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Synthesis and application of *N*-hydroxysuccinimidyl rhodamine B ester as an amine-reactive fluorescent probe

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Abstract

A fluorescent probe (RB−S) containing the *N*-hydroxysuccinimidyl ester in 2 position was prepared from rhodamine B (RB) by one-step condensation reaction. The reactivity of the fluorescent probe with glycine was studied and the products were identified by LC−MS analysis. The excessive equivalent of the RB−S was hydrolyzed to the corresponding carboxylic acids (RB). UV/vis and fluorescence spectra of the fluorescent probe (RB−S), the labeled derivative (RB−Gly) and the hydrolysate (RB) were also studied. Fluorescence and absorption spectra appear as mirror images. The solvent effect on the spectra of the glycine derivative (RB−Gly) was investigated in methanol/water solution.

Keywords: Rhodamine B; *N*-Hydroxysuccinimidyl ester; Fluorescent probe; Glycine; LC−MS

1. Introduction

As there is an increasing need in the determination or characterization of bioactive molecules in trace amounts, more interests are concentrated on new analytical methods with high sensitivity. Compared with other spectrometric methods, spectrofluorimetry has more advantages, as it is a simple, sensitive, rapid method for the determination of bioactive molecules [1]. Particularly, it had been developed as a kind of detector for increasing the sensitivity of high performance liquid chromatography and capillary electrophoresis chromatography [2], and probably, the fluorescent probes are the most important reagents in this method.

Presently, a number of fluorescent probes had been designed and prepared, with the excitation and emission wavelengths ranging between 480 and 780 nm. Most recently, CyDye™ probes were frequently employed in biological studies [3]. They are characterized by a resonance structure comprising a polymethine chain bearing two terminal nitrogen atoms and an overall positive charge. However, the polymethine structures lack good photostability [4] and such kinds of probes were expensive somehow. These factors limit more applications in ordinary labs, so it was still important for chemists to exploit new fluorescent probes with low price, but good stability, reactivity (activity and selectivity) and high fluorescent intensity.

Rhodamine dyes were among the oldest and most commonly used of all synthetic dyes. Generally they were a kind of cheap but widely used dyes. Initially, they were used for cloth coloration. Owing to their unique optical properties, they served as water tracing agents, fluorescent markers for microscopic structure studies, photosensitizers, and as laser dyes [5]. Rhodamine dyes belong to the class of xanthene dyes. They have large molar absorptive coefficients in the visible spectrum which attribute to $\pi \rightarrow \pi^*$ transition. Absorption and emission are influenced by substituents on the xanthene moiety [6]. The *N*-hydroxysuccinimidyl ester was often designed in the structures of fluorescent probes [7,8]

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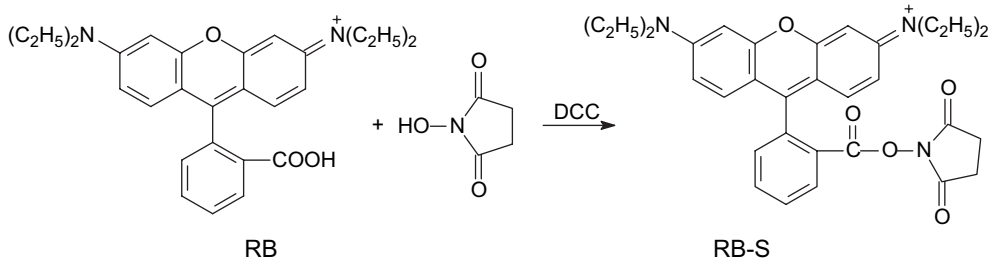

Scheme 1. Synthesis of *N*-hydroxysuccinimidyl rhodamine B ester (RB–S).

