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Highly sensitive determination of nitric oxide in biologic samples by a near-infrared BODIPY-based fluorescent probe coupled with high-performance liquid chromatography

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ABSTRACT

Nitric oxide (NO) acts as an important regulator and mediator in numerous processes of biological systems. In this work, the analytical potential of a novel near-infrared (NIR, >600 nm) BODIPY-based fluorescent probe for NO, 8-(3,4-diaminophenyl)-4,4-difluoro-4-bora-3a,4a-diaza-di(1,2-dihydro) naphtho[b, g]s-indacene (DANPBO-H) has been evaluated in high performance liquid chromatography (HPLC). In 25 mM pH 6.50 borate buffer, DANPBO-H reacted with NO to give the corresponding triazole, DANPBO-H-T, at 35 °C for 20 min. DANPBO-H-T was eluted using a mobile phase of methanol/tetrahydrofuran/50 mM pH 7.00 H_3Cit–NaOH buffer (81:7:12, v/v/v) in 4 min on a C_8 column and detected with fluorescence detection at excitation and emission wavelengths of 621 and 631 nm, respectively. The limit of detection (LOD) (signal-to-noise$=3$) reached to 5.50×10^{-10} M. Excellent selectivity was observed against other reactive oxygen/nitrogen species. Various representative biological matrixes including the whole blood and organs of mice, the pangen and radical of rice, human vascular endothelial (ECV-304) cells and mouse macrophage (RAW 264.7) cells were used to verify the feasibility and resistance to interfering effects from complex biological sample matrixes of the developed DANPBO-H-based HPLC method. Compared to the existing derivatization-based HPLC methods for NO, the proposed method eliminates interfering effects from complex biological sample matrixes efficiently owing to the fluorescence detection in the NIR region, and is more advantageous and robust for the sensitive and selective determination of NO in complex biological samples.

1. Introduction

Nitric oxide (NO) is an important regulator and mediator in numerous processes of biological systems. In mammals, NO plays a significant role in the normal physiological events such as cardiovascular system [1–3], neurotransmission and host immune system defense to pathological states [4–7], including ischemia [8], inflammation [9], cancer and neurodegeneration [10,11]. In plants, NO is a key endogenous signaling molecule involved in different physiological processes, including disease resistance response, immune response [12], seed germination, growth and development processes [13], senescence and programmed cell death [14], and stress resistance [15,16]. Since the function of NO in biological system closely depends on its concentration [17–19], it is of great importance to develop efficient and sensitive methods for the determination of NO in complex biological matrixes.

The determination of NO is challenged by NO's unique chemical and physical properties, including high reactivity, rapid diffusion, and short half-life [20]. Many analytical methods have been developed and well documented in the literatures [20,21], such as absorbance-, fluorescence-, and chemilunescence-based approaches, electron paramagnetic resonance (EPR), electrochemistry, etc. Among these methods, high performance liquid chromatographic combined with fluorescence detection (HPLC-FD) has high sensitivity, excellent selectivity, and good repeatability over other methods [20]. Some ultraviolet–visible fluorescent probes have been used to trap NO in biological samples followed by HPLC-FD, these probes including 2,3-diaminonaphthalene (2,3-DAN) [22,23], 4,5-diaminofluoresceins (DAF-2) [24], 1,3,5,7-tetramethyl-8-(3',4'-diaminophenyl) difluoroboradiaza-s-indacene (DAMBO) [25], and 1,3,5,7-tetramethyl-2,6-dicarbethoxy-8-(3',4'-diaminophenyl)-difluoroboradiaza-s-indacence (TMDCDABODIPY) [26]. In comparison with these ultraviolet–visible fluorescent probes, near-infrared (NIR, >600 nm) fluorescent probes combined with HPLC-FD are characterized by much lower background interference [27]. The absorption and autofluorescence of biological molecules, such as nicotinamide adenine dinucleotide (NADH),

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flavins, oxy- and deoxyhemoglobin, fat and melanin, are not observed in NIR region [28,29]. Moreover, the scattered light from the excitation source will be greatly reduced in NIR region, since the scattering intensity is proportional to the inverse of fourth power of the wavelength [30]. Owing to the advantages of NIR fluorescent probes, a few NIR fluorescent probes, *o*-phenylenediamine cyanine probes (DAC-P and DAC-S) [31], bis(2,2'-bipyridine) (4-(3,4-diami-nophenoxy)-2,2' bipyridine) ruthenium(II) hexafluorophosphate ($[Ru(bpy)_2(dabpy)][PF_6]_2$) [32,33] and {4'-[4-(3,4-diaminophenoxy)phenyl]-2,2':6',2"-terpyridine-6,6"-diyl} bis (methylenenitrilo) tetrakis(acetate)-Eu^{3+} (DATTA-Eu^{3+}) [32,33], have been developed for NO imaging in tissues and cells. However, these NIR fluorescent probes are suffered from common limitations like rapid photo-bleaching and low sensitivity, thus impeding their applications in HPLC.

