

A new BODIPY-based long-wavelength fluorescent probe for chromatographic analysis of low-molecular-weight thiols

Li-Yun Zhang · Feng-Qin Tu · Xiao-Feng Guo · Hong Wang · Peng Wang · Hua-Shan Zhang

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Abstract

A new long-wavelength fluorescent probe, 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-iodoacetamido)difluoroboradiaza-*s*-indacene (DMDSPAB-I), was designed and synthesized for thiol labeling in high-performance liquid chromatography (HPLC). The excitation and emission wavelengths of DMDSPAB-I are 620 and 630 nm, respectively, with a high fluorescence quantum yield of 0.557, which is advantageous in preventing interference of intrinsic fluorescence from complex biological matrices and enabling high sensitivity HPLC. Based on DMDSPAB-I, a reversed-phase HPLC method was developed for measuring low-molecular-weight thiols including glutathione, cysteine, homocysteine, *N*-acetylcysteine, cysteinylglycine, and penicillamine. After the specific reaction of DMDSPAB-I with thiols, baseline separation of all six stable derivatives was achieved through isocratic elution on a C₁₈ column within 25 min, with the limits of detection (signal-to-noise ratio=3) from 0.24 nmol L⁻¹ for glutathione to 0.72 nmol L⁻¹ for penicillamine. The proposed method was validated in part by measuring thiols in blood samples from mice, with recoveries of 95.3–104.3 %.

Keywords 1,7-Dimethyl-3,5-distyryl-8-phenyl-(4'-iodoacetamido)difluoroboradiaza-*s*-indacene · Thiols · long-wavelength · Fluorescence detection · High-performance liquid chromatography

L.-Y. Zhang · F.-Q. Tu · X.-F. Guo · H. Wang (✉) · H.-S. Zhang
Key Laboratory of Analytical Chemistry for Biology and Medicine
(Ministry of Education), College of Chemistry and Molecular
Sciences, Wuhan University, Wuhan 430072, China
e-mail: hongwang@whu.edu.cn

P. Wang
Technology Center, Hubei Entry-Exit Inspection and Quarantine
Bureau, Wuhan 430072, China

Introduction

Low-molecular-weight (LMW) thiols, including glutathione (GSH), homocysteine (HCys), cysteine (Cys), and cysteinylglycine (CysGly), are of great importance in metabolism and cellular homeostasis. For instance, GSH is the most abundant intracellular small-molecule thiol (1–10 mmol L⁻¹) [1], and has an important effect on oxidative stress by maintaining redox homeostasis for cell growth and function. HCys is a thiol compound linked to GSH metabolism via the transsulfuration pathway [2], and is believed to be neurotoxic. Increased HCys is associated with deleterious cellular and vascular events; for example, hyperhomocysteinemia increases the risk of stroke [3] and vascular dementia [4] and is involved in atherosclerosis [5, 6]. Cys is a synthetic precursor of glutathione [7], and disorders of Cys metabolism, including cystinosis and cystinuria, lead to increased cysteine concentration in urine. CysGly is a product of glutathione degradation; elevated plasma or urinary levels of CysGly are observed in patients with rheumatoid arthritis, and may be associated with the extent of inflammation. Penicillamine (PA) is a thiol drug used in treating heavy-metal-poisoning cystinuria and Wilson's disease [8]. Thus, simultaneous determination of multi-thiols in physiological liquids is important for physiological, pathological, and pharmacological studies.

Because of its good repeatability, high sensitivity, and selectivity, HPLC combined with fluorescence detection (FD) is an ideal approach for simultaneous quantification of multi-thiols. Because most thiol compounds have no fluorescence, an excellent fluorescent labeling probe is desired for use in HPLC-FD. Many fluorescent probes for determination of thiols with HPLC have been reported, including monobromobimane (mBrB) [9, 10], 7-fluorobenzo-2-oxa-1,3-diazoloe-4-sulfonamide (SBD-F)

[11], 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) [12], *N*-(1-pyrenyl)maleimide (NPM) [13], 1,3,5,7-tetramethyl-8-phenyl-(4'-iodoacetamido)difluoroboradiaza-*s*-indacene (TMPAB-I) [14], and 1,3,5,7-tetramethyl-8-bromomethyl-difluoroboradiaza-*s*-indacene (TMMB-Br) [15]. However, the excitation (λ_{ex}) and emission (λ_{em}) wavelengths of these probes are all in the UV–Vis spectral region, which does not efficiently prevent interference from intrinsic fluorescence of bio-samples [16, 17] and results in difficulties in HPLC separation. The effective way to overcome this disadvantage is to use long-wavelength (>600 nm) fluorescent probes for labeling. Although some long-wavelength probes, including a 2,4-dinitrobenzenesulfonyl fluorescent probe [18–20], Se–N substituted rhodamine [21], Cy–NiSe, and Cy–TfSe [22], have been designed for thiol imaging, none of them can be used in HPLC because the design strategies and reaction mechanisms of fluorescence imaging and HPLC differ. As far as we are aware, there is no long-wavelength fluorescent probe used in HPLC for multi-thiol determination.

Usually, a derivatizing probe used in HPLC and its derivatives should be stable enough to accomplish the separation and detection procedure. Rhodamine and cyanine-based fluorescent probes are commonly used long-wavelength labeling probes [23, 24]. However, cyanines have poor stability [25], and rhodamine derivatives are pH sensitive and their fluorescence is weak in the pH range of buffers used in HPLC (pH 2.0 to 7.0). Thus, neither is suitable for use in HPLC. Difluoroboraindacene (BODIPY) has gained recognition as one of the most versatile fluorophores because of its excellent thermal and photochemical stability, high fluorescence quantum yield, and chemical robustness. Moreover, creative modification to the BODIPY framework permits its derivatives to not only emit further into the long-wavelength or even near-infrared spectrum, but also retain their high fluorescence quantum yield [26, 27]. Therefore, BODIPY-based long-wavelength labeling probes are more suitable for multi-thiol determination in HPLC. In this study, the first BODIPY-based red-emitting probe for thiol labeling in HPLC, 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-iodoacetamido)difluoroboradiaza-*s*-indacene (DMDSPAB-I), was designed and synthesized, and a corresponding HPLC method was established for the determination of GSH, Cys, HCys, NAC, CysGly, and PA. Separation and derivatization conditions were carefully optimized, and the feasibility of the proposed method was validated by measurement of thiols in blood samples from mice. Through the establishment of this HPLC method, DMDSPAB-I reveals obvious advantages, including low detection limits, smooth and clean baseline, and few interfering peaks from bio-samples.

