A fluorescent diastereoselective molecular sensor for 1,2-aminoalcohols based on the rhodamine B lactone–zwitterion equilibrium

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A simple yet highly sensitive diastereoselective fluorescent assay was developed, which can differentiate diastereomeric 1,2-aminoalcohols such as ephedrine, pseudoephedrine, and methylephedrine using commercially available xanthene dyes. The assay is based on the rhodamine B lactone–zwitterion equilibrium, which is highly sensitive to the presence and structure of 1,2-aminoalcohols. This is in contrast to the majority of rhodamine sensors, which are based on the pH sensitive carboxylate–lactone equilibrium. A model is proposed in which the diastereoselectivity arises from the differences in the strength of an intermolecular lone pair-π interaction within the analyte–rhodamine zwitterion complex. An assay was developed based on this sensing mechanism in which the structure and concentration of a 1,2-aminoalcohol solution can be determined using a mixture of rhodamine B and fluorescein. The selectivity and accuracy of this two-dye system was then demonstrated by the ability to measure the diastereomeric excess of samples of methylephedrine with 98% accuracy.

Introduction

Rhodamine B (RB) is a fluorescent dye that is widely used as a pH indicator[1] and as a fluorophore in many molecular probes.[2] Most rhodamine sensors are based on the pH sensitive equilibrium between the fluorescent carboxylic acid cation (RB) and the non-fluorescent spirolactone (RB-L) (Scheme 1).[3] In this study, we demonstrate that the equilibrium between the neutral rhodamine lactone (RB-L) and zwitterion (RB-Z) forms is also extremely sensitive to the local microenvironment and can be used as a fluorescent probe to identify and measure the diastereomeric purity of 1,2-aminoalcohols. One reason that the lactone–zwitterion equilibrium is not often used is because the equilibrium is strongly shifted toward the non-fluorescent lactone form.[4] However, the few sensing systems based on the lactone–zwitterion equilibrium have shown that it is highly responsive to changes in the probe environment.[5] For example, the lactone–zwitterion equilibrium has been used to measure solvent hydrogen bonding strengths, solvent polarity, metal cations, temperature, and pressure (Scheme 1).[3,4,6] In this work, we demonstrate that the rhodamine lactone–zwitterion equilibrium can be used to discriminate structurally similar 1,2-aminoalcohols and measure their diastereomeric purity with 98% accuracy.

The development of simple, low cost, and accurate sensors for chiral 1,2-aminoalcohols is of great interest, as it is a structural motif that is found in many common drugs, chiral auxiliaries, and asymmetric catalysts.[7] In these applications, the stereochemistry of the 1,2-aminoalcohols is very important in determining their pharmaceutical activity and their synthetic utility. For example, the 1,2-aminoalcohol, ephedrine, is classified as a vasopressor/decongestant; while its diastereomer, pseudoephedrine, is classified as a nasal decongestant.[8] Therefore, the ability to sense and differentiate chiral amino alcohols is important for dosage monitoring, pharmaceutical testing, illicit drug detection, and asymmetric synthesis.

Currently, the methods used to distinguish the enantiomers and diastereomers of amino alcohols are chromatographic analyses such as HPLC and GC.[9] These methods are effective, but are time consuming and require expensive equipment and large volumes of solvent. Another approach employs molecular probes that are selective for the target analyte.[10] Colorimetric and

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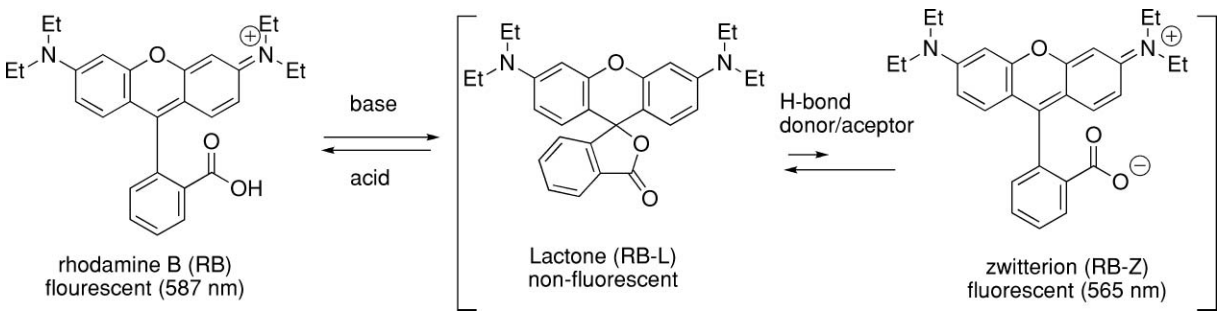