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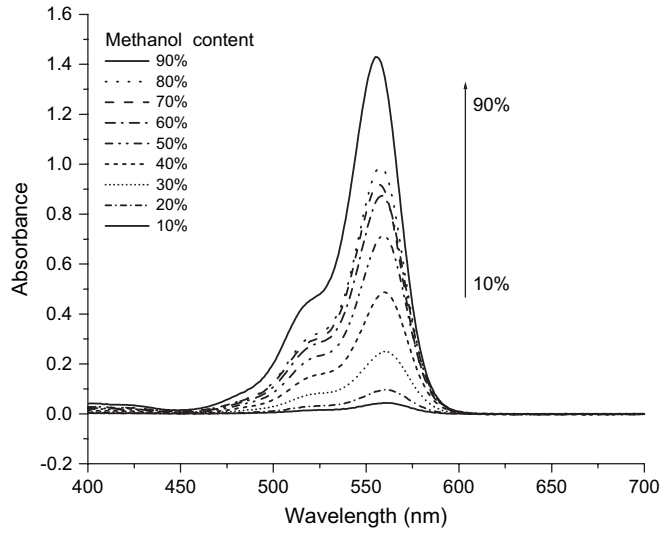


Fig. 1. UV/vis absorptive spectra of RB–Gly in methanol/water (2×10^{-6} mol/L).

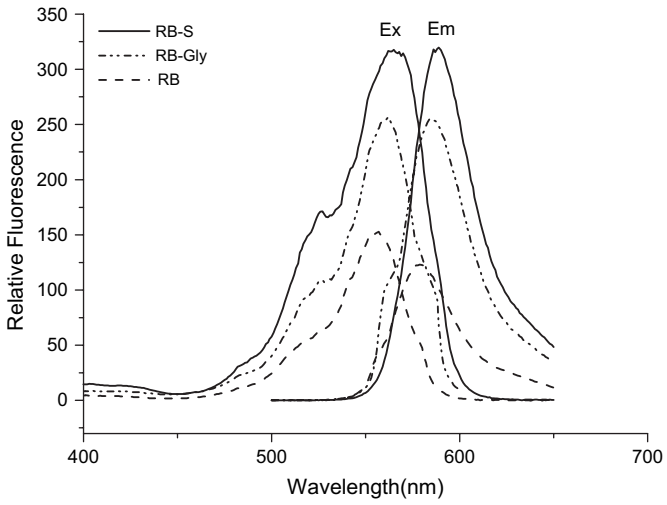


Fig. 2. The fluorescence spectra of the RB–S, RB–Gly, and RB.

and played a role in labeling process [9]. In this paper, a fluorescent probe (RB–S) containing the *N*-hydroxysuccinimidyl ester in 2 position was prepared from rhodamine B (RB) by one-step condensation reaction (Scheme 1). The rhodamine B itself is a commercially available dye in low price and high purity. Subsequently, we studied the reactivity of the fluorescent probe with glycine and the spectral characteristics of the labeled derivative.

2. Experimental

2.1. Materials

The reagents were commercially available and used without further purification. The reagents for analytical purpose were of analytical reagent grade. The H_3BO_3 buffer (pH = 8.5) was prepared by adding 1 mol/L HCl solution to 0.05 mol/L $Na_2B_4O_7$ solution to the required pH value. The H_3BO_3–H_3PO_4–HAc buffer (pH = 3.3) was prepared by mixing 1.24 g H_3BO_3, 1.36 mL 85% H_3PO_4 and 1.18 mL acetic acid, then diluted into 1000 mL by double-distilled water.

