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Rhodamine B Piperazinoacetohydrazine: A Water-Soluble Spectroscopic Reagent for Pyruvic Acid Labeling

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Abstract: A new water-soluble reagent, rhodamine B piperazinoacetohydrazine (RBPH), with improved spectroscopic and reaction properties, has been developed and characterized for pyruvic acid labeling. The reagent RBPH is designed and synthesized by using rhodamine B as a spectroscopic unit, and hydrazine as a carbonyl-specific labeling unit; the two units are connected by a well-chosen linker of piperazine, which prohibits the formation of the nonfluorescent spirocyclic structure of rhodamine B, thereby keeping the spectroscopic response of the reagent in a stable state. Such a design enables RBPH not only to maintain its excellent spectroscopic properties over a wide pH range, but also to exist as a stable cation with high water solubility. Moreover, the hydrazino group of RBPH is expected to react selectively with carbonyl compounds under mild conditions through the rapid formation of hydrazones. These important features make RBPH of great potential use in the labeling of aldehydes or ketones in various biosystems, and such an application of RBPH as a precolumn derivatizing reagent has been successfully demonstrated on the analysis of pyruvic acid in human serum by high-performance liquid chromatography with common UV/Vis detection.

Keywords: derivatizing reagents · fluorescence · pyruvic acid · rhodamine B · UV/Vis spectroscopy

Introduction

Pyruvic acid is a key intersection in the network of metabolic pathways.[1] The exceptional change in its concentration may be associated with metabolic diseases,[2,3] and even tumor conditions.[4] The determination of pyruvic acid is thus of great importance for various biochemical studies as well as the diagnosis of related diseases. However, pyruvic acid itself is spectroscopically inert and lacks any identifiable properties; moreover, many carbonyl compounds often coexist and their properties are similar. This makes pyruvic acid difficult to be determined sensitively and selectively. To overcome the problem, spectroscopic derivatization is often combined with an efficient separation technique, such as high-performance liquid chromatography (HPLC).[5,6] Derivatization improves the spectroscopic response of the analyte and thereby its detection sensitivity, and separation increases selectivity.

So far, two main types of labeling reagents have been proposed for the determination of pyruvic acid by HPLC with precolumn spectroscopic derivatization: one contains an active hydrazino group (e.g., 2,4-dinitrophenylhydrazine),[7] and the other possesses a structural feature of *o*-phenylenediamine,[8–10] such as 4,5-diaminophthalhydrazide. The reaction mechanism of both types is based on the formation of imine derivatives, such as hydrazones and quinoxalines. Although some of the reagents have been applied to biological fluids (e.g., human urine and serum),[7b,10] there are still certain unresolved problems, such as analytical short wavelengths (<450 nm),[7–10] strict reaction conditions (>60 °C, >60 min),[8–10] and the requirement for time-consuming extraction because of poor water solubility[7] (see Table S1 in the Supporting Information for details). Other known reagents[11] containing hydrazino groups appear to have the potential for labeling pyruvic acid, but such attempts have not yet been reported, which may be partly due to the similar problems mentioned above.[12]

An ideal labeling reagent for chromatographic analysis should have the following characteristics: 1) rapid reaction with a target in high yield under mild conditions, 2) good water solubility of both the reagent and its product, and 3) stable spectroscopic response of reaction product with an-

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alytical long wavelength and high absorptivity or fluorescence quantum yield.[13,14] Nevertheless, such a reagent with the above properties suited for pyruvic acid labeling is still lacking. Herein, we report the design, synthesis, and characterization of rhodamine B piperazinoacetohydrazine (RBPH, Scheme 1) as a new spectroscopic reagent for this purpose.