Scheme 1 Equilibria between cationic (RB) and neutral forms (lactone and zwitterion) of rhodamine B.

fluorometric molecular sensors have been developed that target 1,2-aminoalcohols.[11] The RB-L/RB-Z molecular sensor presented, herein, is an example of the second approach. A unique aspect of this system is that the fluorescent signal from RB-Z provides information about the structure and stereochemistry of 1,2-aminoalcohols. To increase the accuracy of the assay, a second responsive fluorophore, fluorescein, was added to measure the concentration of the 1,2-aminoalcohol analytes. This two-dye sensing system has a number of advantages. The first is that rhodamine B and fluorescein are commercially available. Thus, this sensing system is readily accessible to every researcher. Secondly, due to the different emission wavelengths of RB-Z and fluorescein, both dyes can be used simultaneously in a single cuvette to rapidly measure the structural identity, purity, and concentration of 1,2-aminoalcohol samples.

Results and discussion

The development of a fluorescent RB-Z based sensing system was divided up into two separate sections. First, the sensitivity of the rhodamine B lactone-zwitterion equilibrium to the stereochemistry of 1,2-aminoalcohol analytes (Chart 1) was established and characterized. Second, the rhodamine sensing system was combined with fluorescein, and this two-dye sensing system was applied to identify 1,2-aminoalcohols of varying concentrations and to measure the diastereomeric purity of methylephedrine.

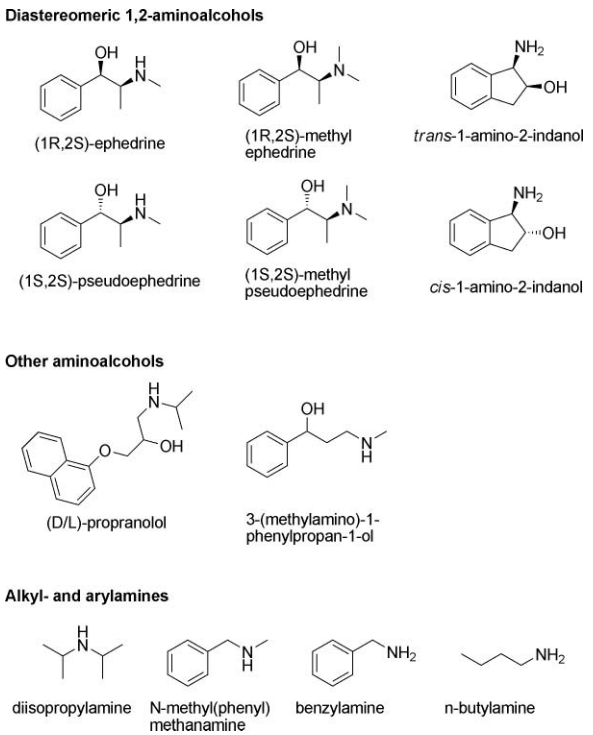


Chart 1 Analytes studied with the two-dye, rhodamine B and fluorescein molecular sensors.

Analysis of rhodamine as a diastereomeric sensor

Our first goal was to characterize the sensitivity of the RB-Z concentration to structural differences in alkylamine analytes. The fluorescence of the cationic ring opened form of rhodamine, RB, is very intense with an emission maximum at 587 nm in acetonitrile (λ_{ex} = 470 nm), as shown by the off-scale spectra in Fig. 1 (dark blue line). The addition of a basic guest such as pseudoephedrine leads to a dramatic decrease in fluorescent intensity as the basic guest deprotonates RB to form the non-fluorescent RB-L. Despite the dramatic changes in emission intensity, the RB–RB-L equilibrium was not able to distinguish structurally similar alkyl amine guests. Instead, the differences in the intensity of the RB 587 nm band were correlated to the differences in basicities of the amine guests, and no differences in RB emission intensities were observed with diastereomeric 1,2-aminoalcohols.