Experimental

Apparatus

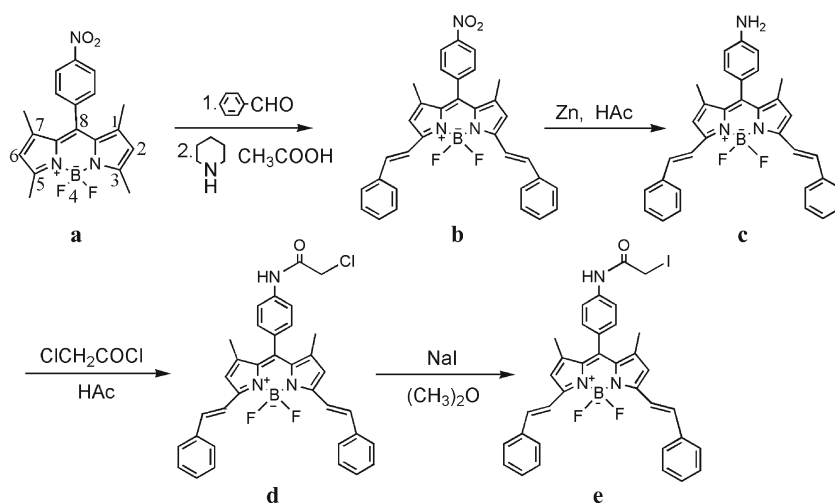
An LC-10A HPLC (Shimadzu, Tokyo, Japan) with LC-10AD dual pumps, CTO-10 column oven, and RF-10AXL detector was used. Separations were performed on a Kromasil C₁₈ column (150×4.6 mm, i.d. 5 μm) at room temperature (25 °C). Fluorescence spectra were recorded on an RF-5301pc spectrofluorimeter (Shimadzu, Tokyo, Japan). All pH values were determined by a Mettler-Toledo (Shanghai, China) Delta 320 meter. Absorption spectra were recorded on a UV-1601 UV–Vis spectrophotometer (Shimadzu, Tokyo, Japan). ¹H NMR spectra were recorded on a Varian Meravry VX-300 MHz spectrometer with chemical shifts reported as ppm (in DMSO-d₆, TMS as internal standard). Mass spectra (MS) data were measured with an LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany).

Chemicals and reagents

Unless otherwise specified, all reagents except DMDSPAB-I were of analytical reagent grade and used without further purification. Benzaldehyde (with a purity of 98 %) and piperidine (with a purity of 99 %) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). ClCH₂COCl was synthesized following the procedure in [28]. GSH, Cys, PA, and NAC were purchased from Sinopharm Chemical Reagent Co., Ltd. CysGly (with a purity of 85 %) and HCys were purchased from Sigma (Shanghai, China). DMDSPAB-I was synthesized in our laboratory. All solutions except DMDSPAB-I were prepared with double-distilled water. A $1.0 \times 10^{-3} \text{ mol L}^{-1}$ DMDSPAB-I stock solution was prepared by dissolving DMDSPAB-I in anhydrous acetonitrile. Standard stock solutions of $1.0 \times 10^{-2} \text{ mol L}^{-1}$ CysGly, GSH, NAC, Cys, and HCys, and $2.0 \times 10^{-2} \text{ mol L}^{-1}$ PA were prepared daily, as was a stock solution of standard thiol mixture containing $1.0 \times 10^{-2} \text{ mol L}^{-1}$ CysGly, GSH, NAC, Cys, and HCys and $2.0 \times 10^{-2} \text{ mol L}^{-1}$ of PA. Because the reaction efficiency of PA with DMDSPAB-I is lower than those of other thiols, a higher concentration of PA was used. Dilution of these stock solutions to appropriate concentrations was performed immediately before use. Na₂B₄O₇ (0.025 mol L⁻¹) and NaOH (0.1 mol L⁻¹) solutions were mixed to the required pH and used as the derivatization buffer. Citric acid (H₃Cit)–NaOH buffer solutions were obtained by mixing 0.1 mol L⁻¹ H₃Cit solution and 0.1 mol L⁻¹ NaOH solution to the required pH.

Synthesis of DMDSPAB-I

DMDSPAB-I was synthesized as shown in Fig. 1, following the steps below.

Fig. 1 Synthesis route of DMDS-PAB-I

Synthesis of 1,3,5,7-tetramethyl-8-phenyl-(4'-nitro)difluoroboradiaza-s-indacene (TMPB-p-N, compound a)

1,3,5,7-Tetramethyl-8-phenyl-(4'-nitro)difluoroboradiaza-s-indacene (TMPB-p-N, compound a) was synthesized following the procedure in [29].

Synthesis of 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-nitro)difluoroboradiaza-s-indacene (DMDS-PB-p-N, compound b)

TMPB-p-N (900 mg, 2.44 mmol) and benzaldehyde (1 mL, 9.76 mmol) were dissolved in 20 mL benzene, into which 2 mL piperidine and 1.7 mL acetic acid were added. The mixture was heated under reflux for 24 h using Dean-Stark apparatus. The solvent was then removed under vacuum and purification was performed by silica-gel column chromatography (eluant: CH_2Cl_2) to obtain a blue powder (800 mg, 60 %). $^1\text{H NMR}$ (300 MHz, DMSO): 1.43 (s 6H), 7.06 (s 2H), 7.42 (d 2H), 7.49 (t 4H), 7.58 (t 2H), 7.64 (d 6H), 7.84 (d 2H), 8.42 (d 2H).

Synthesis of 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-amino)difluoroboradiaza-s-indacene (DMDS-PB-p-A, compound c)

DMDS-PB-p-N (500 mg, 0.92 mmol) was dissolved in 27 mL acetic acid, and then 4.5 g Zn was added. The mixture was stirred at room temperature for 30 min. Later, the solvent was removed under vacuum and purification was performed by silica-gel column chromatography (eluant: CH_2Cl_2) to obtain a blue powder (340 mg, 72 %).