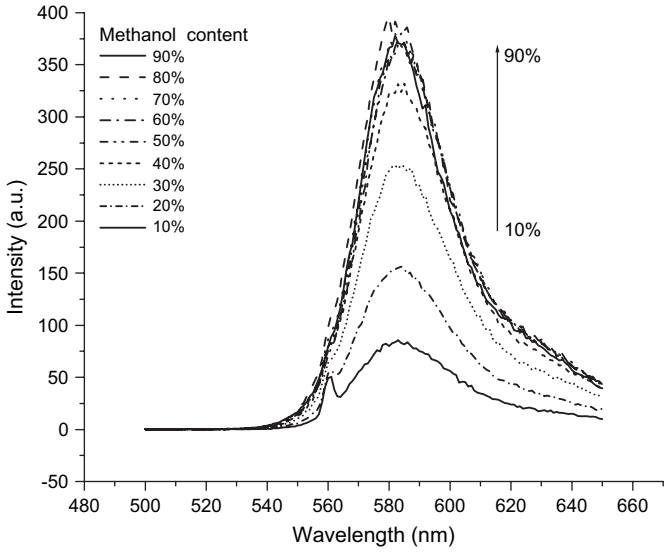
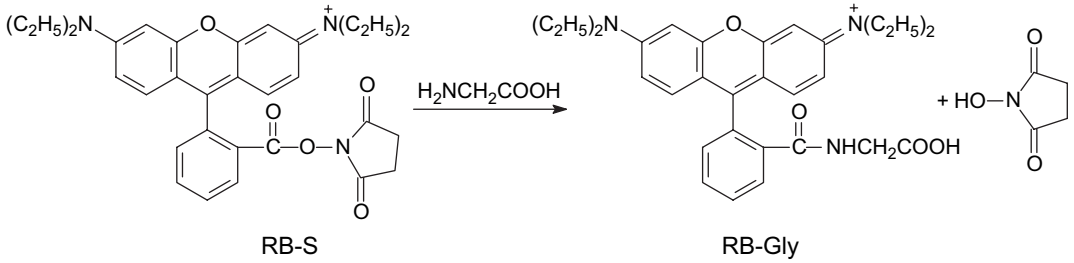
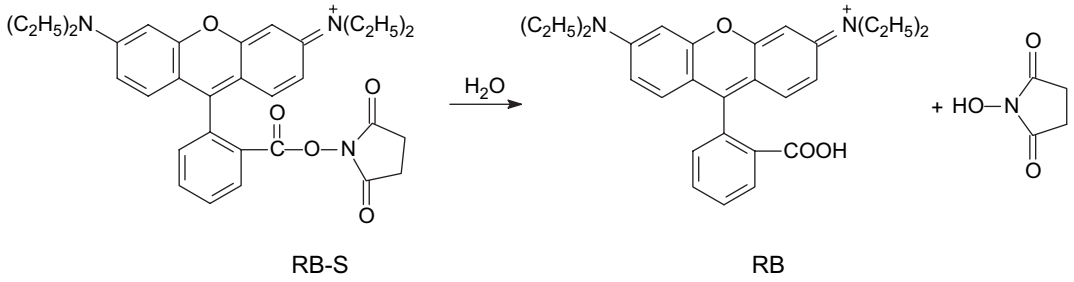


Fig. 3. The fluorescence spectra of RB–Gly in methanol/water (2×10^{-6} mol/L).



Scheme 2. Reaction of the fluorescent probe with glycine.



Scheme 3. Hydrolysis of the fluorescent probe.

inally, the buffer solution was obtained by adjusting the pH value with 0.2 mol/L NaOH solution.

2.2. Apparatus

All melting points were determined with an Mp-500 apparatus (Yanaco) and were not corrected. The pH values were determined with a 320-S acidimeter (Mettler Toledo), which was calibrated with standard aqueous buffer solutions. UV/vis spectra were measured on a Lambda 20/2.0 spectrophotometer (Perkin Elmer). The fluorescence spectra were measured in standard quartz cuvettes on a Cary Eclipse spectrofluorimeter (Varian). Samples were dissolved in solutions where the cationic form for rhodamine dyes was preferably present. IR spectra were recorded on an Avatar360 FT-IR spectrophotometer (Nicolet) in a H_2O-purged environment. All spectra were obtained from compressed KBr pellets in which the samples were evenly dispersed. Thirty-two scans were used to record each FT-IR spectrum in the range of 4000–400 cm^{-1} with 2 cm^{-1} spectral resolution at room temperature. The spectra were corrected for the H_2O and CO_2 content in the optical path. ^1H NMR spectra were recorded in $CDCl_3$ solutions on a Varian Mercury Plus 400 Spectrometer (Varian) using $(CH_3)_4Si$ as an internal standard. LC–MS was performed on an HP 1100 HPLC (Agilent), interfaced with a triple–quadruple mass spectrometer equipped with an ESI ion source and an in-line diode-array ultraviolet–visible detector. The LC separation was performed on a reversed-phase C_{18} column (4.6 × 150 mm, Zobax XDB).

