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1,3,5,7-Tetramethyl-8-aminozide-difluoroboradiaza-*s*-indacene as a new fluorescent labeling reagent for the determination of aliphatic aldehydes in serum with high performance liquid chromatography

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ABSTRACT

A BODIPY-based fluorescent derivatization reagent with a hydrazine moiety, 1,3,5,7-tetramethyl-8-aminozide-difluoroboradiaza-*s*-indacene (BODIPY-aminozide), has been designed for aldehyde labeling. An increased fluorescence quantum yield was observed from 0.38 to 0.94 in acetonitrile when it reacted with aldehydes. Twelve aliphatic aldehydes from formaldehyde to lauraldehyde were used to evaluate the analytical potential of this reagent by high performance liquid chromatography (HPLC) on C_{18} column with fluorescence detection. The derivatization reaction of BODIPY-aminozide with aldehydes proceeded at 60 °C for 30 min to form stable corresponding BODIPY hydrazone derivatives in the presence of phosphoric acid as a catalyst. The maximum excitation (495 nm) and emission (505 nm) wavelengths were almost the same for all the aldehyde derivatives. A baseline separation of all the 12 aliphatic aldehydes (except formaldehyde and acetaldehyde) is achieved in 20 min with acetonitrile–tetrahydrofuran (THF)–water as mobile phase. The detection limits were obtained in the range from 0.43 to 0.69 nM (signal-to-noise = 3), which are better than or comparable with those obtained by the existing methods based on aldehyde labeling. This reagent has been applied to the precolumn derivatization followed with HPLC determination of trace aliphatic aldehydes in human serum samples without complex pretreatment or enrichment method.

1. Introduction

Aldehydes, among a wide variety of chemical species, are identified in various matrices such as atmosphere, water, soils, foods, beverages, and biological fluids. They are acknowledged to be harmful organic pollutants in the environment and also have corrosive, irritant, and carcinogenic effects on biological tissues [1–3]. Nowadays, an urgent need for clinical analysis of aldehydes in biological fluid samples is required [4] because aldehydes are major products of lipid peroxidation [5] and strongly associated with various diseases [6–8], and some aldehydes formed from lipids by oxidative damage have been used as biomarkers [9–11] in order to investigate their roles in the diseases. Therefore, the development of sensitive and selective analytical methods for monitoring aldehyde contents in biological samples targeted in health-related studies is of great interest.

However, due to the lack of intrinsic chromophores or fluorophores as well as their volatility and activity, it is very difficult to detect aldehydes directly in complex matrices. To overcome this problem, chemical derivatization of aldehydes is preferred prior to analysis and various derivatization schemes for the determination of aldehydes have been reviewed [12–20]. As fluorescence derivatization has been found to be one of the most sensitive methods for the determination of analytes at low concentrations, considerable efforts have been directed to the development of new fluorescent labeling reagents. However, by far, only a few fluorescent labeling reagents for aldehydes have been reported including fluoren-9-yl-methoxycarbonylhydrazine (Fmoc-hydrazine) [15], 5-hydrazine-N,N-dimethylnaphthalene-l-sulfonamide (Dns-hydrazine) [16], 6,7-dimethoxy-l-methyl-2-oxo-1,2-dihydroquinoxalin-3-yl-propionohydrazide (DMEQ-hydrazide) [17], 4-(1-methyl-2-phenanthro (9,10-d) imidazol-2-yl)-benzohydrazide (MPIB-hydrazide) [18], 4-(2-carbazoylpyrrolidin-l-yl)-7-(N,N-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-ProCZ) [19] and 1,2-benzo-3,4-dihydrocarbazole-9-ethoxy-carbonylhydrazine (BCEC) [20]. Although some of them are sensitive enough for the determination of aldehydes or ketones in air, water, exhaled breath condensate, wine and so on, some shortcomings have also been found in their applications, such as short detection wavelengths, relatively low sensitivity, poor stability and serious interference for the determination of real biological samples. Therefore, new

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labeling reagents that offer high sensitivity, high selectivity and good fluorescence properties are still desired.