Synthesis of 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-chloroacetamido)difluoroboradiaza-s-indacene (DMDS-PAB-Cl, compound d)

DMDS-PB-p-A (320 mg, 0.62 mmol) was dissolved in 30 mL acetic acid, and 0.5 mL ClCH_2COCl was rapidly added under stirring. Ten minutes later, 80 mL water was added. After precipitation and filtration, the solid product was purified by silica-gel column chromatography (eluant: CH_2Cl_2) to obtain a purple powder (330 mg, 90 %). $^1\text{H NMR}$, 1.48 (s 6H), 4.32 (s 2H), 7.02 (s 2H), 7.41 (d 2H), 7.46 (d 2H), 7.48 (t 6H), 7.53 (d 2H), 7.63 (d 4H), 7.83 (d 2H), 10.58 (s 1H).

Synthesis of 1,7-dimethyl-3,5-distyryl-8-phenyl-(4'-iodoacetamido)difluoroboradiaza-s-indacene (DMDS-PAB-I, compound e)

DMDS-PAB-Cl (330 mg, 0.56 mmol) was dissolved in 25 mL acetone, and NaI (900 mg, 6.00 mmol) was added. The solution was stirred in the dark until complete consumption of DMDS-PAB-Cl, after which the reaction was stopped. The solvent was then removed under vacuum and purification was performed by silica-gel column chromatography (eluant: CH_2Cl_2) to obtain a purple powder (260 mg, 68 %). $^1\text{H NMR}$, 1.48 (s 6H), 3.88 (s 2H), 7.02 (s 2H), 7.41 (d 2H), 7.46 (d 2H), 7.48 (t 6H), 7.58 (t 2H), 7.63 (d 4H), 7.80 (d 2H), 10.59 (s 1H). FT-MS (ESI): cala. For $\text{C}_{35}\text{H}_{29}\text{BF}_2\text{IN}_3\text{O}$ $[\text{M}+\text{H}]^+$ 684.1489, found: $[\text{M}+\text{Na}]^+$ 706.1304.

Preparation of DMDS-PAB-GSH solution

To study the fluorescence properties of DMDS-PAB-I thiol derivatives, solutions of the derivatives had to be prepared. In this study, DMDS-PAB-GSH was selected as the representative of the thiol derivatives. DMDS-PAB-I and DMDS-PAB-GSH both fluoresce strongly, but GSH itself is non-fluorescent. Excess GSH was used to completely convert DMDS-PAB-I to DMDS-PAB-GSH and thus avoid interference from unreacted DMDS-PAB-I. Therefore, 800 μL 1.0×10^{-2} mol L^{-1} GSH solution, 800 μL 1.0×10^{-3} mol L^{-1}

DMDSPAB-I, and 700 μL pH 11.0 $\text{Na}_2\text{B}_4\text{O}_7\text{-NaOH}$ buffer were added into a 4 mL Eppendorf tube. The well-mixed solution was incubated at 45 $^\circ\text{C}$ for 25 min and then diluted to 4 mL with ACN to obtain the DMDSPAB-GSH stock solution. The stock solution was stored at -30 $^\circ\text{C}$ and diluted with different solvents before use.

Derivatization procedure

100 μL acetonitrile, 50 μL pH 11.0 $\text{Na}_2\text{B}_4\text{O}_7\text{-NaOH}$ buffer, 70 μL 8.0×10^{-5} mol L^{-1} DMDSPAB-I solution, and 100 μL of the standard thiol mixtures were transferred into a 0.75 mL Eppendorf tube, and the well-mixed solution was then left to react at 45 $^\circ\text{C}$ for 25 min. After completion of the derivatization, the solution was 10-fold diluted with the mobile phase and 20 μL of the obtained solution was injected into the chromatographic system.

The derivatization optimization experiments were performed by following the above-mentioned procedure and changing the variable under investigation. Investigated variables included DMDSPAB-I concentration, ACN content, buffer pH, and reaction temperature and time.

The potential interferences from the 16 amino acids, Asp, Ala, Asn, Arg, Gly, Gln, Glu, His, Ile, Lys, Met, Phe, Pro, Ser, Tyr, and Val (amino acid:thiol molar ratio=5:20), and six metal ions (ion:thiol molar ratio, Ca^{2+} 300:320, Mg^{2+} 340:380, Fe^{3+} 70:100, Al^{3+} 60:70, Zn^{2+} 25:35, and Cu^{2+} 0.05:0.2) were investigated by mixing them with 100 μL acetonitrile, 50 μL pH 11.0 $\text{Na}_2\text{B}_4\text{O}_7\text{-NaOH}$ buffer, 70 μL 8.0×10^{-5} mol L^{-1} DMDSPAB-I solution, and 100 μL standard thiol mixture before the derivatization. In this experiment, all the potential interferences from amino acids were studied simultaneously, and the potential interferences from metal ions were studied one by one.

Chromatographic method

Before the analysis, the C_{18} column was pre-equilibrated for 30 min using a mobile phase consisting of methanol–water–tetrahydrofuran (THF)–pH 2.0 $\text{H}_3\text{Cit-NaOH}$ buffer (77:12:6:5, v/v) at room temperature. A 20 μL aliquot of the sample solution was injected and the derivatives were eluted at a flow of 0.7 mL min^{-1} with isocratic elution. The detection wavelengths were λ_{ex} and λ_{em} of 620 and 630 nm, respectively. Peak areas were measured for quantitative calculations.

Measurement of fluorescence quantum yield

The fluorescence quantum yields (Φ) of DMDSPAB-I and its thiol derivatives were measured following the procedure in [30]. Rhodamine 101, which has a fluorescence quantum yield in ethanol of 0.97, was chosen as the standard material [31, 32]. The unknown fluorescence quantum yield was

calculated by the formula:

$$\Phi_{\text{u}} = \Phi_{\text{s}} \times (F_{\text{u}} \times A_{\text{s}}) / (F_{\text{s}} \times A_{\text{u}}),$$

in which Φ_{u} and Φ_{s} are the fluorescence quantum yields of the unknown and the standard materials, respectively; F_{u} and F_{s} are the fluorescent peak areas of the unknown and the standard materials, respectively; and A_{u} and A_{s} are the absorbances at the excitation wavelength of the unknown and the standard materials, respectively.

Stability of DMDSPAB-I and its thiol derivatives

The photostability of DMDSPAB-I and of its thiol derivatives was studied as follows. 2 mL 1×10^{-4} mol L^{-1} DMDSPAB-thiol derivative solution was prepared as described in “[Preparation of DMDSPAB-GSH solution](#)”, followed by dilution with ACN to obtain 5×10^{-5} mol L^{-1} derivative solution. The derivative solution and DMDSPAB-I acetonitrile solution (5×10^{-5} mol L^{-1} each) were irradiated by a light source (2 mW 635 laser module, 5 cm). Every ten minutes, 200 μL of each solution was transferred to a colorimetric tube and diluted to 5 mL with ACN, and their fluorescence intensities were measured on the spectrofluorimeter. Control experiments were performed following the same method without irradiation.