2.3. Procedures

2.3.1. Synthesis of N-hydroxysuccinimidyl rhodamine B ester (RB–S)

Rhodamine B (2.4 g) and 0.6 g *N*-hydroxysuccinimide were dissolved in 100 mL acetonitrile and heated to 45 °C. With agitation, a solution of 1.2 g of dicyclohexylcarbodiimide (DCC) in 50 mL acetonitrile under dried condition was added

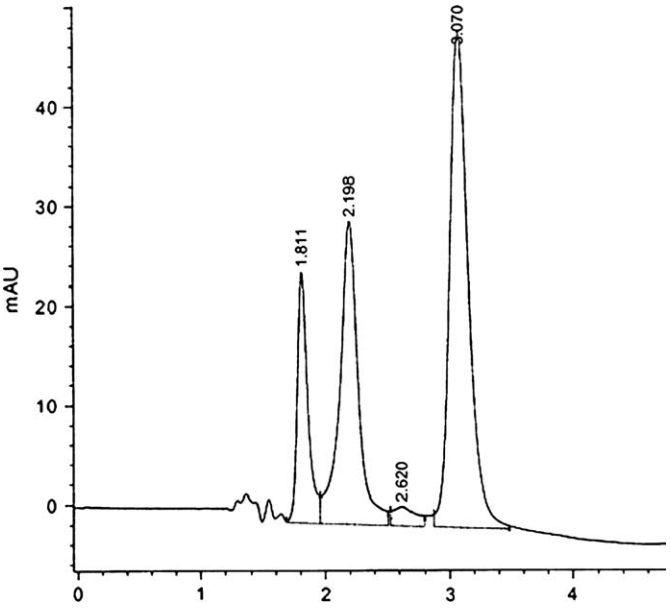


Fig. 4. LC chromatogram of the reaction mixture.

to the previous solution slowly. After continued agitation at 45 °C for 1 h and at room temperature for 20 h, the white precipitate was removed as a by-product from the reaction mixture by filtration. After reduced-pressure distillation of the solvent from the filtrate and recrystallization in absolute ethanol, RB−S was obtained as dark green crystals with a yield of 35%; mp: 202−205 °C. IR (KBr/cm^{-1}): 1768, 1732, 1254, 1173. ^1H NMR ($CDCl_3/\delta$, ppm): 8.39(1H, d), 7.95(1H, t), 7.81(1H, t), 7.44(1H, d), 7.05(2H, d), 6.85(4H, q), 3.61(8H, q), 2.75(4H, s), 1.30(12H, t).

2.3.2. Synthesis of glycidyl rhodamine B amide (RB−Gly)

Glycine (0.5 g) was dissolved in 20 mL H_3BO_3 buffer (pH = 8.5). With agitation, a solution of 0.4 g of RB−S in 20 mL acetonitrile was added to the buffer solution slowly. After continued agitation at room temperature for 4 h, the acetonitrile was removed by reduced-pressure distillation. Violet precipitate was obtained on filtration. After crystallization in ethanol/water, RB−Gly was obtained as bright violet crystals with a yield of 63%; mp: 170−172 °C. IR (KBr/cm^{-1}): 3425, 2640, 2360, 1693. ^1H NMR ($CDCl_3/\delta$, ppm): 13.5(1H, br), 7.96(1H, d), 7.54(3H, m), 7.09(2H, d) 6.91(4H, q), 4.81(1H, br), 3.86(2H, s), 3.49(8H, q), 1.23(12H, t).