Results and Discussion

Design of RBPH and its spectroscopic properties: The design of RBPH takes advantage of the properties of the hydrazine, rhodamine B, and piperazine moieties. First, hydrazine is recognized as a carbonyl-specific labeling unit with high reactivity under mild conditions. Second, rhodamine B, as an excellent fluorochrome,[15] has attracted considerable interest in developing various spectroscopic off–on-type probes by virtue of its easy formation of a colorless and nonfluorescent spirocyclic structure.[16] However, this cyclization (reversible in many cases[16e]) may lead to unstable spectroscopic responses, which unavoidably increases detection error and is unsuitable for labeling analysis.[16f] To circumvent this behavior, we chose piperazine as a linker, not only because its high reactivity would facilitate the connection of labeling and spectroscopic units, but also because its potential ternary nitrogen atoms would prohibit any cyclization[17] of rhodamine B. This prohibition would keep RBPH in a fixed conjugation structure and enable it to exist as a cationic fluorochrome, which is desired for improving both the spectroscopic stability and water solubility of the reagent. As a result, RBPH was synthesized through the reaction of rhodamine B acid chloride with **2** and the subsequent deprotection of the resulting **3** (Scheme 1; see also Figure S1 in the Supporting Information).

The reagent RBPH, as expected, is highly water soluble because of its cationic character, and possesses excellent spectroscopic properties, similar to rhodamine B (Figure 1). The maximum absorption and fluorescence emission of RBPH are located at 565 and 590 nm, respectively, both of which display a 10 nm redshift compared with those of rhodamine B. The high absorptivity ($\varepsilon_{565nm}=8.21\times10^4\,M^{-1}cm^{-1}$) and fluorescence quantum yield ($\Phi=0.19$) of RBPH in aqueous media allow sensitive detection by either UV/Vis absorption or fluorescence spectroscopies. Furthermore, RBPH can retain its strong spectroscopic response over a wide pH range from 3 to 11, with only a small variation in intensity (<10%). The spectroscopic properties of the

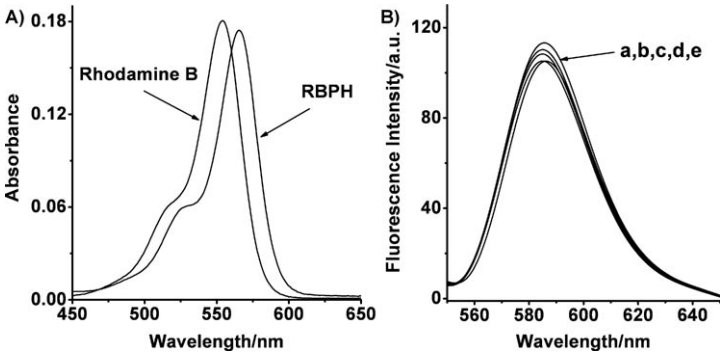
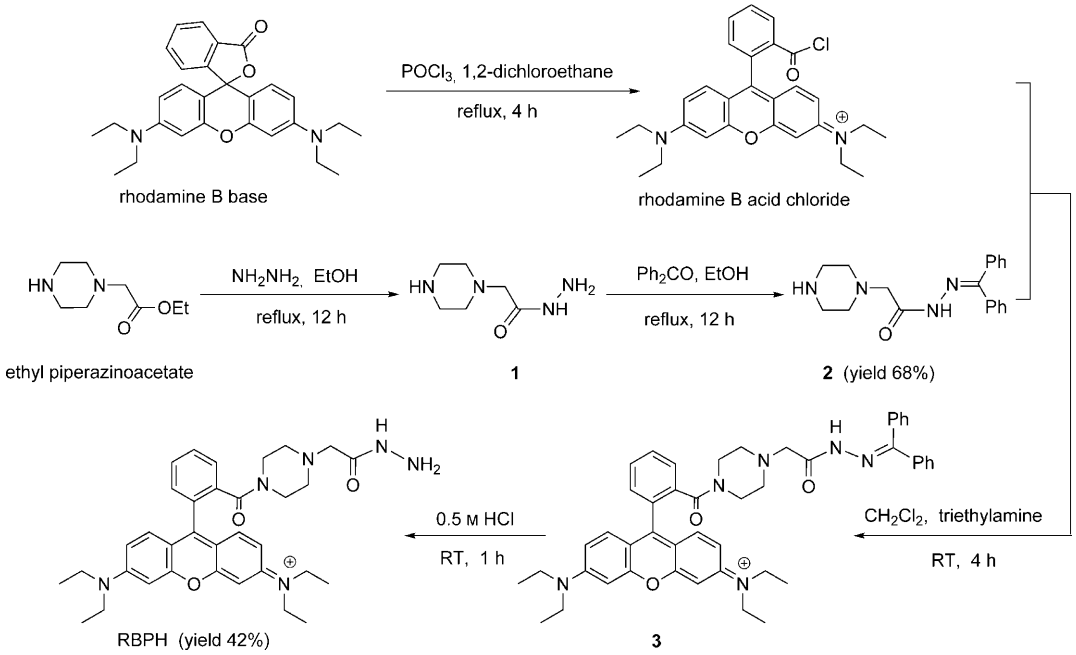