The stability of the thiol derivatives in the acidic mobile phase was also studied as follows. The derivative solution prepared as described in “[Derivatization procedure](#)” was 10-fold diluted with a mobile phase of methanol–water–THF–pH 2.0 $\text{H}_3\text{Cit-NaOH}$ buffer (77:12:6:5, v/v), and 20 μL of the obtained solution was injected into the chromatographic system. The rest of this solution was kept at room temperature in daylight for 24 h, and another 20 μL was then injected into the chromatographic system.

Preparation and determination of samples

All experiments with live animals were approved by the Animal Research Committee of Wuhan University and maintained in accordance with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Male Kunming mice (18–22 g) were obtained from Wuhan University Center for Animal Experiment/A3-Lab (Wuhan, China). The mice were treated with CCl_4 to establish a model of liver damage [33], which was confirmed by tissue imaging. Three groups of mice were treated with different concentrations of CCl_4 (0.25 %, 0.5 %, 1 %; CCl_4 :plant oil v/v), and the control group was fed normally.

Mouse whole-blood samples obtained by tail incision [34] were immediately collected into tubes containing EDTA (0.02 g mL^{-1} , an anticoagulant agent) on an ice bag. 580 μL

0.02 mol L⁻¹ EDTA solution was added to 120 µL anticoagulant whole blood. After vortexing for 1 min, 300 µL 10 % Cl₃CCOOH solution was added into the well-mixed solution to precipitate proteins. After centrifugation at 4250g for 10 min, 200 µL of the supernatant was neutralized with 40 µL 1.0 mol L⁻¹ NaOH. The resulting sample solutions containing only free LMW thiols were stored at -30 °C before use.

Mouse plasma was obtained by collecting the supernatant after centrifugation of anticoagulant mouse whole blood at 3188g for 5 min. Then the plasma was reduced and proteins were removed as described in [14].

The real samples were derivatized as described in “Derivatization procedure”, but substituting 100 µL of the sample solutions obtained above for the standard thiol mixtures, and six parallel experiments were conducted for each sample.

Linearity, detection limits, and recovery study

Different concentrations of standard thiol mixtures (PA: 0.096–48 µmol L⁻¹, other thiols: 0.048–24 µmol L⁻¹; 100 µL) were derivatized and separated, as described in “Measurement of fluorescence quantum yield” and in “Stability of DMDS-PAB-I and its thiol derivatives”, respectively. The linearity regression equations were obtained by examining the linear relationship between thiol concentrations and the corresponding peak areas (seven concentrations for linear analysis). The detection limits of thiols were calculated as the amount of thiol that resulted in a peak height three times higher than that of the baseline noise (S/N=3).

For recovery determination the standard thiol mixtures were added directly to the corresponding samples, including whole blood of normal mouse and liver-injured mouse or plasma of normal mouse, in varying amounts before derivatization.

Results and discussion

Design of DMDS-PAB-I

An ideal labeling probe for HPLC-fluorescence detection should consist of an excellent fluorophore and a labeling moiety. The excellent fluorophore should have pH-independent fluorescence, good photostability, high fluorescent quantum yield, and long wavelengths; and the labeling moiety should react with the target molecules selectively under mild conditions. Because of its nearly ideal fluorescence properties, BODIPY is the best choice of fluorophore for a fluorescent labeling probe. Most BODIPY-based labeling probes in HPLC fluoresce around 500 nm [9–15], which has substantial spectral overlap with auto-fluorescence from

biological samples. BODIPY can be easily modified to red-shift its wavelengths by expanding its conjugated system, and we believe it could be useful to substitute the 3 and 5-methyl groups of 1,3,5,7-tetramethyl-BODIPY (see Fig. 1, compound a) with styryl groups. On the other hand, iodoacetamide is a standard labeling moiety for thiols because of the specific reaction between iodoacetamide and thiols under mild reaction conditions [35]. Taking into account these two points, a new long-wavelength fluorescent thiol-labeling probe, DMDS-PAB-I, was designed and synthesized.

Spectroscopic properties of DMDS-PAB-I and its derivatives

Fluorescence properties of labeling reagents and their derivatives are crucial to fluorescence-based methods. Taking GSH as the representative of thiols, the fluorescence properties of DMDS-PAB-I and its thiol derivative were investigated. The effect of different solvents on their fluorescence intensity was studied first. The effect of the concentration of methanol (MeOH), acetonitrile (ACN), THF, and acetone (CH₃COCH₃) solvents in the organic solvent–water system on the fluorescence intensity of DMDS-PAB-I and of DMDS-PAB-GSH is shown in Fig. 2. The fluorescence intensity of DMDS-PAB-I reaches a maximum and remains constant when the concentration of each organic solvent is above 50 %, because of the lipophilicity of DMDS-PAB-I. In contrast, the fluorescence intensity of DMDS-PAB-GSH plateaus at lower organic-solvent concentrations, and drops rapidly when the concentrations of the non-polar solvents are above 90 %, because of the water-solubility of thiol derivatives. On the basis of these results, the fluorescence spectra of DMDS-PAB-I and DMDS-PAB-GSH were recorded in ACN–H₂O (3:2, v/v). As shown in Fig. 3, the spectra of DMDS-PAB-I and DMDS-PAB-GSH are nearly the same, with λ_{ex} and λ_{em} of 620 and 630 nm, respectively; at these wavelengths it is widely believed to be possible to avoid interference from background fluorescence of bio-samples. The only difference between their fluorescence spectra is that the excitation and emission spectra of DMDS-PAB-GSH are slightly higher than those of DMDS-PAB-I. The fluorescence quantum yields of DMDS-PAB-I and DMDS-PAB-GSH examined in ACN–H₂O (3:2, v/v) are 0.557 and 0.560, respectively, indicating that despite red-shifting to the long-wavelength region, DMDS-PAB-I and DMDS-PAB-GSH maintain high fluorescence quantum yields.

The photostability of DMDS-PAB-I and of DMDS-PAB-GSH was investigated by testing the variation of their fluorescence intensity with irradiation by semiconductor laser (2 mW, 635 nm). Their fluorescence intensity was almost unchanged (variation of less than 3 %) compared with the same solution kept in the dark for at least 120 min, meaning DMDS-PAB-I and its thiol derivatives have excellent photostability for HPLC and CE.