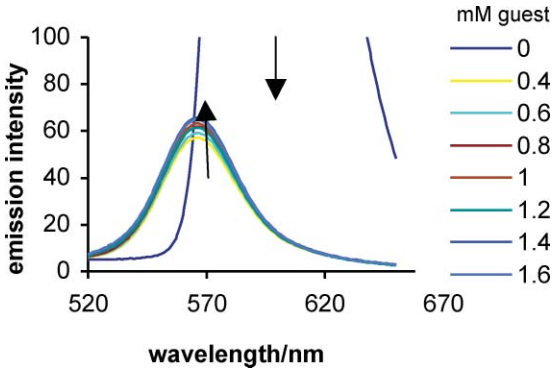


Fig. 1 Emission spectra of 120 μM rhodamine B solutions in acetonitrile (λ_{ex} = 470 nm), containing increasing concentrations (0.0–1.6 mM) of pseudoephedrine.

Careful analysis of the fluorescent spectra of the RB-pseudoephedrine samples revealed the appearance of a second weak band at 565 nm (Fig. 1), which was very sensitive to the structure of the alkylamine guests. For example, two acetonitrile solutions of RB (120 μM) containing diastereomeric ephedrine or pseudoephedrine could be differentiated by the differences in emission intensities of the 565 nm band (Fig. 2). Other diastereomeric 1,2-alcohols such as methylephedrine/methylpseudoephedrine and *cis*-1-amino-2-indanol/*trans*-1-amino-2-indanol showed similar differences in intensity for the 565 nm band. Therefore, the selectivity of the 565 nm band was characterized and studied in more detail.

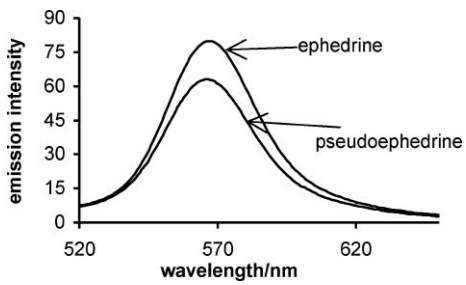


Fig. 2 Emission spectra (λ_{ex} = 470 nm) of 120 μM rhodamine B acetonitrile solutions containing 1.0 mM ephedrine and pseudoephedrine.

The 565 nm band was identified as the zwitterionic RB species by comparison with literature values.[5] This identification helped explain the weak intensity of the 565 nm band. The addition of an excess of a basic analyte to RB yields predominantly the non-fluorescent lactone (RB-L). Only a small concentration

of the zwitterion (RB-Z) is present under these conditions, as the neutral lactone is more stable in organic solvents such as acetonitrile. For example, we attempted to measure the changes in the RB-Z concentration using absorbance. However, the RB-Z concentrations were too low to be accurately observed by UV/vis. The RB-Z concentration, however, could be monitored by fluorescence and was found to be very sensitive to the structure of the amine analyte used to neutralize the RB. Although RB-Z is a minor species in the presence of an excess organic base, it can be easily and accurately measured by fluorescence because the major species formed under these conditions is the non-fluorescent RB-L.

Interestingly, the intensity of the 565 nm band also appeared to be highly specific for 1,2-aminoalcohols. Fig. 3 compares the emission intensities at 565 nm of 120 μM RB acetonitrile solutions in the presence of varying concentrations of 1,2-aminoalcohols (solid lines) and representative alkyl and arylamines (broken lines). The 1,2-aminoalcohols appear to selectively stabilize RB-Z as seen by the much higher emission intensities of the ephedrine and pseudoephedrine measurements. Diols such as ethylene glycol had no effect on the fluorescence of RB, confirming the necessity of both the basic amine and alcohol groups.