In terms of fluorescent labeling-based analytical methods, the sensitivity and detection wavelength of the proposed method are mostly dependent on the fluorescence properties of the fluorophore of the labeling reagent. Difluoro-boraindacene dyes (better known as BODIPY) is a well-known fluorophore characterized by valuable properties such as a high stability toward chemicals and light, high fluorescence quantum yields (typically 0.6–1.0), large molar absorption coefficients (60,000–80,000 L mol^{-1} cm^{-1}) and tunable absorbing and emitting wavelength [21–25]. Several BODIPY-based fluorescent reagents have been developed in our lab for the labeling of NO [26], amines [27] and thiols [28]. Our previous works have demonstrated that BODIPY-based fluorescent reagents are superior as fluorescent labeling reagents.

In this paper, a highly sensitive BODIPY hydrazine reagent, 1,3,5,7-tetramethyl-8-aminozide-difluoroboradiaza-*s*-indacene (BODIPY-aminozide) has been developed from commercial available precursors for the labeling of carbonyl compounds and some valuable spectral properties of the new reagent have been tested. Twelve aliphatic aldehydes from formaldehyde to lauraldehyde were used as models to evaluate the performance of the newly synthesized reagent by HPLC on C_{18} column. The detail derivatization and separation conditions were optimized and the linearities, detection limits, and precision of the proposed method were also determined.

2. Experimental

2.1. Apparatus

Experiments were performed using an LC-6A HPLC system (Shimadzu, Tokyo, Japan) with RF-530 fluorescence detector (Shimadzu, Tokyo, Japan) and 2010 chromatography chemstation (Zhejiang University, Hangzhou, China). Samples were injected manually using a 20 μL sample loop. The separation was performed on a C_{18} column (5 μm, 250 mm × 4.6 mm i.d., Kromasil, Bohus, Sweden). Fluorescence spectra were recorded on a RF-5301PC spectrofluorometer (Shimadzu, Tokyo, Japan) and UV/Vis spectra were recorded on a Lambda 10 UV/Vis spectrometer (Perkin-Elmer) with a 1 cm × 1 cm quartz cell.

2.2. Chemicals and reagents

Aldehyde standards were purchased from Sigma (St. Louis, MO, USA). BODIPY-aminozide (Fig. 1) was synthesized in our lab. Acetonitrile and tetrahydrofuran (THF) of HPLC grade were purchased from Shanghai Chemicals Company (Shanghai, China). Water used for preparing solutions was purified by a Milli-Q ultrapure system (Millipore, Bedford, MA, USA). Unless otherwise specified, all reagents used were of analytical grade.

The BODIPY-aminozide stock solution was prepared by dissolving 3.5 mg BODIPY-aminozide in 10 mL acetonitrile to give a concentration of 1.0×10^{-3} M. The stock solutions of the aldehydes (1.0×10^{-2} M) were prepared by dissolving appropriate aldehydes in acetonitrile, and if necessary, THF was added until the compound dissolved. Dilution of these stock solutions to appropriate concentrations with acetonitrile was performed immediately before use. The phosphoric acid solution (0.1 M, catalyst) for derivatization reaction was prepared by diluting phosphoric acid with double distilled water. When not in use, all standards were stored at 4 °C in a refrigerator.

2.3. Synthesis of BODIPY-aminozide

BODIPY-aminozide was synthesized by the following method (Fig. 1) in two steps.

1,3,5,7-Tetramethyl-8-butyric acid-difluoroboradiaza-*s*-indacene (**1**) was synthesized according to Ref. [29]. ^{1}H NMR ($CDCl_3$, 300 MHz): 6.06 (s, 2H), 3.05–3.00 (m, 2H), 2.57–2.50 (m, 2H), 2.51 (s, 6H), 2.43 (s, 6H), 2.00–1.97 (m, 2H).