Figure 1. Spectroscopic properties of RBPH. A) Absorption spectra of 2 μM of RBPH and rhodamine B in 5 mM phosphate buffer (pH 7.0). B) Fluorescence emission spectra of RBPH (2 μM) in 0.1 M NaCl media with different pH values adjusted by HCl or NaOH: a) pH 3, b) pH 5, c) pH 7, d) pH 9, and e) pH 11.



Scheme 1. Synthesis of RBPH.

RBPH–pyruvic acid derivative were also studied. Compared with RBPH, the product exhibits no wavelength shift in both absorption and fluorescence emission, and shows only minor alterations in molar absorptivity ($\varepsilon_{565nm}=7.84\times 10^4\,M^{-1}cm^{-1}$) and fluorescence quantum yield ($\Phi=0.21$). These results indicate that derivatization barely changes the spectroscopic properties of RBPH, possibly due to the reacting site being far away from the chromophoric skeleton. Thus, both RBPH and its derivatizing product can be detected at the same long wavelength in HPLC. This property is very useful to monitor a precolumn derivatization process in real time by comparing the two corresponding peaks of derivatizing reagent and product.

Identification of RBPH–pyruvic acid derivative: A typical chromatogram of the reaction products of RBPH with pyruvic acid is shown in Figure 2. The reaction products give

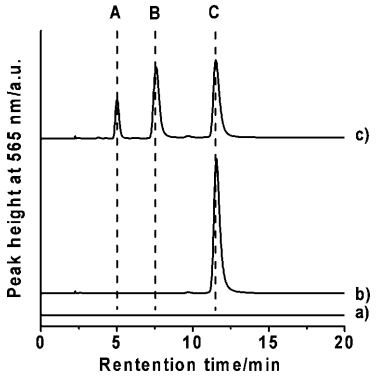
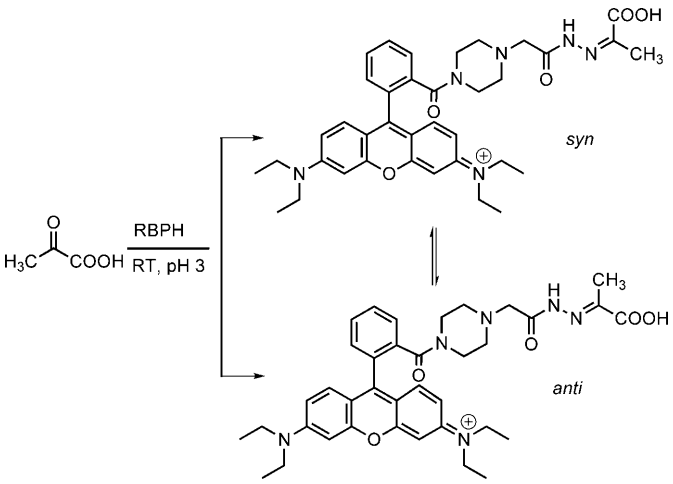