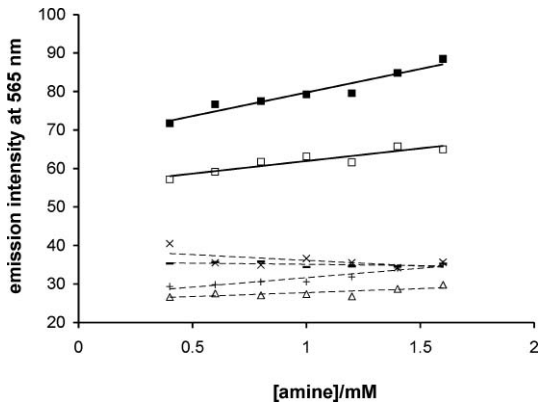


Fig. 3 Emission intensities of rhodamine B (120 μM) in acetonitrile at 565 nm ($\lambda_{ex} = 470$ nm) in the presence of varying concentrations of alkylamine analytes: ephedrine (filled square), pseudoephedrine (open square), diisopropylamine (cross), *N*-methyl-benzylamine (dash), isopropyl amine (triangle), benzylamine (x).

A second interesting characteristic of the 565 nm band was its relative insensitivity to the 1,2-aminoalcohol concentrations. For example, the intensity of the RB-Z band (565 nm) increases only slightly over a four-fold concentration range for both ephedrine and pseudoephedrine (Fig. 3). This insensitivity to analyte concentration makes it much easier to use as a molecular sensor. For example in Fig. 3, even the response of the lowest concentration ephedrine sample is still higher than the highest concentration pseudoephedrine sample. This behavior is in contrast to the majority of fluorescent sensors in which the fluorescence intensity is sensitive to both the structure and the concentration of the analyte. In these systems, a high concentration of a structural analog can often be misidentified as the target analyte because it produces the same response as a low concentration of the target analyte.

To explain the concentration independent behavior of the assay, we hypothesized that the ammonium salt of the 1,2-aminoalcohol that is formed *in situ* is the key species that stabilizes and increases the concentration of RB-Z. We focused on the ammonium salt because its concentration remained constant under the testing conditions. In the presence of an excess of basic 1,2-aminoalcohol, the concentration of the corresponding ammonium salt is limited by the concentration of the proton source, which is RB. Once all the RB has been deprotonated, the ammonium concentration remains constant even with a large excess of 1,2-aminoalcohol. The ammonium salt also has the potential to form stronger complexes with RB-Z, due to its ionic character and stronger hydrogen bond donating ability, than the neutral 1,2-aminoalcohols.

To test whether the ammonium salt was the key species responsible for the stabilization of RB-Z, we carried out experiments under conditions that the ammonium salts could not form. Titration experiments were conducted using RB-L, which is also commonly known as rhodamine B base, in place of RB. In the presence of RB-L, the ammonium salts of the 1,2-aminoalcohols cannot form because RB-L is not acidic. Acetonitrile solutions of RB-L (120 μM) were titrated with aminoalcohols, ephedrine and pseudoephedrine (Fig. 4), and the resulting spectral changes were compared to the previous studies (Fig. 3). In the RB-L solutions, much lower RB-Z concentrations were observed. The emission intensities of the 565 nm band were > 5 times lower, which was consistent with our hypothesis that the ammonium salts were selectively stabilizing RB-Z. Further evidence for the importance of the ammonium salts was provided by the inability of the RB-L solutions to differentiate ephedrine and pseudoephedrine. Finally, the insensitivity of the 565 nm band in the RB-L titration experiments to differences in aminoalcohol concentration was consistent with our hypothesis that the unprotonated aminoalcohols are not able to form effective complexes with RB-Z.