To a solution of 0.6 g (1.8 mmol) of **1** and 0.24 g (2.0 mmol) of N-hydroxysuccinimide in 30 mL of anhydrous THF was added 0.43 g (2.2 mmol) of dicyclohexylcarbodiimide under nitrogen. After stirring at room temperature for 5 h, the mixture was filtered to eliminate the precipitated dicyclohexylurea. Hydrazine hydrate (1.0 mL, 85%) was added to the resulting filtrate and the mixture was stirred at room temperature for 10 min. Then, the solvent was evaporated. The residue was purified by silica gel column chromatography (CH_2Cl_2/methanol) to give 0.55 g (87.3%) of 1,3,5,7-tetramethyl-8-aminozide-difluoroboradiaza-*s*-indacene (**2**) as an orange solid. m.p. 186.4–187.3 °C. ^{1}H NMR ($CDCl_3$, 300 MHz): 6.06 (s, 2H), 3.69 (s, 2H), 3.02–2.96 (m, 2H), 2.52–2.48 (m, 2H), 2.51 (s, 6H), 2.42 (s, 6H), 1.98–1.92 (m, 2H). MS *m*/*z*: 347.19 $(M+H)^+$.

2.4. Measurement of the fluorescence quantum yield

Measurement of the fluorescence quantum yields was carried out according to Ref. [30]. The fluorescence emission spectra of the sample solution (the reagent and its derivatives) and the standard solution (fluorescein in 0.1 M NaOH solution, $\Phi = 0.92$) were recorded at an excitation wavelength of 495 nm. The fluorescence quantum yield was calculated with the expression: $\Phi u = \Phi s \times (Du \times As)/(Ds \times Au)$, where Φu and Φs are the fluorescence quantum yield of samples and the standard solution, respectively; Du and Ds are the areas under the emission curves of the samples and the standard, respectively; Au and As are the absorbance of the samples and the standard, respectively. For fluorescence efficiency measurements, the concentrations of the solutions were adjusted so that the absorbance was less than 0.05, and to minimize error arising from inner filter effects.

2.5. Derivatization procedure and analysis

The derivatization of BODIPY-aminozide with aldehydes proceeded in acetonitrile solution in the presence of phosphoric acid catalyst. A 10 μL of mixed aldehydes (1.0×10^{-4} M each) was added into a vial (1.0 mL), and then successively added 10 μL of 0.1 M phosphoric acid and 100 μL of BODIPY-aminozide solution (1.0×10^{-3} M). The mixture was diluted to the mark with water–acetonitrile (2:8, v/v) and kept at 60 °C for 30 min in a thermostatic water-bath. An aliquot (20 μL) of the reaction mixture was injected directly into the chromatographic system. The reagent blanks without aldehydes were also treated in the same way. The derivatization reaction is shown in Fig. 2.

2.6. Chromatographic separation

The HPLC separations of BODIPY-aminozide and aldehyde derivatives were performed on a Kromasil C_{18} column with a binary gradient. Eluent A was 30 mM formic acid/ammonia buffer (pH 7.50) and eluent B was THF–acetonitrile (2:8, v/v). The flow rate was set at 1.0 mL/min and the column temperature was kept at 20 °C. The fluorescence emission wavelength was set at 505 nm (excitation at 495 nm). Before the analysis, the C18 column was pre-equilibrated for 30 min with the mobile phase composition was 50% A and 50% B. The gradient elution conditions for the separation are shown in Table 1.