8-(3,4-Diaminophenyl)-4,4-difluoro-4-bora-3a,4a-diaza-di(1,2-dihydro) naphtho[b, g]s-indacene (DANPBO-H) is a new NIR fluorescent probe for NO developed in our group (Fig. 1). As a turn-on fluorescent probe, DANPBO-H displays some desirable analytical properties, such as NIR excitation/emission (621/631 nm), good photostability, rapid and linear response to NO with a 400-fold enhancement of fluorescence. Here, the feasibility of DANPBO-H in HPLC for the determination of NO has been evaluated. The separation and derivatization conditions were optimized in detail. The stability and resistance to the interference from complex biological matrixes of DANPBO-H and its NO derivative, DANPBO-H-T, have been investigated. In contrast to the serious background interference detected at excitation and emission wavelengths of DAMBO (496/505 nm), there is almost no background interference from the biological matrixes under the excitation and emission wavelengths of DANPBO-H-T, which are 621 nm and 631 nm, respectively. Furthermore, satisfactory analytical parameters were obtained, indicating that the developed method can serve as a simple and reliable analysis method for the detection of NO in complex biological samples. To the best of our knowledge, DANPBO-H is the first NIR fluorescent probe used in HPLC for the determination of NO.

2. Experimental

2.1. Reagents and chemicals

Dimethyl sulfoxide (DMSO), acetonitrile (ACN), methanol (MeOH), tetrahydrofuran (THF), citric acid (H_3Cit), sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), sodium hypochlorite (NaClO), sulfuric acid (H_2SO_4), and so on, were of analytical grade, and were obtained from Sinopharm Chemical Reagents (Shanghai, China). Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco (USA). L-Glutamine was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was taken from Tianhang Biological Technology (Zhejiang, China). Penicillin, streptomycin and trypsin were obtained from Amresco (USA). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA).

Phosphate-buffer saline (PBS) solution consisted of 8.00 g/L NaCl, 0.20 g/L KCl, 0.20 g/L KH_2PO_4, 2.78 g/L $Na_2HPO_4 \cdot 12H_2O$ (pH 7.40), and pH values were adjusted with 1.0 M HCl and 1.0 M NaOH. H_3Cit–NaOH buffer was prepared by mixing 0.10 M H_3Cit solution with 0.10 M NaOH solution and adjusted to the required pH values. The preparations of H_3PO_4–Na_3PO_4 buffer and $Na_2B_4O_7$–H_3BO_3 buffer were similar to that of H_3Cit–NaOH buffer.

NO-saturated solution was prepared according to a previous method, and the concentration of NO-saturated solution is 1.8 mM at 25 °C [34]. DANPBO-H [35] and DAMBO [36] were synthesized in our lab. 1×10^{-3} M DANPBO-H stock solution was prepared by dissolving DANPBO-H with DMSO. The DAMBO stock solution was prepared similar to that of DANPBO-H.

2.2. Instruments

HPLC analyses were conducted using a LC-20A HPLC system (Shimadzu, Tokyo, Japan) with RF-10AXL fluorescence detector (Shimadzu), LC-20AD dual-pump (Shimadzu), manual injection (20 μL), and LabSolutions/LCsolution Lite chromatography chemstation (Shimadzu) in the experiments. The separation was performed on a C_8 column (5 μm, 150 mm × 4.6 mm id, Kromasil, Bohus, Sweden). Fluorescence excitation and emission spectra were recorded at a RF-5301PC spectrofluorometer (Shimadzu, Tokyo, Japan). The pH values of solutions were measured by a DELTA 320 pH meter (Mettler-Toledo, Shanghai, China). The samples were centrifuged by a TGL-16G centrifuge (Anke, Shanghai, China). The cells were kept in a Forma Series II 3111 Water-Jacketed CO_2 Incubators (Thermo Scientific, USA).

2.3. Biological samples

The human vascular endothelial (ECV-304) cells and mouse macrophage (RAW 264.7) cells were obtained from the China Center for Type Culture Collection (Wuhan, China). ECV-304 cells were firstly kept in the RPMI 1640 medium, in which there were 12% fetal bovine serum, 2% glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL) [37]. Then, the cells were transferred into a humidified incubator at 37 °C with 5% CO_2. The RAW 264.7 cells were grown in DMEM supplemented with 10% fetal bovine

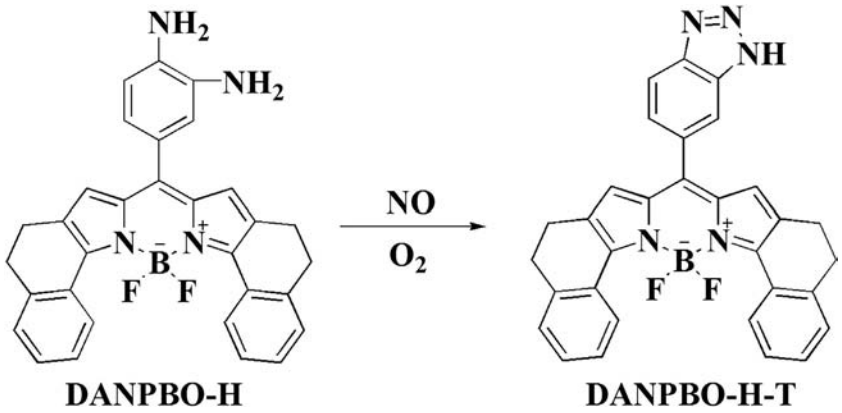

Fig. 1. Reaction of DANPBO-H with NO.

serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator containing 5% CO_2 [38].

Male Kunming mice (18–22 g) were obtained from Wuhan University Center for Animal Experiment/A3-Lab (Wuhan, China). All experiments with live animals in this report were performed according to the principles of laboratory animal care and Chinese national law.