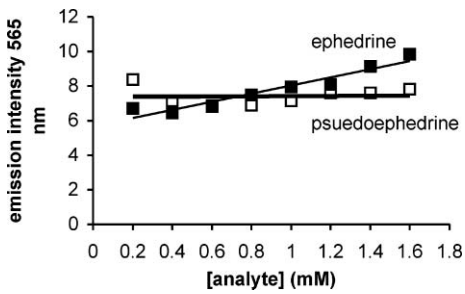
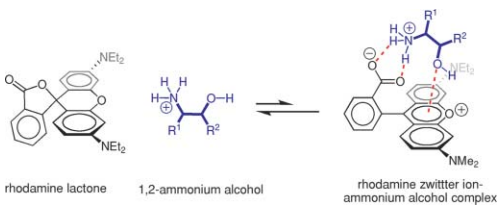


Fig. 4 Emissions intensities of RB-L (120 μM) in acetonitrile ($\lambda_{ex} =$ 470 nm) measured in the presence of increasing concentrations of ephedrine and pseudoephedrine.

Based on the above observations, we hypothesize that the ammonium salts of the 1,2-aminoalcohols complex with RB-Z, leading to an increase in [RB-Z]. The primary interactions in the complex are hydrogen bonding and electrostatic interactions between the cationic ammonium groups and the anionic carboxylates of the RB-Z, as shown in Scheme 2. However, the results in Fig. 3 show that analytes with alcohol groups further stabilize the ammonium–RB-Z complexes. One explanation is the formation of an additional supramolecular interaction between the lone pair of the OH group and the cationic π-system of the xanthene dye. Examples of similar lone pair-π interactions have been recently observed in solid-state[12] and in solution.[13] Furthermore, the lone pair-π interactions are only formed with strongly electron poor and cationic aromatic surfaces,[14] similar to



Scheme 2 Proposed complex between 1,2-ammonium alcohol and RB-Z involving electrostatic, hydrogen bonding, and lone pair-π interactions.

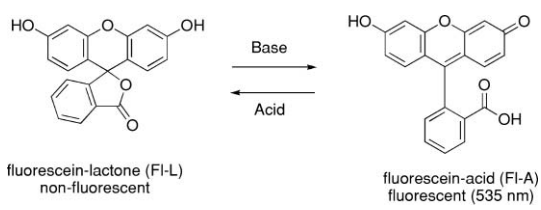
those of the cationic xanthene surface in the ring opened RB-Z structure. The lone pair-π interaction also provides an explanation for the diastereoselectivity of the 565 nm band. Diastereomeric 1,2-alcohols could form different strength lone pair-π interactions due to steric and conformational differences, leading to the observed differences in the RB-Z concentrations.

These studies confirm that the RB-Z concentration is sensitive to the presence and structure of the ammonium salts of 1,2-aminoalcohols and is easy to monitor. In addition, the RB-Z concentration is relatively insensitive to differences in 1,2-aminoalcohol concentrations. This facilitated the differentiation of structurally similar and diastereomeric alcohols by monitoring the emission intensity of the RB-Z at 565 nm.

Development of qualitative and quantitative 1,2-aminoalcohol assays

Next, two separate assays were developed utilizing the ability of RB-Z to differentiate 1,2-aminoalcohols. First, an assay was developed that could classify structurally similar 1,2-aminoalcohols. Second, an assay was developed that could quantitatively measure the diastereomeric excess (de) of binary mixtures of 1,2-aminoalcohols.

The first challenge was to augment the ability of RB-Z to differentiate aminoalcohols. Although the initial studies showed that the RB-Z concentration was more sensitive to differences in structure than to differences in concentration, there was still some variation in the emission intensities at different analyte concentrations that could lead to misidentifications. To mitigate these effects, a second molecular probe, fluorescein, was introduced to provide complementary information about the 1,2-aminoalcohol concentrations. Fluorescein, like rhodamine, is commercially available and can be used to sense changes in a local environment by monitoring its ring opening and closing equilibrium (Scheme 3).[15] Fluorescein exists in a fluorescent ring opened carboxylic acid (Fl-A) form and in a non-fluorescent ring closed lactone (Fl-L) form. This equilibrium is very sensitive to the pH of the solution. Acids shift the equilibrium toward the ring closed Fl-L form; whereas, bases shift the equilibrium toward the



Scheme 3 Equilibrium between the non-fluorescent lactone (Fl-L) and fluorescent carboxylic acid (Fl-A) forms of fluorescein.

ring opened Fl-A form.[16] Fluorescein was particularly attractive because it turns on in the presence of base and because its emission maxima at 535 nm is sufficiently shifted from RB-Z at 565 nm that both dyes can be monitored simultaneously.