Table 1
Identification of the ingredients

Peak number	Retention time (min)	Molecular ion mass (*m/z*)	Ingredients
1	1.811	500.3	RB−Gly ($C_{30}H_{34}N_3O_4$)
2	2.198	540.3	RB−S ($C_{32}H_{34}N_3O_5$)
3	3.070	443.2	RB ($C_{28}H_{31}N_2O_3$)

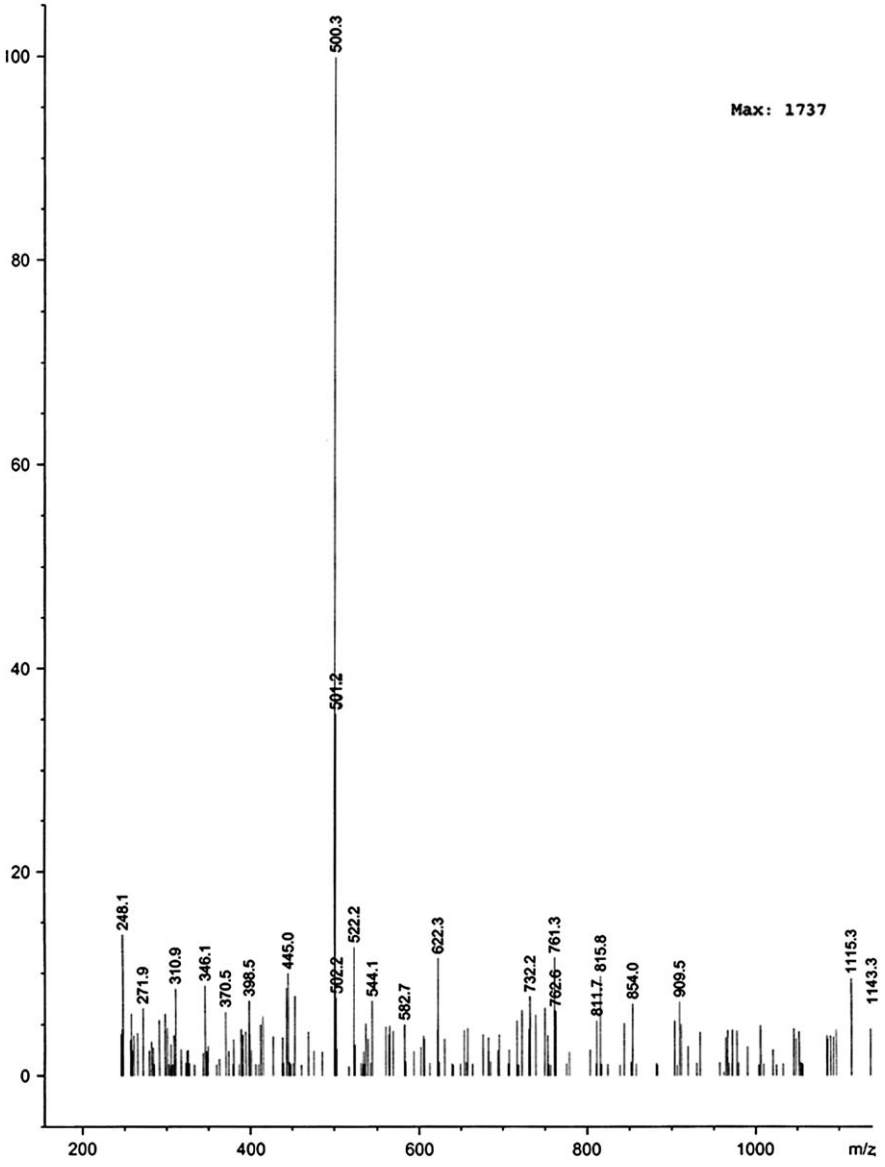


Fig. 5. ESI mass spectrum of Peak 1 (RB−Gly).