Rice seeds were sterilized with 5% (v/v) NaClO solution for 15 min, rinsed for 10 times with water. Then, they were put between moisture filter papers in Petridishes at 25 °C for 3 days for germination [39]. The germs with 2-cm-long radicles grew under controlled conditions in greenhouse at photo-flux density of 240 μmol $m^{-2}s^{-1}$ (12/12 h day/night period), and at relative humidity of 55–60% and temperature of 25 ± 2 °C after they had been put into the modified Hoagland solution, which consisted of 1 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 0.5 mM KCl, 0.5 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1 μM $MnSO_4$, 5 μM $ZnSO_4$, 0.1 μM $(NH_4)_6 \cdot MO_7O_{24}$, 10 μM H_3BO_4, 0.1 μM $AlCl_3$ and 20 μM Fe-EDTA [39].

2.4. Chromatographic method

HPLC separation of the NO derivative was carried out on a reverse-phase C_8 column. Before analysis, the C_8 column was pre-equilibrated with the mobile phase for 30 min. Sample solution (20 μL) was then injected to the chromatograph system. The flow rate was 0.7 mL/min and the detection wavelengths were set at $\lambda_{ex}/\lambda_{em}$=621/631 nm. The column temperature was 25 °C. All measurements were repeated for at least three times. The pH values of mobile phase were referred to the pH values of buffer solutions before mixing with organic modifier. The mobile phase was composed of methanol/tetrahydrofuran/50 mM pH 7.00 H_3Cit–NaOH buffer (81:7:12, v/v/v). Prior to use, the mobile phases were filtered through a G-4 fritted glass funnel and degassed in an ultrasonic bath for about 5 min. All the solvents were filtered with a 0.45 μm membrane filter before use.

2.5. Derivatization reaction of DANPBO-H with NO

2 mL of 0.1 M pH 6.50 $Na_2B_4O_7$–H_3BO_3 buffer, 40 μL solution of 1×10^{-3} M DANPBO-H in DMSO and an appropriate amount of NO solution were added to a 10-mL vial. The whole solution was diluted to 10 mL with ACN and then kept at 35 °C for 20 min. The reaction mixture was diluted two-fold with the mobile phase and injected into the chromatographic system.

2.6. Reactions of DANPBO-H with different interferents

Solid KO_2 was used to provide superoxide (O_2^-). Hydroxyl radical ($HO^\bullet$) was generated by the Fenton reaction of H_2O_2 and Fe^{2+} [40]. Peroxynitrite ($ONOO^-$) was synthesized by infusing acidified hydrogen peroxide with sodium nitrite [41]. 100-Fold excess of $\cdot OH$, NO_2^- and 1000-fold excess of NO_3^-, $ONOO^-$, $HO^\bullet$, H_2O_2, ClO^-, O_2^- and ascorbic acid (AA) were added individually to 10-mL vials containing 2 mL of 0.1 M pH 6.50 $Na_2B_4O_7$–H_3BO_3 buffer, 40 μL solution of 1×10^{-3} M DANPBO-H in DMSO. The obtained solutions were then diluted to 10 mL with ACN and kept at 35 °C for 20 min. The reaction mixture was diluted two-fold with the mobile phase and injected into the chromatographic system.

2.7. Biological sample preparation

2.7.1. Standard-spiked groups

40 μL DANPBO-H or DAMBO solution (1×10^{-3} M in DMSO) was added to a certain amount of biological samples, including the organs (kidney, heart, spleen, liver, and lung) and blood of mice, the pangen and radical of rice. Then, different amounts of NO PBS saturated solution were added to the above solutions. The mixture was homogenized and adjusted to 10 mL with the ACN/borate buffer (80/20, v/v), incubated at 35 °C for 20 min and then centrifuged at 16000 rpm for 15 min. The solutions containing 5×10^6 ECV-304 cells or 5×10^6 RAW 264.7 cells were centrifuged at 3000 rpm for 10 min. After the removal of the supernatant, 10 mL ACN/borate (80/20, v/v) mixed solution containing 4.0×10^{-6} M DANPBO-H and 2.0×10^{-7} M NO, was added. The mixtures were incubated at 35 °C for 20 min, ultrasonicated for 5 min and then centrifuged for 15 min at 16000 rpm. The supernatants were collected and diluted two-fold with mobile phase for the subsequent chromatographic analysis.

2.7.2. Sample groups

The pretreatment procedures of the samples were the same as those of the standard-spiked groups except without the addition of NO standard solution.

2.7.3. Blank groups

The pretreatment procedures of the samples were the same as those of the standard-spiked groups except without the addition of NO solution and the fluorescent probes (DANPBO-H or DAMBO).

3. Results and discussion

3.1. Chromatographic separation

The conditions used for the chromatographic separation of DANPBO-H and DANPBO-H-T were examined. DANPBO-H and DANPBO-H-T can be rapidly separated in a simple way with the full use of their different chemical properties. Since DANPBO-H and DANPBO-H-T are hydrophobic, C_8 column and high organic composition in the mobile phase are recommended. In order to match with the high level of organic modifier, H_3Cit–NaOH buffer was chosen to control the pH value of the mobile phase, which can avoid salt precipitation in high organic composition. When the pH values of H_3Cit–NaOH buffer are lower than 3.00, DANPBO-H starts to fluoresce because of the protonation of the amino groups [31] and a relatively high peak appears in the chromatogram, which affects the separation of DANPBO-H and DANPBO-H-T. Therefore, the pH values from 3.50 to 7.50, at which the fluorescence intensity of DANPBO-H is negligible, were selected to investigate the effect of pH in the mobile phase on the peak areas of DANPBO-H-T while the organic composition and salt concentration were kept at 80% and 50 mM, respectively. The peak areas of DANPBO-H-T increase with the increase of pH values, and reach to the highest at the pH value of 7.00. Therefore, pH 7.00 H_3Cit–NaOH buffer was selected for further experiments. The methanol content, organic modifier and salt concentration of the mobile phase for the separation were also optimized. Finally, the optimal separation conditions are determined to be methanol/tetrahydrofuran/50 mM pH 7.00 H_3Cit–NaOH buffer (81:7:12, v/v/v).