The ability of fluorescein to measure the concentrations of 1,2-aminoalcohols was tested with pseudoephedrine. The addition of pseudoephedrine to a 70 μM acetonitrile solution of fluorescein yielded a steadily increasing emission at 535 nm (Fig. 5). This was consistent with the expected sensing mechanism in which the basic aminoalcohol deprotonates the non-fluorescent Fl-L phenol, yielding the ring opened fluorescent Fl-A. A linear fluorescent response curve at 535 nm was measured over the concentration range of 0.1 to 1.6 mM (Fig. 5, inset, open squares). A similar linear response was observed for ephedrine with a slightly larger slope (Fig. 5, inset, filled squares). This shows that the fluorescein response at 535 nm is dominated by the concentration of the basic aminoalcohol and not its structure. Thus, the fluorescein 535 nm band provides information about the 1,2-aminoalcohol analyte concentration that is complementary to the structural information provided by the RB-Z 565 nm band.

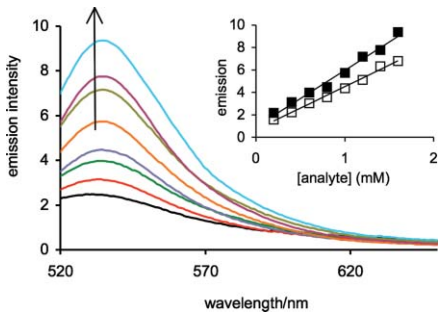


Fig. 5 Emission spectra for increasing concentrations (0.0–1.6 mM) of pseudoephedrine in 70 μM fluorescein acetonitrile solutions (λ_{ex} = 470 nm). (Inset) titration data plotted showing emission of fluorescein at 535 nm with increasing concentrations of pseudoephedrine (open squares) and ephedrine (filled squares).

Carrying out two separate analyses using fluorescein and rhodamine was time consuming and increased the error of the analysis. Therefore, we explored the possibility of simultaneously monitoring the signal from Fl-A and RB-Z in a single solution. To test the viability of this strategy, an acetonitrile solution containing 70 μM fluorescein and 120 μM rhodamine B was titrated with pseudoephedrine (Fig. 6). The results were analogous to the separate experiments carried out with the individual dyes (Fig. 1 and Fig. 5). A rapid disappearance in the fluorescence

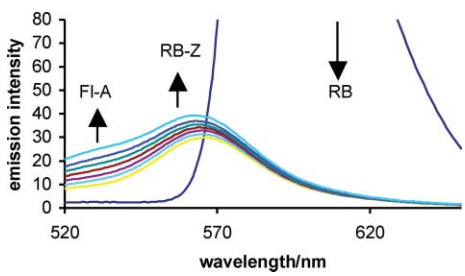


Fig. 6 Emission spectra for increasing concentrations (0.4–1.6 mM) of pseudoephedrine in an acetonitrile solution of 120 μM rhodamine B and 70 μM fluorescein (λ_{ex} = 470 nm).

from RB (587 nm) was observed between 0.0 and 0.4 mM of pseudoephedrine. From there, increases in the RB-Z 565 nm and Fl-A 535 nm bands were observed.