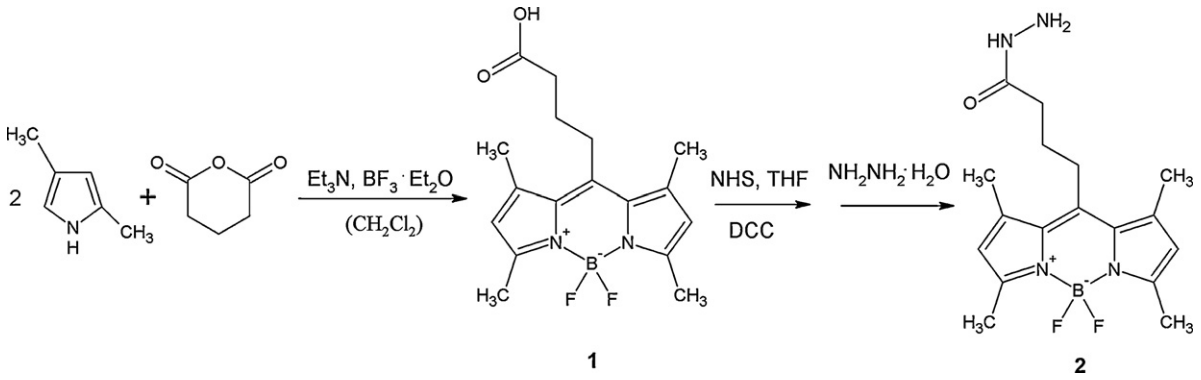

Fig. 1. The synthesis scheme of BODIPY-aminozide.

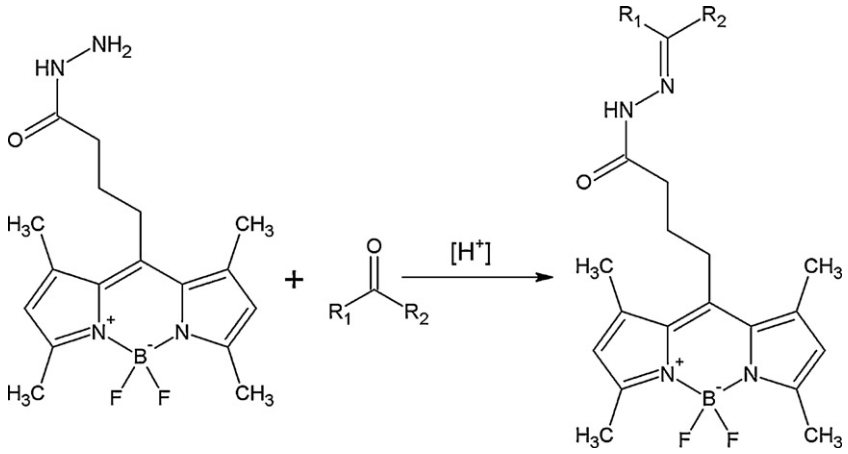

Fig. 2. The derivatization reaction of BODIPY-aminozide with aldehydes.

2.7. Sample preparation

Blood samples were collected from the Hospital of Wuhan University (Wuhan, China). The preparation for serum was performed according to Ref. [31]. In brief, the blood samples were centrifuged at 4500 rpm (4 °C) for 15 min and the supernatant was further deproteinized by mixing it with acetonitrile at the volume ratio of 1:4 (200 μL of serum was placed in a vial filled with 800 μL acetonitrile). And then, the resultant was centrifuged at 4500 rpm (4 °C) for 15 min. The supernatant was collected for the derivatization directly. The serum samples were stored at −20 °C when they were not used.

3. Results and discussion

3.1. Design strategy of BODIPY-aminozide

BODIPY fluorophore has spectral characteristics that are often superior to those of fluorescein, tetramethylrhodamine, Texas Red and some other longer-wavelength dyes [32]. As a result, BODIPY-based fluorescent derivatization reagents have attracted increasing attention in recent years [21]. For carbonyl labeling, an asymmetric BODIPY hydrazine reagent (Fig. 3), 4,4-difluoro-5,7-dimethyl-bora-3a,4a-diaza-*s*-indacene-3-propionohydrazide (BODIPY FL hydrazine), has been developed by Invitrogen Company (USA). This reagent showed good sensitivity for the determination of progesterone by HPLC [33]. It also has good reactivity with heparan sulfate disaccharides [34]. However, the reactive group (–RCONHNH$_2$) of this reagent is introduced to 3-position of BODIPY core in relatively low yields which involves asymmetric synthesis using two different pyrroles, one of which is obtained from multiple synthetic steps [35].