Under the optimized chromatographic conditions, the chromatograms of DANPBO-H and DANPBO-H-T are presented in Fig. 2. The peak of DANPBO-H disappears because the separation and derivatization are both carried out in near neutral solutions, thus effectively inhibiting the fluorescence of DANPBO-H. Good peak shape of DANPBO-H-T and clean baseline are both obtained.

3.2. Optimization of derivatization conditions

For a pre-column derivatization HPLC-FD method, the derivatization efficiency is very significant for the detection sensitivity. In order to achieve the best derivatization efficiency, derivatization conditions including the kinds, pH values, and salt concentrations

of buffer solution, reaction temperature and time, ACN amount, and DANPBO-H dosage, were investigated in detail.

In acidic condition, the *o*-phenylenediamine group can react with nitrite in samples [42], leading to the interference to the determination of NO. For this reason, the pH values from 6.00 to 9.00 of three different buffers, PBS, H_3PO_4–Na_3PO_4 and $Na_2B_4O_7$–H_3BO_3 were selected and the effects of these pH values on the derivatization yield which is expressed as the peak areas of derivative were investigated. As shown in Fig. 3A, at pH 6.50 all the three buffers obtained the maximum peak areas and $Na_2B_4O_7$–H_3BO_3 buffer has the highest derivatization yield. Therefore, pH 6.50 $Na_2B_4O_7$–H_3BO_3 buffer was chosen for the subsequent derivatization optimization. The concentration of $Na_2B_4O_7$–H_3BO_3 buffer from 10 to 35 mM was also examined and it is found that at 25 mM the highest derivatization yield was achieved (Fig. 3B).

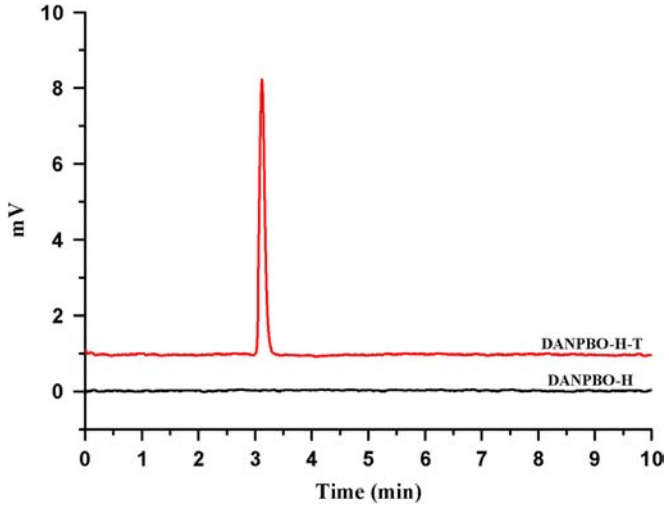

Fig. 2. Chromatograms of DANPBO-H and DANPBO-H-T. Mobile phase: methanol/tetrahydrofuran/50 mM pH 7.00 H_3Cit–NaOH buffer (81:7:12, v/v/v). Fluorescence detection: $\lambda_{ex}/\lambda_{em}$=621/631 nm. Flow rate: 0.7 mL/min. Injection volume: 20 μL. Standard DANPBO-H-T concentration: 1.0×10^{-7} M.

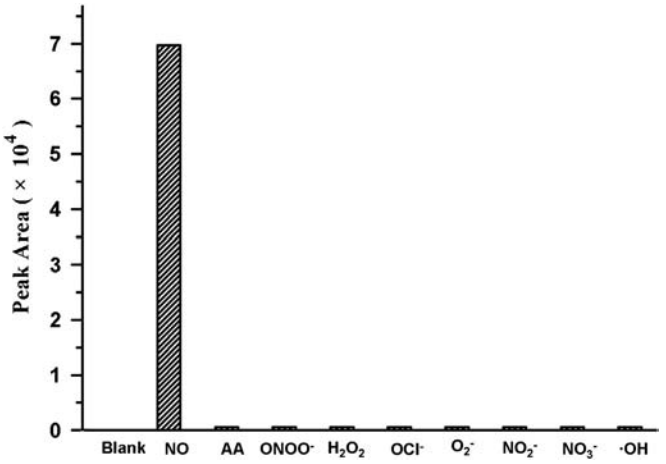

Fig. 4. Peak areas of DANPBO-H after the reaction with NO and interferents. 100 equiv. for ·OH, NO_2- and 1000 equiv. for other interferents. Detection conditions are the same as in Fig. 2.

Table 1
Comparison of the proposed method with the other HPLC methods using fluorescent probes for NO derivatization.