To test the discriminating ability of the two-dye mixture, fluorescence spectra were measured in the presence of six structurally similar aminoalcohols at eight different concentrations from 0.2 to 1.6 mM. The six analytes were ephedrine, pseudoephedrine, methylephedrine, methylpseudoephedrine, D,L-propranolol and 3-(methylamino)-1-phenylpropan-1-ol (Chart 1). The first four analytes are diastereomeric pairs of 1,2-aminoalcohols. The last two analytes were selected to demonstrate that other types of aminoalcohols could also be differentiated. Linear discriminant analysis (LDA), as implemented in Systat, was applied to the emission spectra from 495–595 nm measured in 10 nm increments. Even though eight different concentrations (0.2 mM to 1.6 mM) were tested for each analyte, the analysis was able to differentiate the six different aminoalcohols with 94% accuracy, using the leave-one-out analysis. To test whether the discriminating abilities of the two-dye mixture were due to the orthogonal concentration and structural information provided by fluorescein and rhodamine, the LDA analysis was reapplied using the fluorescent maxima for the open forms of the two dyes under the testing conditions (535 nm and 565 nm). The accuracy of the analysis dropped only slightly to 92%, demonstrating that the majority of the discriminating ability arises from the orthogonal information provided by the emission intensities of the two dyes. This can be seen by plotting the emission intensities at 535 nm and 565 nm for each sample (Fig. 7). The complementarity of the two wavelengths even allows differentiation of analytes of different concentrations. For example, the diastereomeric analytes ephedrine (filled squares) and pseudoephedrine (open squares) fall along parallel lines in the plot and are easily distinguished. The ephedrine samples all have consistently higher 565 nm (*y*-axis) values than the pseudoephedrine samples. The sensitivity of the 535 nm band to concentration can be seen by the responses being spread out along the 535 nm (*x*-axis), with the concentrations increasing left to right. The sensitivity of the 565 nm band can be seen by the responses for each analyte being spread out along the *y*-axis. There is some overlap in the information provided by the two bands. This can be seen by the gradual increase in the 565 nm values for each analyte with increasing concentration. This is due to the influence from the shoulder of the concentration dependent 535 nm band.

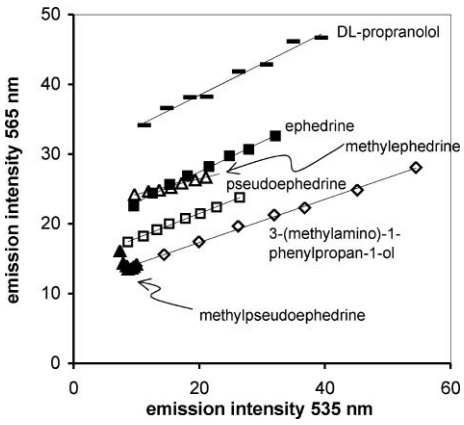


Fig. 7 Emissions titrations of a mixture of rhodamine B (120 μM) and fluorescein (70 μM) with amino alcohols 0.2–1.6 mM in acetonitrile (λ_{ex} = 470 nm). The first point shown is a concentration of 0.2 mM. Each point going left to right represents a 0.2 mM increase. Amine analytes were also tested but are not shown in this plot.

Due to the orthogonal information provided by the two dyes, most of the analyte samples can be easily differentiated and identified. Using the plot in Fig. 7 as a calibration matrix, an unknown sample can be easily identified in the presence of the two-dye mixture from the measured 535 nm and 565 nm intensities. For example, intensities of 38 (535 nm) and 22 (565 nm) for an unknown would lead to a classification as 3-(methylamino)-1-phenylpropano-1-ol. Analytes that occupy similar regions of the 535 nm/565 nm plot, however, could be readily misidentified such as ephedrine/methylephedrine and methylpseudoephedrine/3-(methylamino)-1-phenylpropan-1-ol. These analytes were also the most commonly misclassified samples in the LDA analysis.

Measuring diastereomeric excess

Finally, an assay based on the discriminating ability of RB-Z was developed to measure the diastereomeric excess (de) of 1,2-aminoalcohol mixtures (Fig. 8). Diastereomeric methylephedrine and methylpsuedoephedrine were selected as analytes for this study due to the large differences in their 565 nm emission intensities. First, we prepared mixtures of known concentrations of methylephedrine and methylpseudoephedrine in varying ratios. The fluorescent responses of these samples were measured with the dye mixture. For each measurement, 5 replicates were run (Fig. 8). We initially thought that accurate de measurements would require both the rhodamine and fluorescein responses. However, analysis of the data for the dye mixtures show that accurate de measurements could be made by monitoring just the rhodamine 565 nm peak. Monitoring the fluorescein 535 nm peak was not necessary since the total concentration of the diastereomers was held constant. Estimates of de based on the fluorescent response of the samples at 565 nm were highly reproducible with each point varying less than 2% from the trend line. This plot demonstrates the potential utility of this simple yet robust detection method.