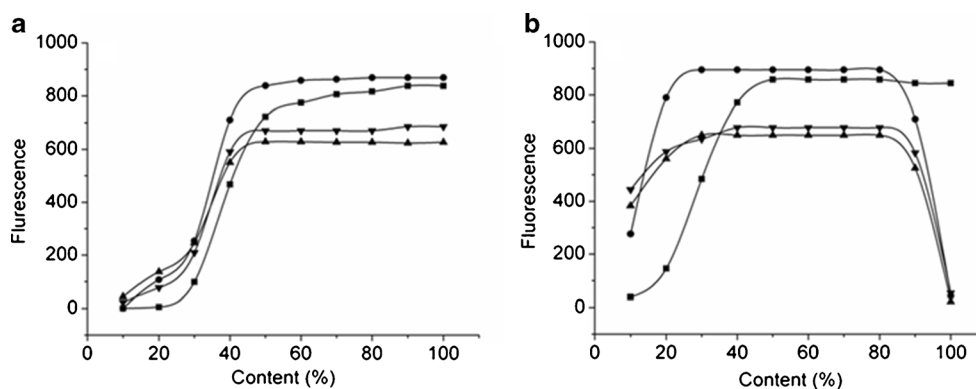


Fig. 2 Effect of different solvents and their concentration on fluorescence intensity of DMDSPAB-I (**a**) and DMDSPAB-GSH (**b**). $C_{\text{DMDSPAB-I}}=5.0 \times 10^{-7} \text{ mol L}^{-1}$, $C_{\text{DMDSPAB-GSH}}=5.0 \times 10^{-7} \text{ mol L}^{-1}$.

The pH dependence of the fluorescence of DMDSPAB-I and DMDSPAB-GSH was also investigated. Their fluorescence remained almost the same for pH values ranging from pH 2.0 to 12.0, revealing DMDSPAB-I to be suitable for use in both acidic mobile phase in HPLC and acidic or alkaline background electrolyte in CE.

Optimization of derivatization conditions

High derivatization efficiency is the premise of accuracy and sensitivity. According to previously reported data on iodoacetamide probes [36, 37], several conditions affect the reaction of DMDSPAB-I and thiols, including concentration of probe, buffer pH, buffer volume, and derivatization time and temperature.

An excess concentration of DMDSPAB-I was used to obtain a constant yield from the derivatization reaction. The relationship between the derivatization yields, assessed by the derivative peak areas, and DMDSPAB-I concentrations in the range 8×10^{-7} – $1.6 \times 10^{-6} \text{ mol L}^{-1}$ was investigated with the concentration of each thiol compound at $1 \times 10^{-7} \text{ mol L}^{-1}$

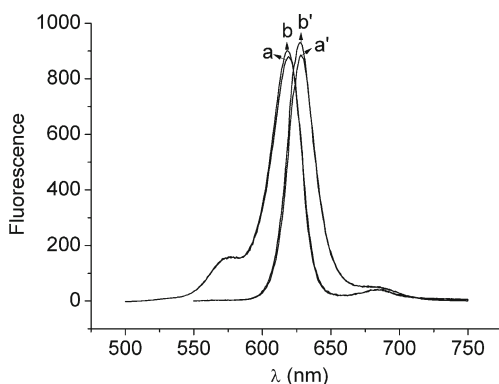


Fig. 3 Fluorescence spectra of DMDSPAB-I (*a*, *a'*) and DMDSPAB-GSH (*b*, *b'*) in ACN–H₂O (3:2, *v/v*). $C_{\text{DMDSPAB-I}}=5.0 \times 10^{-7} \text{ mol L}^{-1}$, $C_{\text{DMDSPAB-GSH}}=5.0 \times 10^{-7} \text{ mol L}^{-1}$. Slit width: 3 and 1.5 nm for excitation and emission spectra, respectively

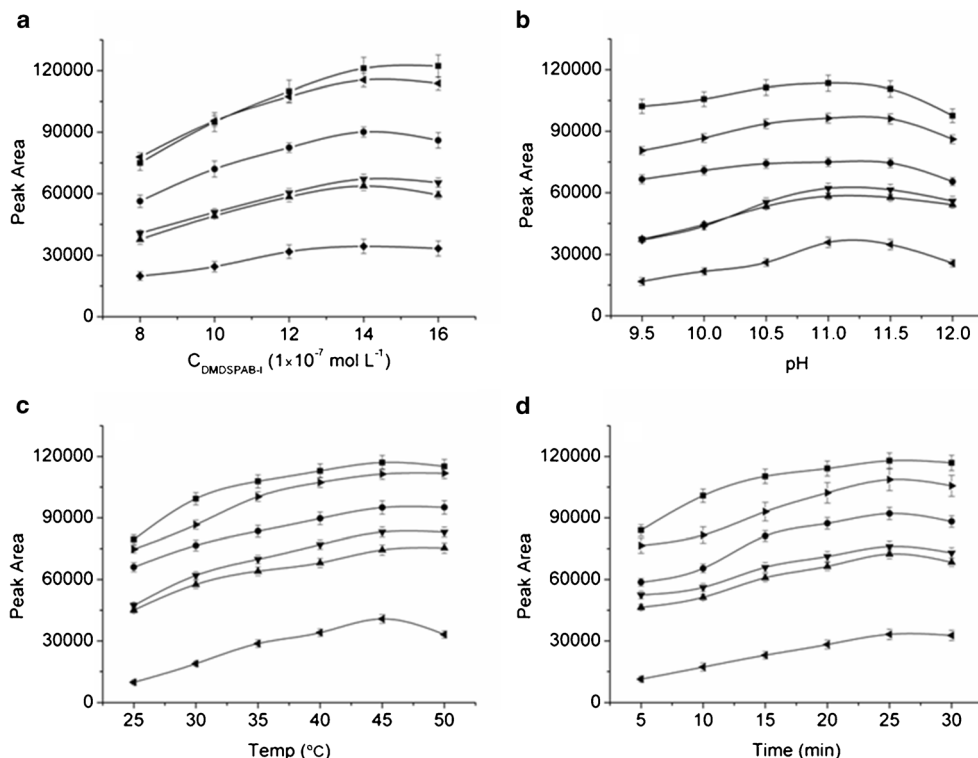
Symbol assignment: (*square*) MeOH; (*circle*) ACN; (*upward-pointing triangle*) THF; (*downward-pointing triangle*) CH₃COCH₃. Slit width: 3 and 1.5 nm for excitation and emission spectra, respectively

(Fig. 4a). The peak areas of six thiol derivatives are at a maximum when the DMDSPAB-I concentration is $1.4 \times 10^{-6} \text{ mol L}^{-1}$. When the concentration of DMDSPAB-I increases further, the peak areas of Cys, PA, and CysGly derivatives remain stable, but those of GSH, HCys, and NAC derivatives decrease. Therefore, $1.4 \times 10^{-6} \text{ mol L}^{-1}$ of DMDSPAB-I was chosen as the best derivatization concentration. In this situation, the molar ratio between DMDSPAB-I and each thiol is 14, which means that there are still $8 \times 10^{-7} \text{ mol L}^{-1}$ excess DMDSPAB-I in the resulting solution after the derivatization.