Figure 2. Typical HPLC chromatograms of reaction products of RBPH with pyruvic acid detected at 565 nm. a) 0.1 mM pyruvic acid, b) 0.2 mM RBPH, c) reaction products of 0.1 mM pyruvic acid and 0.2 mM RBPH according to the derivatization procedure. The assignment of the peaks: A) 5.01 and B) 7.58 min, two RBPH–pyruvic acid derivatives; C) 11.58 min, RBPH.

two peaks at 5.01 and 7.58 min, while RBPH displays a peak at 11.58 min. To identify the products, the two components corresponding to the peaks A and B were collected separately, and subjected to ESI mass spectrometry analysis. Surprisingly, the two components show an identical molecular ion peak at *m*/*z* 653.5 (Figure S2 in the Supporting Information), which suggests that the two chromatographic peaks A and B might arise from the isomerization of the RBPH–pyruvic acid derivative (Scheme 2).

To further prove the above assumptions, an attempt was made to purify each component of the two derivatization products for NMR spectroscopy analysis, but was unsuccessful. The reason for this is that the single component in solution, initially obtained by octadecylsilanized silica gel column chromatography, is readily changed into the original two-component mixture based on TLC analysis, which supports the existence of a possible isomerization equilibrium. This isomerization might be caused by warming and the presence of water.[7b] Then, the two-component mixture was



Scheme 2. Proposed isomerization of RBPH–pyruvic acid derivative.

directly subjected to ^{1}H NMR spectroscopy analysis. Two methyl signals were detected at $\delta=2.1$ and 2.4 ppm (Figure S3 in the Supporting Information), respectively, clearly confirming that peaks A and B shown in Figure 2 correspond to the *syn* and *anti* isomers of the RBPH–pyruvic acid derivative.

Optimization of experimental conditions

Derivatization conditions: As noted above, the high water solubility of RBPH allows the derivatization reaction to proceed in an aqueous medium, which is extremely desirable for biological sample analyses.[18] Thus, various possible factors affecting the derivatization efficiency in aqueous media were examined, including the concentration of RBPH, pH, reaction temperature, and time. The experimental results showed that, with the increase in the stoichiometric ratio of RBPH to pyruvic acid, the rate of the derivatization reaction increased, but the removal of the excess reagent would be rather time consuming. In this work, two equivalents of RBPH was used to react with one equivalent of pyruvic acid, since at this ratio a sufficient reaction rate can be achieved. The study of the pH effect on derivatization reveals that the optimal pH is about 3.0 (Figure S4 A in the Supporting Information). Room temperature is found to be suitable for the present system (Figure S4 B) and the derivatization reaction is completed in about 10 min (Figure S4 C); whereas a higher temperature of 50 °C led to a decrease in the derivative peak area, which may be caused by an accelerated hydrolysis of the product. Therefore, the derivatization of pyruvic acid can be achieved by reacting it with two equivalents of RBPH at room temperature for 10 min at pH 3, which has a considerable advantage of mild reaction conditions over other approaches.[7–10]

Separation conditions: The separation conditions of RBPH and its pyruvic acid derivatives by HPLC were also optimized. Methanol and acetonitrile as organic modifiers were

examined first. In combination with potassium citrate buffer, acetonitrile was found to be superior to methanol, since the latter caused a higher column pressure. The influence of pH values from 3 to 7 on the mobile phase upon separation was then studied. The best separation could be achieved at pH 6, which was maintained with 10 mM potassium citrate buffer. The pH values below six caused a partial overlap between the chromatographic peaks of RBPH and its pyruvic acid derivatives, because the decrease in pH reduced the retention time of RBPH, but scarcely affected those of the RBPH–pyruvic acid derivatives. In addition, isocratic elution was employed to get a good reproducibility. As a result, RBPH and its pyruvic acid derivatives could be separated by using a mobile phase of acetonitrile/water (80:20, v/v) containing 10 mM potassium citrate buffer (pH 6.0), and each chromatographic run could be completed within 15 min.