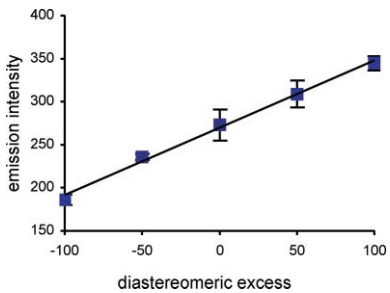


Fig. 8 Fluorescent emission measurements of the diastereomeric excess for the diastereomers of methylephedrine with 120 μM rhodamine B and 70 μM fluorescein in acetonitrile (λ_{ex} = 470 nm). Error bars represent one standard deviation for the average measurement of five individual solutions. Averaged measurements vary less than 2% from calculated value.

Conclusions

In this work, we have shown that a commercially available, inexpensive dye (rhodamine B) can be employed to yield dramatic and accurate differentiation of 1,2-aminoalcohols. The assay is

sufficiently sensitive that diastereomeric 1,2-aminoalcohols are distinguishable over a range of concentrations. The selectivity of the assay appears to be due to the ability of structurally different 1,2-aminoalcohols to stabilize the RB-Z (565 nm) to differing degrees.

The proposed origins of this differential response are consistent with previous studies of the equilibrium states of rhodamine B and fluorescein. The primary difference is that we have taken advantage of the sensitivity of the lactone–zwitterion equilibrium, which has not been extensively studied in organic solvents because of the predominance of the lactone form under these conditions. However, the zwitterion is sufficiently fluorescent and spectroscopically distinct from the lactone form so that changes in its concentrations can be easily and accurately measured in organic solvents. In closing, a system has been developed that affords an easy and accurate method to differentiate diastereomeric amino alcohols *via* the emission intensities of a commercial dye or dye mixture.

Experimental

Instrumentation and materials

HPLC grade acetonitrile was purchased from Fisher Scientific and was used as received. Rhodamine B was purchased from Acros and was purified by recrystallization twice from ethanol. Fluorescein was purchased from Sigma Aldrich and was purified by flash chromatography using 10% methanol in dichloromethane. All of the analytes were used in their free base forms. For those analytes purchased as salts, the free base was attained by washing the analyte with sodium bicarbonate solution and then extracting the free amine into methylene chloride. Fluorescence measurements were made using a Jasco PF-6500 on the low setting using a 10 nm bandwidth for both excitation and emission. Fluorescence data was collected by exciting at 470 nm. The measured fluorescence intensities were normalized to the response at 535 nm of a control solution of the dye mixture measured in the presence of 0.2 mM diisopropylamine, unless otherwise stated.

Procedure for titration plot

A solution of 3.3 mM analyte and in 120 μM rhodamine and/or 70 μM fluorescein was prepared by mixing the analyte and dye solution at a ratio of 1 to 2 respectively. This solution was then incrementally added to a cuvette containing a solution of 120 μM rhodamine and 70 μM fluorescein to create concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 1.4, and 1.6 mM. After each addition of analyte, the solution in the cuvette was mixed and a fluorescence measurement was taken within 1 min. The concentration of the xanthene dye was held constant throughout to prevent dilution effects.

Diastereomeric excess plot

A solution of 3.3 mM methylephedrine and methylpseudoephedrine in 120 μM rhodamine and 70 μM fluorescein was prepared as previously described. These solutions were then used to make six replicate solutions with varying ratios. The v/v percentages of methylephedrine to methylpseudoephedrine used for the six solutions were of 100% to 0%, 75% to 25%, 50% to 50%, 25% to 75%, and 0% to 100%. The fluorescence spectra of each sample were then run under the same condition as previously described. The emission intensities for this study were not normalized to a control sample, in contrast to all of the previous studies.

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