear. The prediction of a linear relationship was confirmed experimentally by examining mixed enantiomeric compositions of 1,1′-binaphthyl-2,2′-diylhydrogen phosphate in the presence of β- and α-cyclodextrin. The enantiomeric compositions of four solutions of mixed enantiomers were determined based on a 2-point calibration with an average absolute error of less than 2%.

Introduction

The rapid development of combinatorial synthesis and the pervasiveness of chiral drug development demand simple, fast, reliable and sensitive methods for the determination of enantiomeric composition.[1] Significant effort has been made to address this challenge, including the improvement of conventional methods and the development of new techniques for *ee* determination.[2] Fluorescence spectroscopy can provide high sensitivity, and significant progress has been made by various approaches that have been used to examine enantiomeric composition.[3] Despite this progress, the importance of enantioselective sensing necessitates the development of new methods to determine enantiomeric composition. We recently reported the use of fluorescence anisotropy to examine chiral recognition based on the observed correlation between the anisotropy and chiral selectivity and are developing a methodology to examine the thermodynamic parameters of enantioselective binding.[4] These recent investigations have prompted us to evaluate the possibility of using fluorescence anisotropy to quantitatively determine enantiomeric composition. Herein we describe the use of fluorescence anisotropy for the determination of enantiomeric composition, which is demonstrated by theoretically modeling and confirmed experimentally.

Theoretical Approach

The dependence of the fluorescence anisotropy on the chiral recognition is based on the difference in rotational diffusion rates of the bound and free forms of the chiral molecule in the presence of a chiral selector. In a solution of mixed enantiomeric composition, the anisotropy will vary between two extreme values, based on the enantiomeric composition and the chiral selectivity expressed within the system. Thus, for a given chiral compound in the presence of a chiral selector, the measured anisotropy will be a function of the enantiomeric composition. To elucidate and best exploit the relationship between fluorescence anisotropy and enantiomeric excess, a mathematical model was evaluated based on the additive nature of anisotropy[5] and the associated host−guest equilibria of complexation.

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For a solution containing a single enantiomer, A_R, and a selector, S, the association reaction is given by eq 1,

$$A_R + S = A_RS \tag{1}$$

where A_R represents the *R*-enantiomer of the analyte (guest) and S represents the chiral selector (host). Under equilibrium conditions the observed anisotropy (steady-state) is the weighted average of the anisotropy of the bound ($r_{b,R}$) and free analyte ($r_{f,R}$) (eq 2).

$$r_R = f_{b,R}r_{b,R} + (1 - f_{b,R})r_{f,R} \tag{2}$$

The term $f_{b,R}$ represents the molar fractions of bound and free species, which can be expressed in terms of the concentration (eq 3).

$$f_{b,R} = \frac{[A_RS]}{[A_R] + [A_RS]} \tag{3}$$

Thus, the observed anisotropy can be represented by eq 4.

$$r_R = \frac{[A_RS]r_{b,R}}{[A_R] + [A_RS]} + \frac{[A_R]r_{f,R}}{[A_R] + [A_RS]} \tag{4}$$

In most cases, the anisotropy of the free fluorophore approaches zero such that its contribution to the total anisotropy is negligible and the overall anisotropy is approximated by that of the bound species (eq 5).

$$r_R = \frac{[A_RS]r_{b,R}}{[A_R] + [A_RS]} \tag{5}$$

Considering the expression of the association constant of the complexation reaction,

$$K_R = \frac{[A_RS]}{[A_R][S]} \tag{6}$$

Equation 5 can be arranged to represent the dependence of the measured anisotropy on the association constant and the anisotropy of the bound species (eq 7). The analogous relationship for the *S*-enantiomer is given in eq 8.

$$r_R = \frac{K_R[S]r_{b,R}}{1 + K_R[S]} \tag{7}$$

$$r_S = \frac{K_S[S]r_{b,S}}{1 + K_S[S]} \tag{8}$$

It is thereby apparent that the magnitude of the measured anisotropy is dependent on the selector concentration, [S], the association constant, *K*, and the anisotropy of bound spices. For a given system the association constant and the anisotropy of the bound species are constant. Furthermore, if the selector concentration is high relative to the analyte concentrations the concentration of free selector is essentially a constant.