Fluorescent probe	Detection wavelengths (nm)	Detection limit (M)	Reference (s)
DAN	375/415	2.1×10^{-8}	[23]
DAF-2	486/521	5×10^{-9}	[24]
DAMBO	496/505	2.00×10^{-11}	[25]
TMDCDABODIPY	500/510	9.00×10^{-11}	[26]
DANPBO-H	621/631	5.50×10^{-10}	This paper

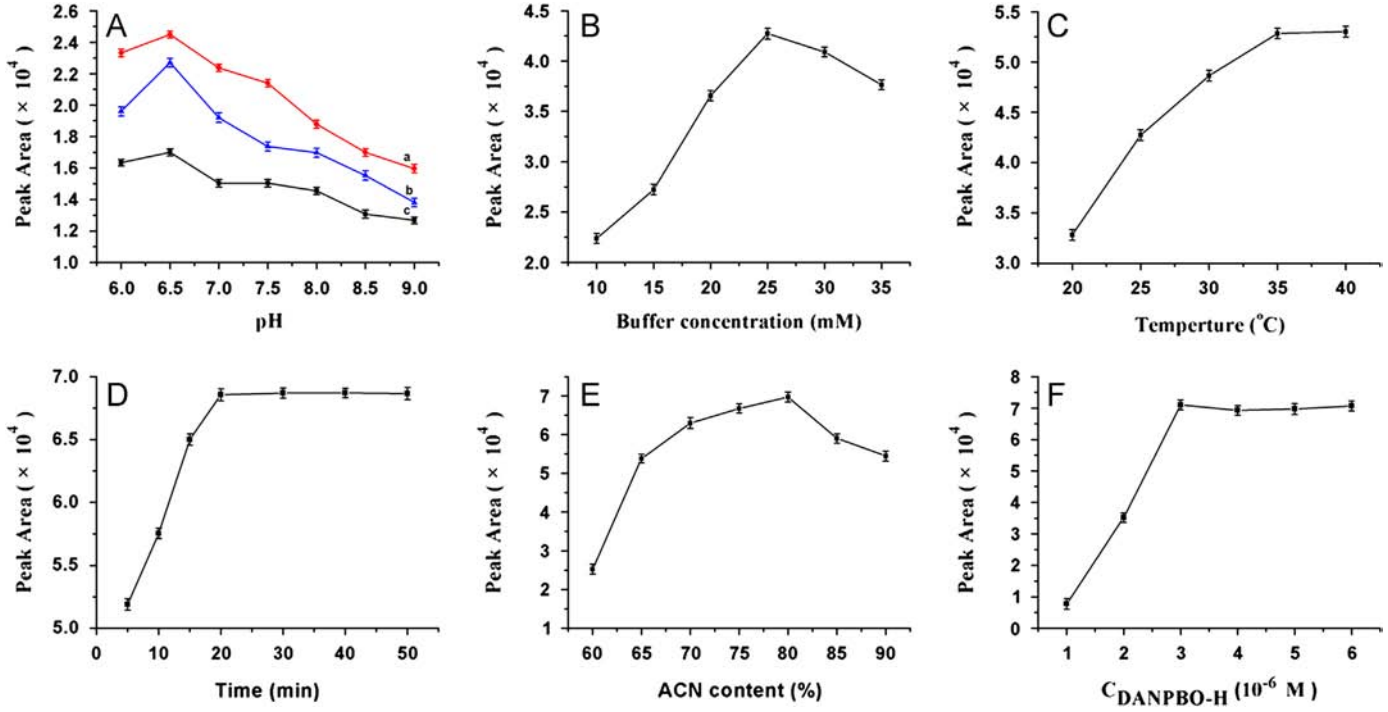

Fig. 3. Effect of the derivatization conditions on the peak areas of DANPBO-H-T. (A) buffer pH, (a) $Na_2B_4O_7$–H_3BO_3 buffer; (b) H_3PO_4–Na_3PO_4 buffer; (c) PBS buffer; (B) $Na_2B_4O_7$–H_3BO_3 buffer concentration; (C) reaction temperature; (D) reaction time at 35 °C; (E) ACN content; and (F) DANPBO-H concentration. Detection conditions are the same as in Fig. 2.

To a great extent, the derivatization yield is also influenced by the reaction temperature and time. Since NO will escape from the reaction system at high temperature, 20–40 °C was chosen as the derivatization temperature. The effect of derivatization temperature and time (from 5 to 50 min) on the peak area of DANPBO-H-T are shown in Fig. 3C and D, from which it is found that the highest derivatization yield can be achieved when the temperature is 35 °C and the reaction time is 20 min.

Due to the hydrophobicity of DANPBO-H and DANPBO-H-T, an organic solvent should be added to the derivatization media. The effect of ACN content on the derivatization reaction has been investigated. As shown in Fig. 3E, the derivatization efficiency rapidly increases as the ACN content increases from 60% to 80%. However, as the ACN content continues to increase from 80% to 90%, the derivatization efficiency obviously decreases, which maybe results from the low solubility of borate salt under high ACN content condition and thus the pH value in reaction media is probably changed. Considering the above analysis, 80% of ACN (v/v) is determined to be the optimized content.

The DANPBO-H dosage is a crucial factor to the derivatization. The effect of DANPBO-H dosage from 1.0×10^{-6}–6.0×10^{-6} M on the derivatization efficiency was assessed while keeping the NO concentration at 2.0×10^{-7} M unchanged. Fig. 3F indicates that the derivatization efficiency reaches to its plateau when DANPBO-H concentration is higher than that of 3.0×10^{-6} M. Therefore, 4.0×10^{-6} M DANPBO-H was selected for our experiments.