HI is a product of the derivatization reaction and could cause the reaction to become irreversible in the alkaline buffer. The effect on peak areas of pH in the range 9.5 to 12.0, using a Na₂B₄O₇–NaOH buffer, is shown in Fig. 4b. The peak areas of GSH and PA derivatives remain almost unchanged from pH 9.5 to 11.5, but reduce at pH 12.0; and the peak areas of the other thiols reach a maximum at pH 11.0 and are constant from pH 11.0 to pH 11.5. The optimum pH for derivatization was concluded to be pH 11.0. The effect of the Na₂B₄O₇–NaOH buffer volume on the derivatization was also investigated, for volumes from 20 μL to 60 μL . The peak areas of six thiols change slightly for volumes from 20 μL to 60 μL , and the maximum peak areas appear at 50 μL . Therefore 50 μL was chosen as the optimum buffer volume.

Subsequently, the effect of derivatization temperature in the range 25 $^{\circ}\text{C}$ to 50 $^{\circ}\text{C}$ on the peak areas of the derivatives was studied. As shown in Fig. 4c, the peak areas of PA and CysGly derivatives reach a maximum at the derivatization temperature of 45 $^{\circ}\text{C}$, and those of the others remain constant from 45 $^{\circ}\text{C}$ to 50 $^{\circ}\text{C}$. Hence 45 $^{\circ}\text{C}$ was selected as the optimum temperature. The effect of derivatization time in the range 5 min to 30 min on the peak areas of the derivatives was also investigated; results are shown in Fig. 4d. The peak areas of derivatives increase with increased derivatization time and reach a maximum at 25 min. The best derivatization time was thus 25 min (Fig. 5d).

Fig. 4 Effect of concentration of DMDS-PAB-I (a), buffer pH (b), derivatization temperature (c), and derivatization time (d) on peak areas. The concentration of CysGly, GSH, HCys, Cys, PA, and NAC is $1.0 \times 10^{-7} \text{ mol L}^{-1}$. Symbol assignment: (square) CysGly; (circle) GSH; (upward-pointing triangle) HCys; (downward-pointing triangle) Cys; (left-pointing triangle) PA; (right-pointing triangle) NAC



Optimization of separation conditions

Isocratic elution was used in this HPLC method because of its simplicity. To achieve baseline separation of the six mixed derivatives and DMDS-PAB-I within a short time, the following aspects of separation conditions were investigated: the content of MeOH and THF (used as organic additive), pH value, and volume of buffer in the mobile phase.

Buffer pH is critical to the separation properties of each derivative containing easily protonated groups. Using $\text{H}_3\text{Cit-NaOH}$ buffer, the relationship between the retention time of

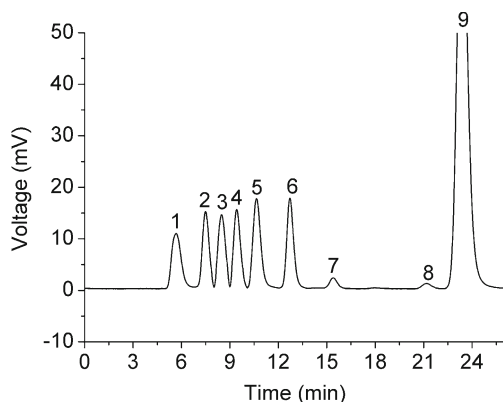


Fig. 5 Typical chromatogram of thiol derivatives. Mobile phase: methanol–water–THF–pH 2.0 $\text{H}_3\text{Cit-NaOH}$ buffer. Flow: 0.7 mL min^{-1} . Injection volume: $20 \mu\text{L}$. Standard thiol compounds concentration: $0.6 \mu\text{mol L}^{-1}$ for PA and $0.3 \mu\text{mol L}^{-1}$ for the others. Peaks: (1) CysGly; (2) GSH; (3) HCys; (4) Cys; (5) PA; (6) NAC; (7) (8) hydrolysates of DMDS-PAB-I; (9) DMDS-PAB-I

the derivatives and buffer pH from 2.0 to 7.0 was investigated. All six derivatives could be separated when buffer pH was lower than 2.4. Accordingly, a thorough investigation of the separation between pH 2.0 and pH 2.4 was performed, after which pH 2.0 was selected as the optimum buffer pH because it enabled reproducible retention time and baseline separation of all the analytes. The buffer content of the mobile phase was investigated in the range 2 % to 6 % (v/v). In this range the retention times of the six thiol derivatives change only slightly, but a significant change to the retention time of one hydrolysate of DMDS-PAB-I is observed. The peaks of NAC derivative and the hydrolysate overlap when the buffer content is larger than 5 %. Thus, 5 % (v/v) $\text{H}_3\text{Cit-NaOH}$ buffer was used to ensure sufficient buffer capacity and prevent the interference of the hydrolysate of DMDS-PAB-I.