It has been noted by us that the *meso* position of BODIPY dyes has often been used as a connection onto which various functionalities can be easily introduced [36] and a higher fluorescence quantum yields has been observed when a alkyl at the BODIPY *meso* position instead of an aryl. For example, 1,3,5,7,8-pentamethyl-substituted

Table 1
Chromatographic gradient condition.

Time (min)	A[a] (%)	B[b] (%)
0–3	50	50
3–5	30	70
5–7	20	80
7–8	10	90
8–30	0	100

[a] 30 mM formic acid/ammonia buffer (pH 7.50).
[b] Tetrahydrofuran–acetonitrile (2:8, v/v).

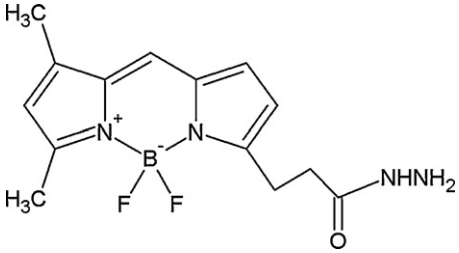

Fig. 3. BODIPY FL hydrazine synthesized by Invitrogen Company (USA).

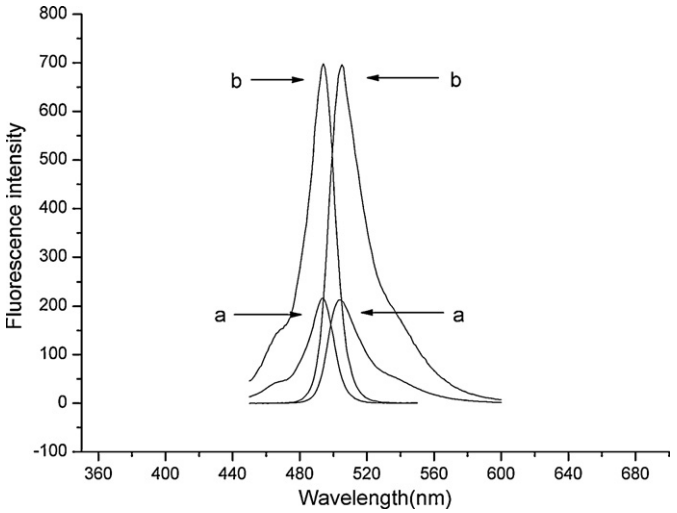

Fig. 4. Fluorescence spectra of BODIPY-aminozide (A) and its formaldehyde hydrozone (B). Slit widths: 1.5 and 1.5 nm for excitation and emission spectra. Concentrations of BODIPY-aminozide and formaldehyde hydrozone were 1.0 μM each.

BODIPY (PM 546) has a high fluorescence quantum yields up to 95% in menthol [21], while the BODIPY dyes introduced a phenyl on 8-position exhibit considerably lower emission yields (0.5–0.6) [37]. Besides, the introduction of the reactive group to the *meso* position of BODIPY dyes can avoid asymmetric synthesis.

Based on the above considerations, a new symmetric BODIPY hydrazine reagent 1,3,5,7-tetramethyl-8-aminozide-difluoroboradiaza-*s*-indacene (BODIPY-aminozide) (BODIPY-aminozide) has been developed in our lab. Compared to commercial BODIPY FL hydrazine, the reactive group (–RCONHNH$_2$) in BODIPY-aminozide is introduced on *meso* position of a symmetric BODIPY core instead of on 3-position of an asymmetric BODIPY core. This reagent can be easily synthesized from common commercially available precursors in only two steps which give the total yield up to 20.8% whereas BODIPY FL hydrazine is synthesized from two different pyrroles, one of which is obtained from multiple synthetic steps [35].