In summary, the optimized derivatization reaction of DANPBO-H with NO should be performed at 35 °C for 20 min using ACN/25 mM pH 6.50 H_3BO_3–$Na_2B_4O_7$ buffer (8/2, v/v) which contained 4.0×10^{-6} M DANPBO-H.

3.3. Stability of DANPBO-H and DANPBO-H-T

The stability of DANPBO-H and its corresponding triazole derivative is very important for its application. As observed, after DMSO solution of DANPBO-H is stored at 4 °C for 1 week or at room temperature for 3 days, the derivatization yield of NO with DANPBO-H has no obvious change, which is measured by the peak area of DANPBO-H-T. Once formed, DANPBO-H-T is stable for at least 2 weeks at 4 °C and 1 week at room temperature, with the variations of DANPBO-H-T peak areas within 3% ($n=6$). The results

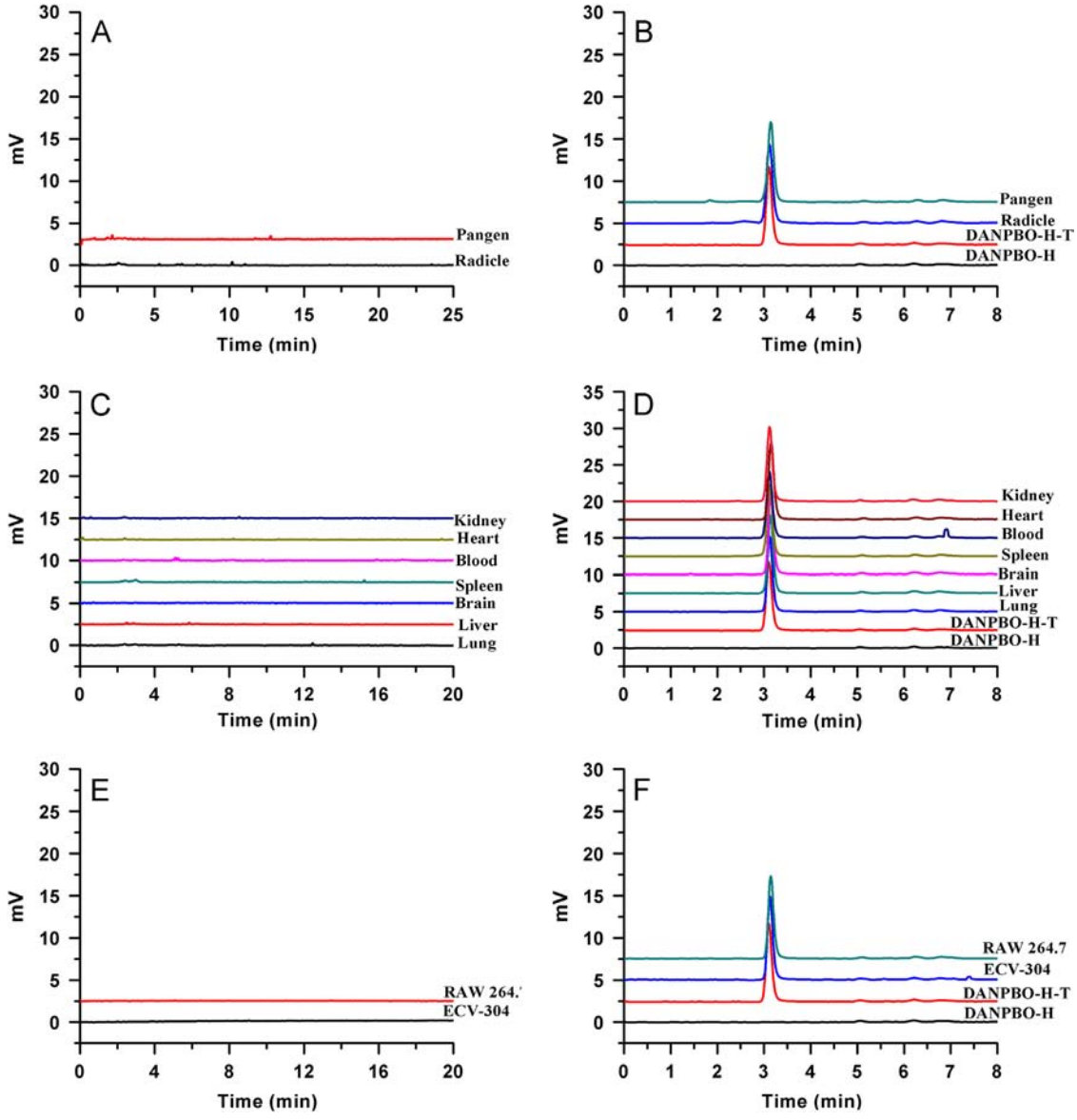

Fig. 5. Chromatograms obtained at the detection wavelengths of $\lambda_{ex}/\lambda_{em}=621/631$ nm. (A, C, E) blank groups; (B, D, F) standard-spiked groups with the addition of DANPBO-H and NO. The other detection conditions are the same as in Fig. 2. Chromatograms were shifted along the vertical axis with a space of 2.5 mV for visual separation.

reveal that DANPBO-H and DANPBO-H-T are stable enough to allow quantitative analysis of NO.