Linearity: The relationship between the total peak area of the RBPH–pyruvic acid derivatives and the concentration of pyruvic acid was investigated under the above optimized derivatization and separation conditions by a common UV/Vis absorption detector at 565 nm. A perfect linearity was observed over the concentration range of pyruvic acid from 5 to 500 μM, and the regression equation was determined to be $A_{(\text{total peak area})}=2899\times C$ (μM pyruvic acid)-7854 ($n=8$, $\gamma=0.9994$), with a detection limit of 1.2×10^{-7} M ($S/N=3$). More sensitive analysis may be expected if a fluorescence detector is employed. Reproducibility tests ($n=6$) showed that the relative standard deviation of the peak area was 0.9% for 1×10^{-4} M pyruvic acid.

Application to analysis of pyruvic acid in human serum: The proposed method was used to determine pyruvic acid in human serum directly. A typical chromatogram of the serum sample is shown in Figure 3. As can be seen, no interference from other peaks was observed (Figure 3d). The concentrations of pyruvic acid in serum samples from two healthy individuals were determined to be 47.9 and 71.5 μM (normal level of serum pyruvic acid: 30 to 100 μM[19]). The recoveries of the serum samples were also studied to examine the validity of the method. The results obtained are summarized in Table 1, from which it can be seen that the recoveries range from 92.1 to 97.1%, and the relative standard deviations are below 2%.

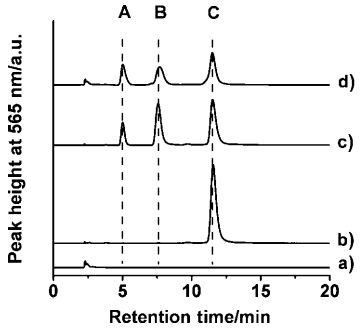


Figure 3. Typical HPLC chromatogram of human serum sample. a) Serum sample before derivatization, b) RBPH, c) standard pyruvic acid after derivatization by RBPH, and d) serum sample after derivatization by RBPH. The assignment of the peaks: A) 5.01 and B) 7.58 min, *syn* and *anti* isomers of the RBPH–pyruvic acid derivative; C) 11.58 min, RBPH.

Table 1. Analytical results of pyruvic acid in human serum samples.

Sample	Pyruvic acid added [μM]	Current method[a] [μM]	Recovery[b] [%]	Enzymatic fluorometric method[b,c] [μM]
1	0	47.9 ± 0.5	–	46.4 ± 2.2
	75	117 ± 1	92.1 ± 0.7	–
	200	242 ± 2	97.1 ± 0.8	–
2	0	71.5 ± 0.8	–	69.1 ± 3.8
	75	141 ± 2	92.7 ± 1.6	–
	200	259 ± 3	93.8 ± 1.1	–

[a] Pyruvid acid detected by using the present method. Mean of three determinations ± standard derivation. [b] Mean of three determinations ± standard derivation. [c] Pyruvic acid detected by using the enzymatic fluorometric method previously published.[20,21]

Comparison with other methods: Several methods for determining pyruvic acid have been developed recently, including mass spectrometric methods,[22] amperometric biosensors based on pyruvate oxidase,[23] indirect amperometric capillary analysis,[24] and enzymatic fluorescence capillary analysis.[20] Amperometric biosensors are rapid and sensitive, but suffer from interference.[23] The mass spectrometric and enzymatic methods show good selectivity and sensitivity; both of them would be more promising if their relative high cost can be reduced.[20] Because of the improved spectroscopic properties and labeling conditions of the reagent RBPH, our proposed method may offer a new choice for the simple, selective, and sensitive determination of pyruvic acid. Moreover, to further evaluate the validity of the proposed method, a comparative experiment was also conducted by an enzymatic fluorometric method (Table 1).[20,21] The results from the two methods are in good agreement with each other, and no significant difference between them is found at 90% confidence level when using the paired *t* test. In addition, the relative standard derivation (about 1%) of the present method is lower than that (about 5%) of the enzymatic fluorometric assay, indicating that the RBPH-based method has better precision.