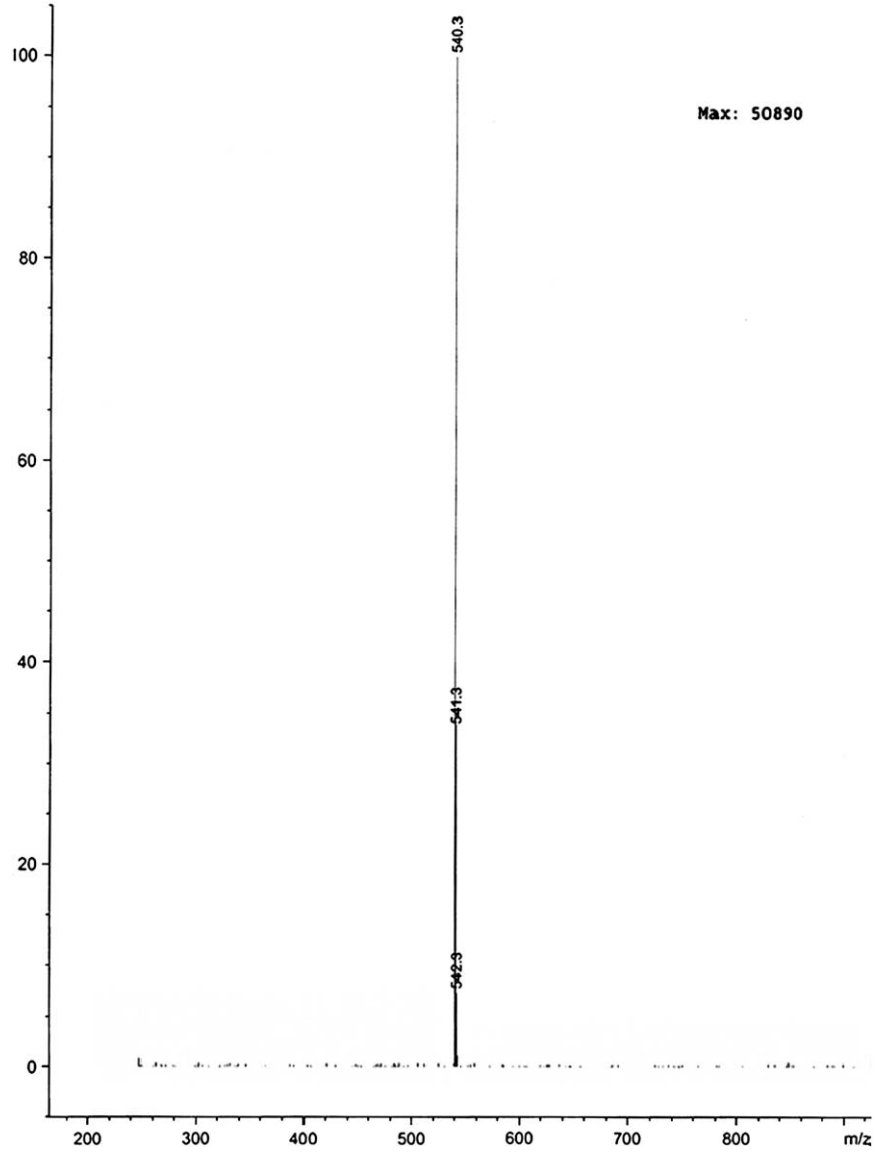


Fig. 6. ESI mass spectrum of Peak 2 (RB–S).

2.3.3. Labeling and analyzing procedure

Glycine solution (0.5 mL of 10 mM) was mixed with 4.5 mL H_3BO_3 buffer (pH = 8.5). Then 5 mL acetonitrile solution containing 0.4 mM RB–S was added to the mixture. The reaction was continued at room temperature for 4 h and diluted to 25 mL with the mobile phase solution. The mixture was ultra-sonicated for 3 min and separated by centrifugation at 2500 rpm for 10 min. The clear supernatant liquid was filtered through 0.45 μm membrane filter prior to LC–MS determination.