The MeOH– H_2O system was selected as the mobile phase and the effect of methanol content on the separation was studied. Usually, the retention times of the analytes are shortened with increased methanol content. However, when the methanol content is higher than 85 % (v/v) the peaks of GSH and HCys derivatives overlap. When the methanol content is lower than 82 %, the separation time is longer than 60 min. To improve the separation efficiency and peak shape, THF was added to the mobile phase to replace some of the methanol and thus reduce the retention time of all components. The effect on retention times of THF content from 4 % to 8 % (v/v) was investigated. With increased THF content, the retention times of analytes decreased without overlap until the THF content reached 6 %. Therefore, 77 % MeOH and 6 % THF (v/v) were

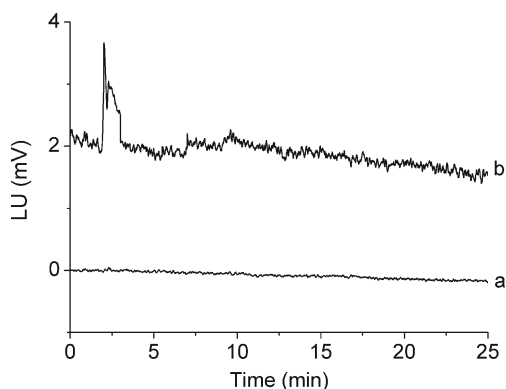


Fig. 6 Baseline of bio-samples. (a) Detected at λ_{ex} and λ_{em} of 620 and 630 nm, respectively; (b) Detected at λ_{ex} and λ_{em} of 500 and 510 nm, respectively

used as the organic solvents in the mobile phase. As shown in Fig. 2b, the fluorescence intensity of DMDS-PAB-GSH remains at a maximum when the content of MeOH is higher than 50 %. Regarding detection sensitivity and separation resolution, 77 % MeOH is suitable for the determination.

The stability of DMDS-PAB-thiol derivatives at a low pH of HPLC mobile phase was also investigated. In the mobile phase of methanol–water–THF–pH 2.0 H₃Cit–NaOH buffer (77:12:6:5, v/v), the change in the peak areas of thiol derivatives is less than 3 % within 24 h. Therefore, DMDS-PAB-thiol derivatives are stable at a low pH of HPLC mobile phase.

Finally, the optimum separation conditions were determined to be methanol–water–THF–pH 2.0 H₃Cit–NaOH buffer (77:12:6:5, v/v) with a flow of 0.7 mL min⁻¹. Figure 5 shows a typical chromatogram under the optimum separation conditions; the baseline separation was achieved within 25 min with λ_{ex} and λ_{em} of 620 nm and 630 nm, respectively.

Interference

The main interference in blood samples may come from coexistent substances, including proteins, ions, and amino acids. Proteins can be removed by pretreating samples with CCl₃COOH, so only potential interferences from ions and

amino acids were studied, using standard thiol solutions. The interference results indicate that amino acids at concentrations 10-fold higher than thiols (molar ratio, amino acid:thiol) do not interfere with the determination, and that the maximum amounts of metal ions allowable in determination of thiol compounds (molar ratio, ion:thiol) are Ca²⁺ 315; Mg²⁺ 360; Fe³⁺ 80; Al³⁺ 63; Zn²⁺ 31.5, and Cu²⁺ 0.1 in the absence of EDTA.

The excitation and emission wavelengths of DMDS-PAB-I and its derivatives are 620 and 630 nm, respectively, at which wavelengths it is widely believed to be possible to avoid interference of auto-fluorescence from bio-samples. The chromatograms of a treated whole-blood sample obtained with fluorescence detection at 620 and 630 nm and at 500 and 510 nm are given in Fig. 6. It can be seen from the chromatograms that better baseline and less-interfering peaks are obtained at higher wavelengths (620 and 630 nm). This was confirmed by a long-wavelength NO probe [38].

Method validation

Method validation encompassed linearity, limit of detection, recovery, and precision. After the systematical investigation, the linear calibration ranges, regression equations, detection limits, and RSDs of the migration time and peak areas were calculated and are listed in Table 1. The correlation coefficients for these thiols were in the range 0.9984 to 0.9997, indicating a good linearity; and the intraday and interday RSDs were below 3.3 % and 3.5 %, respectively, revealing excellent precision and repeatability.

Comparison of the proposed method based on DMDS-PAB-I with reported HPLC methods based on other fluorescent probes for thiols was made, and is presented in Table 2. Compared with non-BODIPY-based fluorescent probes, DMDS-PAB-I can achieve the lowest detection limits. With the excitation and emission wavelengths in the near-infrared region, the detection limits obtained by DMDS-PAB-I are comparable to those of TMPAB-I, a BODIPY-based fluorescent derivatizing probe emitted at $\lambda=510$ nm. These

Table 1 Linear calibration ranges, regression equations, and detection limits of DMDS-PAB-thiol derivatives

Thiol	Linear range (nmol L ⁻¹)	Regression equation (nmol L ⁻¹) ^a	R ²	Intraday RSD (%) n=6	Interday RSD (%) n=6	Detection limits (nmol L ⁻¹) ^b
CysGly	1.2–600	y=1408x – 1306	0.9984	2.2	2.4	0.26
GSH	2.3–600	y=1470x – 1522	0.9987	2.1	2.3	0.24
HCys	2.3–600	y=1539x – 2266	0.9989	3.2	3.5	0.31
Cys	2.3–600	y=1548x – 2672	0.9991	3.3	3.4	0.30
PA	2.3–1200	y=1832x – 1933	0.9991	1.2	1.4	0.72
NAC	1.2–600	y=1682x – 524	0.9993	0.9	1.0	0.26

^a x, concentration of mercapto compound (nmol L⁻¹); y, peak area of mercapto compound derivatives

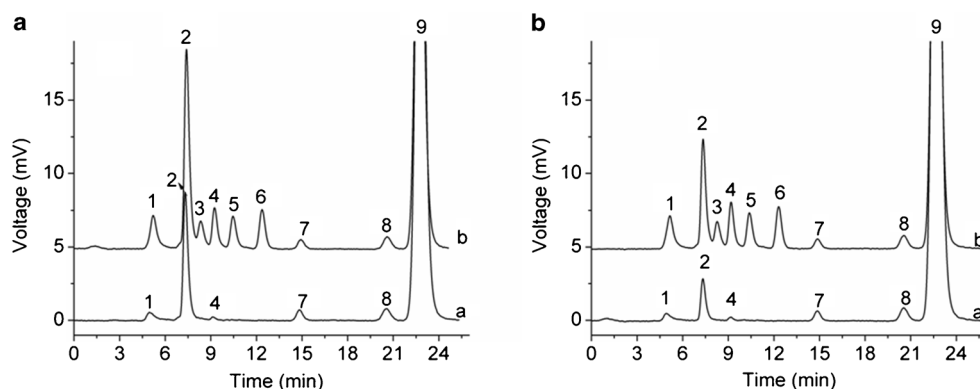
^b Signal-to-noise ratio=3

Table 2 Comparison of DMDSPAB-I with other fluorescent probes used in HPLC for thiol determination