Conclusion

A novel water-soluble reagent, RBPH, with improved spectroscopic and reaction properties has been developed for labeling of carbonyl compounds, and its use as a precolumn derivatizing reagent has been successfully demonstrated on the analysis of pyruvic acid in human serum by HPLC. The excellent properties of RBPH described herein may provide a promising future for its wide use in labeling aldehydes or

ketones in various biosystems. Clearly, RBPH might also apply to regioselective labeling of proteins through N-terminal transamination,[25] which is now under study in our laboratory.

Experimental Section

Chemicals and instruments: Ethyl piperazinoacetate (Fluka), rhodamine B base (Aldrich), pyruvic acid (Acros), benzophenone (Beijing Chemical Reagents Co.), L-lactic dehydrogenase from rabbit muscle (LDH, Sigma), and β-nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH, Sigma) were used as received. Acetonitrile and methanol (HPLC grade) were purchased from Fisher (USA). Other reagents were of analytical reagent grade. Deionized and distilled water was used throughout. The stock standard solutions (10 mM) of both pyruvic acid and RBPH were prepared by dissolving appropriate amounts of the compounds in water; they were stored at 4 °C in dark and were found to be stable for at least one month. For enzymatic fluorimetric analysis, the solutions of NADH (2 mM) and LDH (5 KUL^{-1}; one unit of the enzyme activity was defined as the amount of the enzyme that reduced 1.0 μmol of pyruvate to L-lactate per min at pH 7.5 at 37 °C were prepared daily by dissolving appropriate amounts of the compounds in 0.2 M phosphate buffer (pH 7.5) and stored at 4 °C.[20,21]

^1H and ^{13}C NMR spectra were measured on a Bruker DMX-300 spectrometer at 300 and 75 MHz, respectively, in D_2O. Electrospray ionization (ESI) mass spectra were recorded with a Shimadzu LCMS-2010. High-resolution SIMS analysis was performed by using a Bruker-Daltonics APEX II FT-ICR instrument. Fluorescence spectra were recorded on a Hitachi F-2500 fluorescence spectrophotometer in 10×10 mm quartz cells (Tokyo, Japan), with excitation and emission slit widths of 10 nm; fluorescence quantum yields were determined in 5 mM phosphate buffer (pH 7.0) by using rhodamine B in ethanol ($\Phi = 0.69$) as standard.[26] Absorption spectra were recorded in 1 cm cells with a TU-1900 spectrophotometer (Beijing, China). HPLC analyses were performed on a HiQ sil C18W (4.6×200 mm) column by using a Jasco HPLC system consisting of a PU-2080-plus pump and a UV-2075-plus detector. A model HI-98128 pH-meter (Hanna Instruments Inc.) was used for pH measurements.