Five microlitres of this sample solution was injected into LC–MS system operated both in diode-array and ESI detecting modes to confirm the identities of the ingredients. The mobile phase consisted of a methanol–water–HAc solution (70:29.4:0.6, v/v) and the flow rate was 1 mL/min at 25 °C. The electrospray ionization (ESI) mass detector was operated at a capillary voltage of 3 kV, a sample cone voltage of 50 V, a source temperature of 120 °C, and at a desolvation temperature of 250 °C. The desolvation gas flow-rate was 5 L/min for both negative and positive ion generation. The mass range scanned was 200–1500 *m/z* and UV/vis spectrum was scanned in the range of 200–700 nm for the entire chromatographic run.

3. Results and discussion

3.1. UV/vis spectrum

Rhodamine dyes exist in solution in several forms as ionized forms, lactones or as aggregates depending on solvent effects (proton activity, solvent polarity, temperature or concentration) [10–12]. Each form is characterized by its spectrum. Therefore, spectroscopic properties of rhodamines are still the subject of numerous research studies and could be

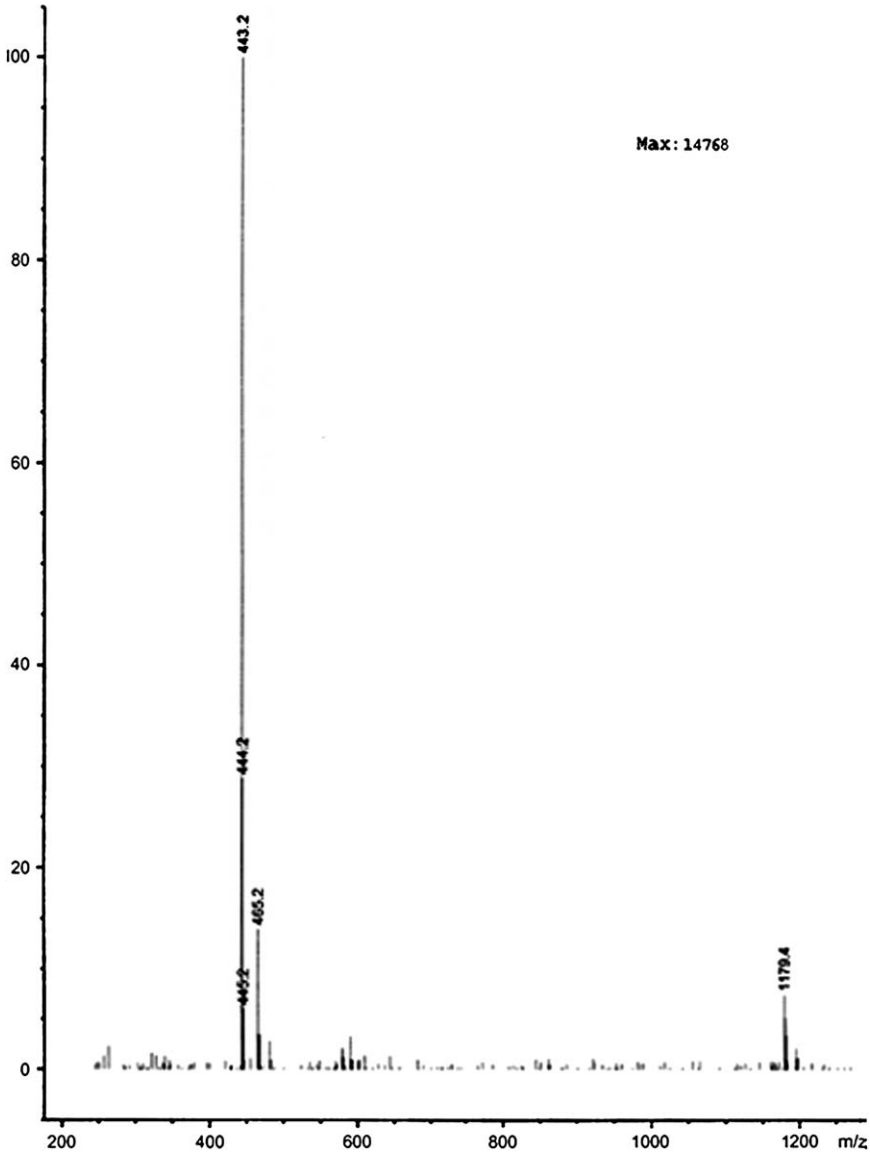


Fig. 7. ESI mass spectrum of Peak 3 (RB).