In the case of a solution containing a mixture of the two enantiomers of a chiral analyte (A_R and A_S) in the presence of chiral selector (S), the average anisotropy (r_{av}) is the sum of the anisotropy of the bound species (A_RS and A_SS) weighted by the molar fractions of each species (eq 9), where ϕ_R represents the fraction of *R*-enantiomer in the mixture.

$$r_{ave} = \phi_R r_R + (1 - \phi_R)r_S \tag{9}$$

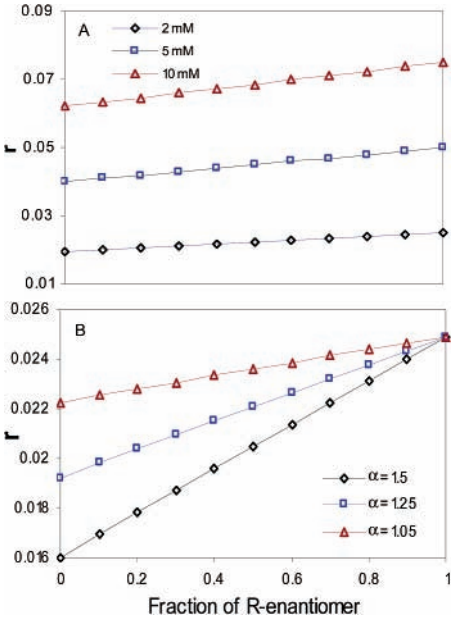

Figure 1. Calculated anisotropy as a function of enantiomeric composition at (A) various selector concentrations ($K_R = 100\ M^{-1}$, $K_S = 80\ M^{-1}$, $r_{b,R} = 0.15$, $r_{b,S} = 0.14$, $[A_{total}] = 50\ \mu M$ ($\alpha = 1.25$) and (B) various selectivities ($K_R = 100\ M^{-1}$, $r_{b,R} = 0.15$, $r_{b,S} = 0.14$, [S] = 2 mM, $[A_{total}] = 50\ \mu M$).

The anisotropy of a solution of mixed enantiomers can therefore be given by eq 10, which is obtained by combining eqs 7, 8, and 9.

$$r_{ave} = \frac{\phi_R K_R[S]r_{b,R}}{(1 + K_R[S])} + \frac{(1 - \phi_R)K_S[S]r_{b,S}}{(1 + K_S[S])} \tag{10}$$

Considering that K_R, K_S, $r_{b,R}$, and $r_{b,S}$ are constant, as is [S] if the concentration of the selector is sufficiently high compared to the analyte concentration (typically millimolar vs micromolar), a linear relationship is expected between the average anisotropy and the molar fraction of enantiomers. To illustrate this more clearly, let

$$\gamma_R = \frac{K_R[S]r_{b,R}}{1 + K_R[S]} \tag{11}$$

and

$$\gamma_S = \frac{K_S[S]r_{b,S}}{1 + K_S[S]} \tag{12}$$

Upon combination with eq 11 and eq 12 and rearrangement, eq 13 is obtained, which clearly shows the linear form of the equation.

$$r_{ave} = \phi_R(\gamma_R - \gamma_S) + \gamma_S \tag{13}$$

Thus, a plot of the measured anisotropy as a function of enantiomeric composition should yield a line whose intercept is equal to γ_S and slope is equal to the difference between γ_R and γ_S.

The predicted linear relationship was examined by modeling the major parameters of eq 10. Figure 1 shows the anisotropy as a function of enantiomeric composition and the effect of varying the chiral selectivity and the concentration of free selector, [S]. The resulting plots were linear and changes in the selectivity and selector concentration affected the plots as

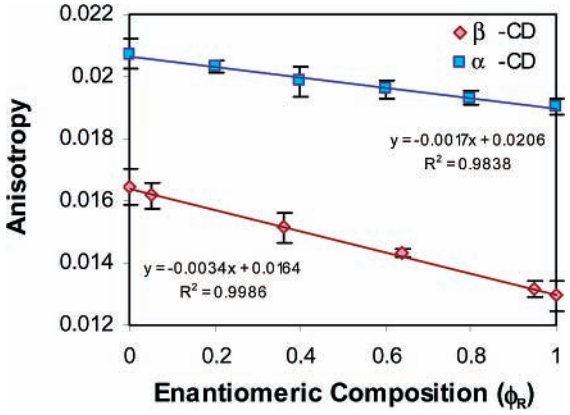

Figure 2. Anisotropy of BNP (31 μM) as a function of enantiomeric composition in the presence of β-cyclodextrin and α-cyclodextrin.

expected. Inspection of eqs 10−13 reveals that large differences in association constants (chiral selectivity, $\alpha = K_R/K_S$) will result in a larger slope and better precision in determining enantiomeric composition. It is also clear that the concentration of free selector significantly affects the intercept; therefore successful development of analytical assays will depend on operating in a concentration regime such that the free selector concentration remains constant. Importantly, the average anisotropy has a minimal dependence on the total analyte concentration. This insensitivity to analyte concentration allows method development for determination of enantiomeric composition without the need to precisely control the analyte concentration.