3.4. Interference from reactive oxygen or nitrogen species

The interference from reactive oxygen and nitrogen species including · OH, ClO^-, H_2O_2, O_2^- and $ONOO^-$, ascorbic acid (AA), and oxidized forms of NO (NO_2^-, NO_3^-) to the reaction selectivity of DANPBO-H for NO has been investigated. As shown in Fig. 4, the fluorescence intensity of DANPBO-H has no obvious change when these interferents are added with the amounts tested, which demonstrates that DANPBO-H has good selectivity for NO.

3.5. Analytical calibration

Test solutions containing NO at different concentrations from 6.0×10^{-9} to 2.00×10^{-7} M were prepared and analyzed with the use of the optimized derivatization procedure and separation conditions. The calibration curve is plotted by peak areas of DANPBO-H-T versus NO concentrations and exhibits good linear relationship with satisfactory squared regression coefficients (R^2=0.9998). The linear regression equation is Y=704.209X +46.995, in which Y is the peak area of DANPBO-H-T and X (nM) is the concentration of NO. The limit of detection (LOD) is 5.5×10^{-10} M, which was calculated through 3 times of the baseline noise. Precision was assessed by repeating six sequential runs within-day and between-day, which is expressed as relative standard deviation (RSD). The intra-day and inter-day RSDs are 3.1% and 4.8% (n=6), respectively, indicating the good reproducibility of the proposed method. The comparison of the proposed method with other HPLC methods using fluorescent probes for NO derivatization is given in Table 1, which shows that the LOD of NO using DANPBO-H, i.e. 5.5×10^{-10} M, is lower than those obtained using DAN and DAF-2 by HPLC-FD, i.e. 2.1×10^{-8} and 5.0×10^{-9} M, respectively.

3.6. Investigation of interfering effects from biological sample matrixes

It is very significant to decrease interfering effects from biological sample matrixes for the purpose of improving detection sensitivity and selectivity of target molecules in complex biological

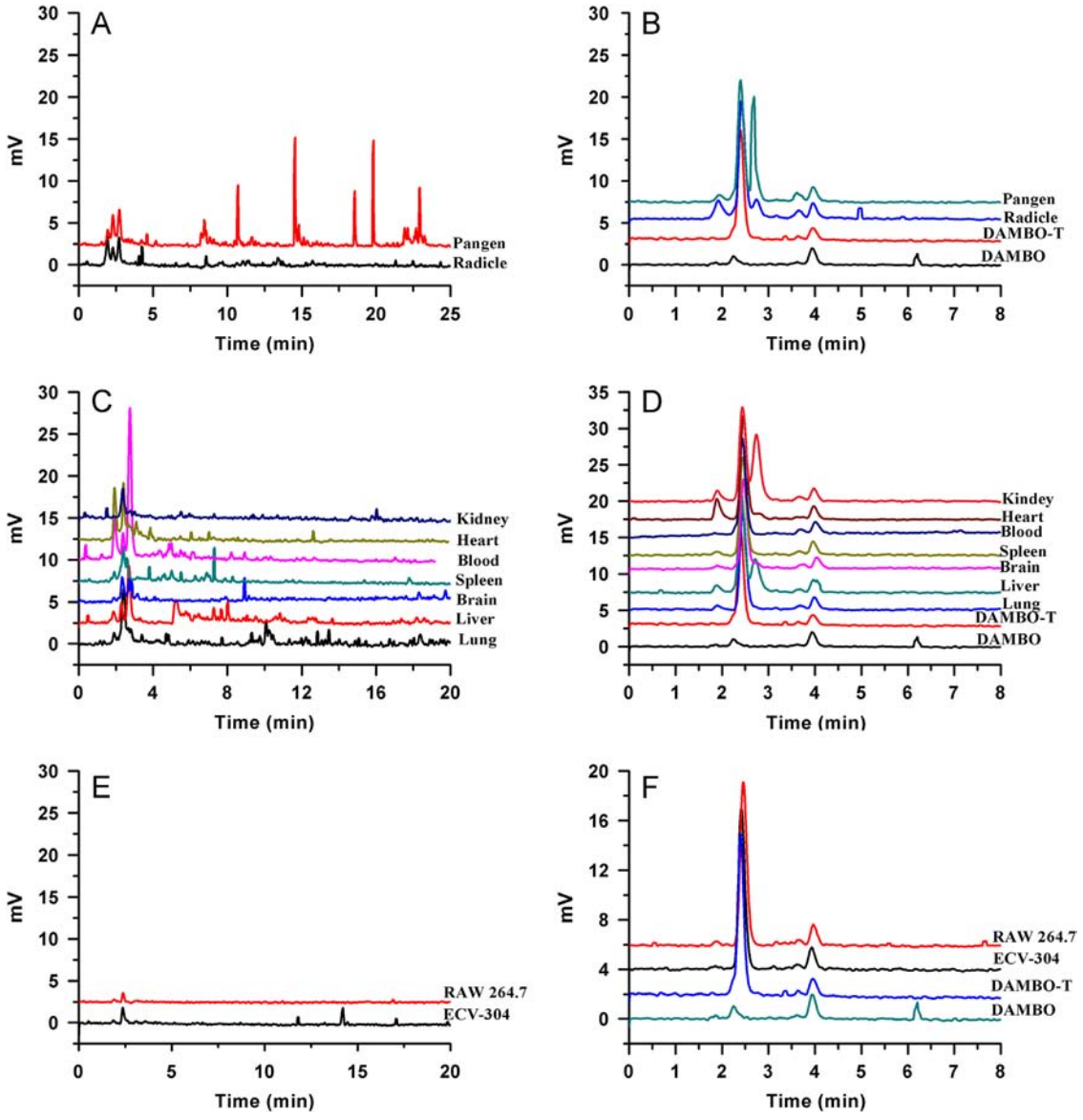

Fig. 6. Chromatograms obtained at the detection wavelengths of $\lambda_{ex}/\lambda_{em}$=496/505 nm. (A, C, E) blank groups; (B, D, F) standard-spiked groups with the addition of DAMBO and NO. The other detection conditions are the same as in Fig. 2. Chromatograms were shifted along the vertical axis with a space of 2.5 mV for visual separation.