very useful for analytical application. In this work, the UV/vis spectrum and the solvent effect of the glycine derivative (RB–Gly) labeled by RB–S were studied in methanol/water solution, which would be used as mobile phase in LC separation.

UV/vis spectrum and the solvent effect of RB–Gly are shown in Fig. 1. This dye indicated the typical spectroscopic properties of rhodamine dyes. In the spectrum, 564 nm was considered as the wavelength of the monomer absorption maximum and 520 nm could be assigned to the aggregate absorption maximum. The UV/vis spectrum was further studied in methanol/water mixtures of various volume ratios (Fig. 1). By increasing the volume ratio of methanol in the mixture, nearly no shift in absorption maximum, but a significant increase in intensity of the absorption band was noted. In the meanwhile, it had a further effect on the sensitivity of spectrometric detectors. On account of both the solvent action and the spectrometric sensitivity, the volume ratio of methanol/water in the flow phase was recommended to be 70:30 (v/v).

3.2. Fluorescence spectra

The fluorescence spectra of the fluorescent probe (RB–S), the labeled derivative (RB–Gly) and the hydrolysate (RB) are shown in Fig. 2. The $\lambda_{ex}/\lambda_{em}$ for them were 564/589 nm, 559/582 nm and 556/579 nm, respectively. Fluorescence and absorption spectra appear as mirror images. Generally, they exhibited native fluorescence characteristics of rhodamine dyes except for a little shift of $\lambda_{ex}/\lambda_{em}$, which can be explained in terms of the different substitutes joined to the xanthene moiety. However, the Stokes shift for each fluorescence spectrum kept nearly the equal value as 23–24 nm. As Stoke's shift is usually associated with the vibrational relaxation in the fluorescence process, perhaps the substitutes had

inconsiderable effects on the vibrational energy levels of rhodamine moiety.

The fluorescence spectrum of RB–Gly was further studied in methanol/water mixtures of various volume ratios, too. Like the UV/vis spectrum, by increasing the volume ratio of methanol, nearly no shift in emission maximum (582 nm), but an increase in intensity of the emission band was observed (Fig. 3). However, the intensity seemed reaching its maximum when the methanol ratio was over 50% (v/v).

3.3. Reactivity of the fluorescent probe

Glycine is a primary amino molecule and could react with the fluorescent probe (RB–S) (Scheme 2). Excessive equivalent of the RB–S was utilized for the purpose of labeling completeness of the glycine. Some of the excess RB–S was hydrolyzed to the carboxylic acids (RB) as a by-product (Scheme 3) in the solution. The fluorescent probe (RB–S), labeled derivative (RB–Gly) and the hydrolysate (RB) in reaction system were separated by liquid chromatography (LC). According to the separation result, a typical chromatogram is given in Fig. 4. The ingredients were separated on the baseline within 8 min. Each peak was identified by the corresponding ESI mass spectrum (Table 1, Figs. 5–7). In the LC–MS analysis, no evidence indicated the fluorescent probe's reaction with the –COOH in glycine, owing to the high amine-reactive specificity of *N*-hydroxysuccinimidyl ester moiety in the structure of RB–S.

4. Conclusions

The fluorescent probe (RB–S) containing the *N*-hydroxysuccinimidyl ester in 2 position was prepared. The reactivity of the fluorescent probe with glycine was identified by LC–MS analysis. The UV/vis and fluorescence spectra were studied, too. As a result of fact, this probe could be applied in labeling analysis appropriately, not only for its fluorescence characteristics but also for its reactive specialty.

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