Rhodamine B piperazinoacetohydrazine (RBPH): RBPH can be synthesized through the route depicted in Scheme 1, wherein rhodamine B acid chloride was obtained following the known procedure[27] and **2** was prepared from ethyl piperazinoacetate. Briefly, hydrazine hydrate (0.3 mL; 5 mmol) was added to a stirred solution of ethyl piperazinoacetate (0.4 mL, 2.3 mmol) in ethanol (3 mL). The mixture was heated at reflux for 12 h. Then, the solvent and the excess hydrazine were removed by rotary evaporation under reduced pressure to give **1** as a colorless oil, to which benzophenone (1.0 g, 5 mmol), ethanol (3 mL), and acetic acid (0.5 mL) were added successively. The resulting solution was heated at reflux for 12 h, during which time the color of the solution changed from colorless to yellow brown. The reaction mixture was evaporated under reduced pressure to give a brown oil, which was then purified by silica gel column chromatography with CH_2Cl_2/methanol (20:1, v/v) as the eluent, affording **2** as a yellowish powder (1.1 g, 68%). Compound **2** (170 mg, 0.5 mmol) was dissolved in CH_2Cl_2 (3 mL) containing triethylamine (0.3 mL). Rhodamine B acid chloride (0.5 mmol) in CH_2Cl_2 (1 mL) was then added dropwise to the resulting solution. After stirring for 4 h at room temperature, the reaction mixture was washed with water (1 mL). The separated organic phase was concentrated under reduced pressure to give a glassy purple solid **3**, to which 0.5 M HCl (20 mL) was added. The reaction mixture was sonicated for 5 min, followed by stirring for 1 h at room temperature. The resulting solution was neutralized to pH 3–4 with 1 M NaOH, saturated with NaCl, washed several times with ethyl acetate and CH_2Cl_2 successively, and finally extracted three times with isopropanol/CH_2Cl_2 (1:1, v/v). The combined organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated to near dryness under reduced pressure. The residue was recrystallized from isopropanol, producing RBPH chloride as a red–purple solid (yield 42%). It should be noted that the preparation of RBPH by the direct reaction of ethyl piperazinoacetate with rhodamine B acid chloride, followed by hydrazinolysis, was unsuccessful, because in such a procedure the spirocyclic structure of rhodamine B hydrazide was formed preferably. In other words, due to the strong nucleophilicity of the hydrazino group, our synthetic route to a rhodamine tertiary amide derivative by modifying at position 2′ is different from others.[17] The reagent RBPH is highly soluble in water and alcohols. Water solubility: >0.6% (w/v) at 20 °C; ^1H NMR (300 MHz, D_2O): $\delta = 1.26$–1.30 (t, $J = 7.0$ Hz, 12H), 3.05 (br s, 2H), 3.20 (br s, 2H), 3.63–3.77 (m, 12H), 3.87 (s, 2H), 6.88 (s, 2H), 6.97–7.00 (d, $J = 9.2$ Hz, 2H), 7.20–7.23 (d, $J = 9.5$ Hz, 2H), 7.64–7.72 (m, 2H), 7.82–7.93 ppm (m, 2H); ^{13}C NMR (75 MHz, D_2O): $\delta = 12.2$, 44.3, 46.0, 51.9, 52.4, 55.5, 96.5, 112.5, 114.3, 127.5, 130.1, 130.4, 131.0, 131.2, 133.3, 153.1, 155.6, 157.1, 163.7, 169.8 ppm; HRMS (FT-ICR-SIMS): *m/z* calcd for $[C_{34}H_{43}N_6O_3]^+$: 583.3390907; found: 583.3394560.

Derivatization procedure

Derivatization of standard pyruvic acid: In a 1 mL vial, an appropriate volume of standard solution of pyruvic acid was mixed with water (0.6 mL), followed by the addition of RBPH (its final molar concentration was about twice that of pyruvic acid) and 0.1 M HCl (10 μL). Then, the reaction solution was diluted to 1 mL with water. After 10 min at room temperature with shaking occasionally, aliquots of the reaction solution (20 μL) were injected into the chromatographic system.

Derivatization of pyruvic acid in human serum: Human sera from two healthy individuals were provided by Peking University People's Hospital, and an informed consent was obtained from each donor. The human sera (typically 1 mL) were first deproteinized by adding an equal volume of methanol. After shaking for 5 min, the mixture was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatants were collected as the serum samples. The derivatization of pyruvic acid in human serum was performed by using the procedure described above, in which 0.3 M HCl (10 μL) instead of 0.1 M HCl was used to acidify the reaction system to about pH 3.

Chromatographic method: HPLC analysis was performed on a C18 separation column at ambient temperature with acetonitrile/water (80:20, v/v) containing 10 mM potassium citrate buffer (pH 6.0). A flow rate of 0.80 mL min^{-1} was used, and all the solvents were filtered with a 0.45 μm membrane filter before use. The detection wavelength was set at $\lambda = 565$ nm, and peak areas were measured for the quantization of the analyte.

Acknowledgements

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