Experimental Section

Materials. β-Cyclodextrin was a gift from Cerestar USA, Inc. (Hammond, IN), and α-cyclodextrin was purchased from Acros Organics (Fair Lawn, NJ). Pure enantiomers of 1,1′-binaphthyl-2,2′-diylhydrogen phosphate (BNP) were purchased from Aldrich (Milwaukee, WI) and were used as received. Sodium phosphate was obtained from Fisher Scientific (Fair Lawn, NJ). Water used in all experiments was purified to at least 18 MΩ resistivity by a Millipore Milli-Q system (Milford, MA). Sample solutions were prepared using phosphate buffer (0.25 mM NaH_2PO_4, 0.25 mM Na_2HPO_4, pH = 6.9).

Fluorescence Measurements. A modular spectrofluorometer (Photon Technology International Inc., London, Ontario) equipped with double excitation and emission monochromators, a single-photon counting PMT detector, and large aperture Glann−Thompson polarizers were used for all fluorescence anisotropy measurements. All anisotropy measurements were corrected for instrumental polarization bias by applying a *G*-factor correction. A Xe lamp was used as an excitation source, and temperature controlled 1 cm quartz cuvettes were used for all fluorescence measurements. Error bars on experimental measurements represent standard deviations of replicate measurements derived from co-averaged data during each measurement.

Results and Discussion

To evaluate and confirm the theoretically predicted response, the fluorescence anisotropy of 1,1′-binaphthyl-2,2′-diylhydrogen phosphate (BNP) was measured at various enantiomeric compositions in the presence of β-cyclodextrin (CD) as a chiral selector. Figure 2 shows the linear response that was observed ($R^2 = 0.999$). The same analyte was also examined in the case of α-CD, which allowed evaluation of the effect of selectivity and association constant (Figure 3). The slope of the plot with β-CD is larger than that with α-CD, while the intercept with

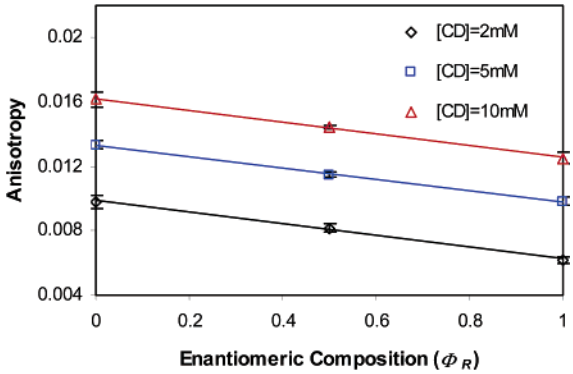

Figure 3. Measured anisotropy of BNP at various β-cyclodextrin concentrations (31 μM BNP).

TABLE 1: Determination of Enantiomeric Composition for BNP (% S)

% (actual)	% (measured)	error
95	92.3	−2.7
64	62.9	−1.1
36	33.7	−2.3
5	5.71	0.71

α-CD is higher than that with β-CD. This result is in agreement with those from modeling, which predict an increase in slope with increasing selectivity. To further test the results predicted in Figure 1a, a series of solutions of various enantiomeric composition and CD concentration were examined (Figure 3). The change in CD concentration significantly affects the intercept with little influence on the slope

Table 1 shows the determination of the enantiomeric composition of four BNP solutions following a two-point calibration (pure *R* and *S* enantiomers). The determined enantiomeric compositions are in good agreement with the actual value, with an average absolute error of 1.7%. In contrast to methods based on optical rotation, the measurement error of the method is consistent across the range of enantiomeric compositions. This is relevant to applications involving screening applications (e.g., chiral catalysts), as most targets may only exhibit moderate enantiomeric excess prior to optimization.

Conclusion

Theoretical modeling and experimental results showed a linear relationship between the average anisotropy and enantiomeric composition. Under typical conditions, the average anisotropy depends primarily on the difference in association constants (chiral selectivity), the difference in anisotropy of the bound enantiomers, and the enantiomeric composition. Fluorescence anisotropy is inherently insensitive to analyte concentration, and the enantioselective response has no requirement of spectral perturbation, making the method quite versatile. Furthermore, the insensitivity of the anisotropy to analyte concentration will enable rapid determination of enantiomeric excess without significant regard to the analyte concentration. While the precision of the method is not expected to be as good as circular dichroism at high enantiomeric purity, it is competitive in cases where the enantiomeric excess is low, such as in the case of screening potential catalysts. Furthermore, the advantages of higher sensitivity, relative simplicity, and comparatively low cost make this fluorescence-based method a very complementary tool, especially since enantiomeric composition can be determined on extremely small quantities of sample. For example, the analysis shown in Table 1 was performed on ∼3 pg of material. In addition, because the method does not require

chromatographic separation, it is well suited for high throughput screening applications.

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