Fluorescent derivatization probes	Analytes	Derivatization conditions	Elution time	$\lambda_{\text{ex}}/\lambda_{\text{em}}$ (nm)	Linear range ($\mu\text{mol L}^{-1}$)	LOD (nmol L^{-1})	Ref.
DBD-F	HCys, Met, Cys	pH 8.5, 80 °C, 15 min	Isocratic elution 12 min	400/570	HCys, Met 1–100; Cys 5–500	20–130	[12]
ABD-F	GSH, Cys, CysGly, HCys	pH 9.3, 35 °C, 10 min	Isocratic elution 15 min	390/510	Cys 6.3–800; CysGly, HCys, GSH 0.8–50	30–200	[42]
SBDF	HCys, Cys, CysGly, GSH	pH 9.5, 60 °C, 60 min	Isocratic elution 6 min	385/515	Cys 15–500; CysGly 5–80; HCys 2–50; GSH 0.5–7.5	10–90	[43]
MIPBO	Cys, GSH, NAC, PA, 2-ME	pH 9.0, 40 °C, 35 min	Isocratic elution 30 min	310/375	NG ^a	0.175–0.75	[44]
IPBT	Cys, GSH, NAC, PA, 2-ME	pH 8.8, 40 °C, 30 min	Isocratic elution 21 min	330/383	GSH 0.02–4.0 NAC 0.05–4.0; Cys 0.06–4.0; PA 0.05–6.0; 2-ME 0.009–0.2	2.5–10	[36]
TMPAB-I	CoA, GSH, NAC, HCys, Cys, 6-MP	pH 10.5 45 °C, 20 min	Isocratic elution 20 min	500/510	HCys 0.002–0.4; others 0.001–0.2	0.1–0.7	[14]
DMDSPAB-I	GSH, HCys, Cys, NAC, CysGly, PA	pH 11.0, 45 °C, 25 min	Isocratic elution 25 min	620/630	CysGly, NAC 0.0012–0.6; GSH, HCys, Cys 0.0023–0.6; PA 0.0012–1.2	0.24–0.72	

^a Not given

Fig. 7 Representative chromatograms for the determination of thiols in mouse blood using the proposed method. Chromatographic conditions and peaks 1–9 as in Fig. 5: (a) Blood of normal mouse (A) and with specific amount of standard thiol solutions (B); (b) Blood of liver-injured mouse 3 (A) and with specific amount of standard thiol solutions (B)



facts indicate that the emission red-shift of DMDS-PAB-I to long wavelengths is not accompanied by decreased sensitivity.

Sample analysis

To further validate the proposed DMDS-PAB-I derivatization HPLC method for thiols, it was used for analysis of whole-blood samples from normal and liver-injured mice, which were treated and analyzed as described in “Preparation of

DMDS-PAB-GSH solution”. The chromatograms of mouse whole-blood samples and of the same samples spiked with a specific amount of standard thiols mixture are shown in Fig. 7, and the analytical results for samples are listed in Table 3. GSH, Cys, and CysGly were detected in both normal and liver-injured mice, and the concentration of GSH (Fig. 7, peak 2) in blood of liver-injured mice is clearly lower than that in blood of normal mice. Because of the importance of HCys, the total concentrations of each thiol in plasma of a normal mouse

Table 3 Analytical results for blood samples of mice

Sample	CysGly	GSH	HCys	Cys	PA	NAC
Normal mouse						
Added (10^{-5} mol L $^{-1}$)	0	8.8	0	8.8	0	8.8
Found ^a (10^{-5} mol L $^{-1}$)	2.7±0.1	11.2±0.2	84.2±1.6	93.3±2.9	0	8.7±0.3
RSD (% , n=6)	2.3	1.4	1.9	3.4	–	3.2
Recovery (%)	–	96.6	–	102.3	–	98.9
Liver-injured mouse 1						
Added (10^{-5} mol L $^{-1}$)	0	12.8	0	12.8	0	12.8
Found ^a (10^{-5} mol L $^{-1}$)	1.3±0.1	14.2±0.2	73.2±2.3	86.2±3.7	0	12.2±0.4
RSD (% , n=6)	2.5	1.2	3.2	4.3	–	3.6
Recovery (%)	–	100.8	–	101.6	–	95.3
Liver injured mouse 2						
Added (10^{-5} mol L $^{-1}$)	0	18.4	0	18.4	0	18.4
Found ^a (10^{-5} mol L $^{-1}$)	0.9±0.1	18.5±0.7	69.1±2.6	88.3±3.0	0	17.9±0.8
RSD (% , n=6)	2.8	3.8	3.7	3.4	–	4.2
Recovery (%)	–	95.7	–	104.3	–	97.3
Liver-injured mouse 3						
Added (10^{-5} mol L $^{-1}$)	0	11.8	0	11.8	0	11.8
Found ^a (10^{-5} mol L $^{-1}$)	0.7±0.1	12.1±0.2	65.7±0.8	77.3±2.1	0	11.5±0.4
RSD (% , n=6)	1.7	1.8	1.2	3.5	–	3.6
Recovery (%)	–	96.6	–	98.3	–	97.5
Normal mouse plasma						
Added (10^{-5} mol L $^{-1}$)	0	4.8	0	4.8	0	4.8
Found ^a (10^{-5} mol L $^{-1}$)	0.3±0.1	5.1±0.1	3.5±0.1	8.4±0.4	1.2±0.1	6.1±0.2
RSD (% , n=6)	4.3	2.2	2.7	3.6	4.4	2.8
Recovery (%)	–	97.6	–	102.9	–	102.8

^a SD values lower than 0.1 are all listed as 0.1

were also determined, and HCys was detected at $12 \mu\text{mol L}^{-1}$. All thiol levels of the samples are in good agreement with those of literature [39–41]. Recoveries from 95.3 to 104.3 % were obtained for the detected thiols, with satisfactory analytical precision ($\text{RSD} \leq 4.8 \%$). [42–44]

Conclusions

The first long-wavelength emission BODIPY-based fluorescent probe for HPLC-fluorescence detection of thiols, DMDS-PAB-I, was designed and synthesized, and an HPLC method based on DMDS-PAB-I for the determination of six LMW thiol compounds was developed. The proposed method was validated, with good specificity, linearity, accuracy, precision, and recovery, in real samples. Compared with other derivatizing probes for HPLC-fluorescence detection of thiols, DMDS-PAB-I has unique advantages including smoother and cleaner baseline with less-interfering peaks, longer excitation and emission wavelengths of above 600 nm, and higher sensitivity, which is preferable for analysis of complex biological samples.

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