samples. Under the optimized conditions, interfering effects from biological sample matrixes have been investigated. The samples include tissues, blood and cells, which are the typical samples in biological and medical analysis. The results are given in Fig. 5. Firstly, the blank biological sample groups have been examined. As shown in Fig. 5A, C and E, the baseline is smooth and clean on all chromatograms of the target samples in 20–25 min, which indicates that there are no interference peaks from these biological sample matrixes in blank groups. Accordingly, no other interference peaks appear besides DANPBO-H-T peak on the chromatograms of the standard-spiked groups for which the derivatization procedure has been carried out with the addition of DANPBO-H and NO, as shown in Fig. 5B, D and F.

As a comparison, using DAMBO for NO derivatization and 496/505 nm for fluorescence detection, the same experimental procedure has been performed and the results are shown in Fig. 6. Serious interfering effects are observed through the chromatograms from rice pangen and radical sample matrixes in blank groups (Fig. 6 A). Some obvious interference peaks from the blank biological sample matrixes of ECV-304 cells, RAW 264.7 cells and the organs and blood of mice are found, with the retention time in the range of 2.0–3.5 min (Fig. 6C and E). These interferential peaks affect the separation and detection sensitivity in HPLC-FD for biological samples based on DAMBO derivatization for NO (as shown in Fig. 6 B, D and F), as well as DAF-2 derivatization for NO owing to the similar detection wavelengths in visible region (Table 1).

Table 2
Analysis results of NO in biological samples.

Samples	Added	Found	RSDs (%, $n=6$)	Recoveries (%)
Rice radical (10^{-10} mol/g)	0	6.484	1.7	-
	2.898	9.530	1.3	105
	7.246	14.310	2.1	108
Rice pangen (10^{-10} mol/g)	0	6.405	1.1	-
	2.390	8.818	1.6	101
	5.975	12.739	2.3	106
Kidney (10^{-10} mol/g)	0	0	–	–
	3.211	3.243	0.8	101
	16.056	16.859	0.9	105
Heart (10^{-10} mol/g)	0	0	–	–
	8.929	8.750	1.1	98
	44.643	45.089	1.2	101
Spleen (10^{-10} mol/g)	0	0	–	–
	11.261	11.599	1.7	103
	56.306	55.743	1.5	99
Brain (10^{-10} mol/g)	0	0	–	–
	2.171	2.128	1.4	98
	10.855	11.072	1.6	102
Liver (10^{-10} mol/g)	0	0	–	–
	1.381	1.436	1.2	104
	6.906	6.975	1.3	101
Lung (10^{-10} mol/g)	0	0	–	-
	6.817	7.022	0.9	103
	34.083	35.787	1.1	105
Blood (10^{-8} M)	0	0	–	–
	1.000	0.981	1.2	98
	5.000	5.104	1.5	102
ECV-304 cells (10^{-10} mol)	0	0	–	–
	1.002	0.956	1.4	95
	5.010	5.261	1.3	105
RAW 264.7 cells (10^{-10} mol)	0	0	–	–
	1.002	1.029	1.7	103
	5.010	4.910	1.4	98

These investigations reveal that the interfering effects from biological sample matrixes have been efficiently removed with the proposed HPLC-FD method based on DANPBO-H derivatization. The use of NIR fluorescent probe DANPBO-H can avoid the interfering effects from biological sample matrixes and achieve easy separation and high sensitivity for NO detection in complex biological samples.

3.7. Sample analysis

The proposed method has been validated by the determination of NO in various biological samples. Samples were treated and analyzed as described in the "Experimental" section. The spiked recoveries were determined by comparing the calculated amounts of NO in the samples (using calibration curve) with the spiked amounts. The analytical results are summarized in Table 2. The spiked recoveries range from 95 to 108% with RSDs below 2.3%, demonstrating the excellent accuracy and precision of the proposed method.

In plants, NO is a key endogenous signaling molecule and often involved in many different physiological processes [43]. Correspondingly, NO has been detected in rice samples. However, NO has not been found in the normal organs and blood of mice, and unstimulated ECV-304 cells and RAW 264.7 cells, for the reason that only endothelial nitric oxide synthase is active under normal conditions and NO exists at very low level in mammals [44]. Inducible nitric oxide synthase is activated under pathological conditions, such as cancer, inflammation and angiocardiopathy, thus producing sustained amounts of NO [44,45].

4. Concluding remarks

DANPBO-H is the NIR fluorescent probe first used for the sensitive and selective determination of NO in HPLC. Compared to the previous fluorescent probes for NO in HPLC, DANPBO-H exhibits excellent resistance to the interfering effects from complex biological sample matrixes, which results in a flat and clean baseline in sample analysis favorable for the separation and detection. The developed method has been verified by the analysis of NO in real biological samples from plants and mammals and presents powerful potential in the simple and rapid detection of NO in different complex biological samples.

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