3.2. Fluorescence and ultraviolet absorption of BODIPY-aminozide and its derivatives

The derivative of formaldehyde with BODIPY-aminozide was used as the model to investigate the fluorescence characteristics and absorption of the aldehyde derivatives, which was prepared according to the method described in Section 2.5 by adding a great

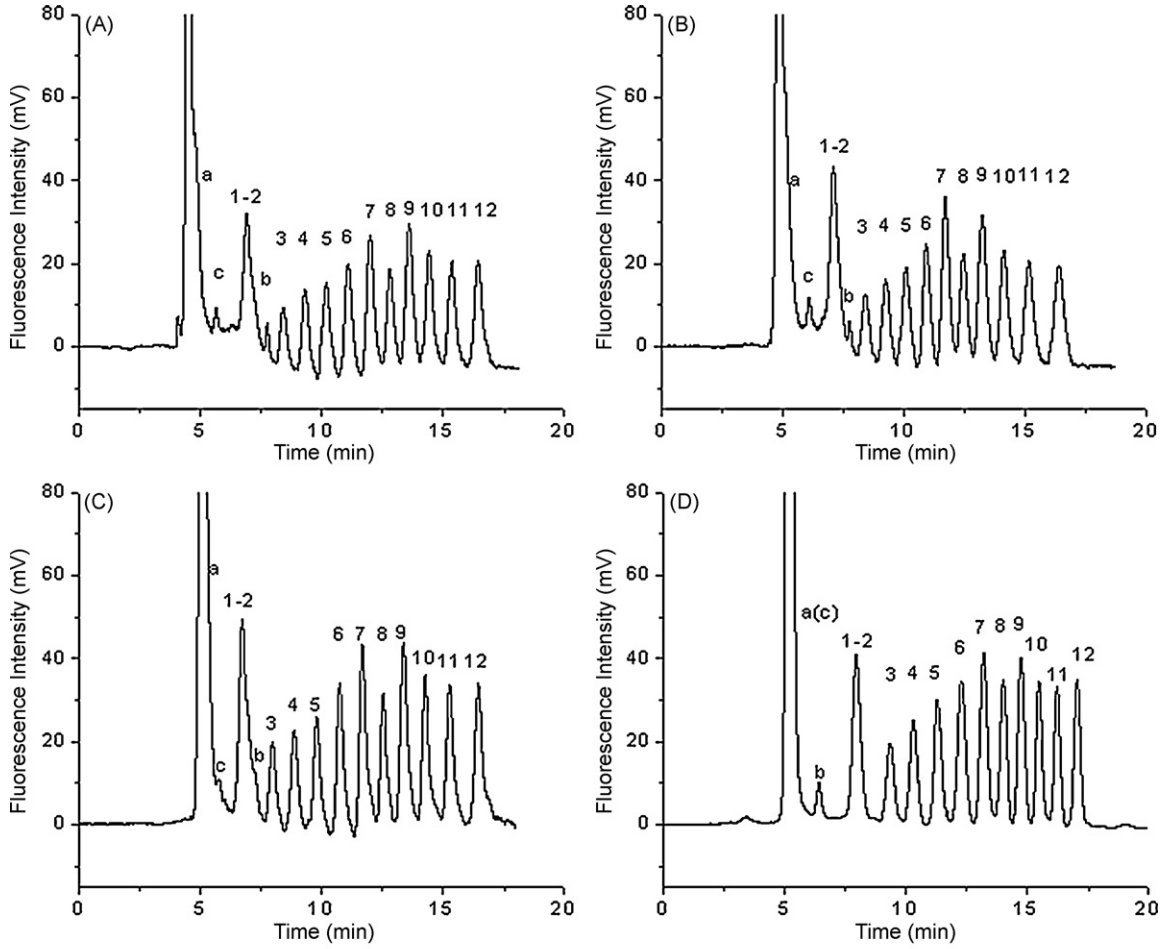

Fig. 5. Effect of pH value on the separation. Mobile phase: A was 30 mM formic acid/ammonia buffer; B was tetrahydrofuran (THF)–acetonitrile (2:8, v/v). Flow rate: 1.0 mL/min. Gradient: 0–3 min, 50% B; 3–5 min, 70% B; 5–7 min, 80% B; 7–8 min, 90% B; 8–30 min, 100% B. pH values of buffer: (A) pH 4.50; (B) pH 5.50; (C) pH 6.50; (D) pH 7.50. Peaks: (1 and 2) formaldehyde and acetaldehyde, (3) propanal, (4) butanal, (5) pentanal, (6) hexanal, (7) heptanal, (8) octanal, (9) nonaldehyde, (10) decanal, (11) undecanal, (12) lauraldehyde, (a) reagent, (b) and (c) unknown.

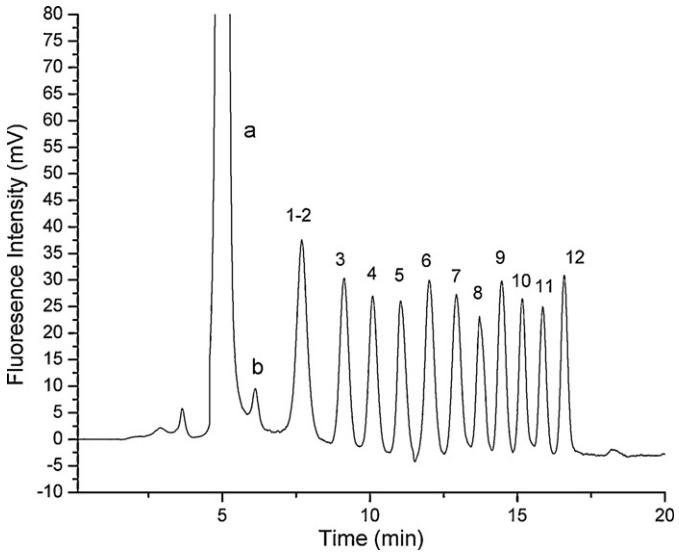

Fig. 6. Typical chromatogram of BODIPY-aminozide and its aldehyde derivatives. Mobile phase: A was 30 mM formic acid/ammonia buffer (pH 7.50); B was tetrahydrofuran (THF)–acetonitrile (2:8, v/v). Flow rate: 1.0 mL/min. Gradient: 0–3 min, 50% B; 3–5 min, 70% B; 5–7 min, 80% B; 7–8 min, 90% B; 8–30 min, 100% B. Standard aldehydes concentration: 1.00 μM. Peaks: (1 and 2) formaldehyde and acetaldehyde, (3) propanal, (4) butanal, (5) pentanal, (6) hexanal, (7) heptanal, (8) octanal, (9) nonaldehyde, (10) decanal, (11) undecanal, (12) lauraldehyde, (a) reagent, (b) unknown.

excess of formaldehyde to ensure that the reagent was completely consumed, which was checked by HPLC.

The fluorescence spectra of BODIPY-aminozide and its formaldehyde derivative both exhibited the excitation maximum at 495 nm and emission maximum at 505 nm in acetonitrile (Fig. 4). The excitation and emission wavelengths in acetonitrile–water solution have no obvious difference compared to those in methanol–water solution. The fluorescence intensity in acetonitrile is 12.5% stronger than that in methanol.

The fluorescence quantum yield of formaldehyde derivative is 0.94, while that of the reagent is only 0.38. For BODIPY-aminozide, a intramolecular photoinduced electron transfer (PeT) from hydrazine group to the BODIPY core, which inhibits the fluorescence of BODIPY core; after the reaction with aldehyde, the formation of Schiff base interrupt the PeT, and a higher fluorescence quantum yield is observed. Similar phenomena has also been reported and well explained in Refs. [38,39]. This property (from a low fluorescence quantum yield to a higher fluorescence quantum yield after labeling) is favorable for the following determination because when the excessive labeling regent is used in the derivatization, a relative low fluorescent peak would be obtained from the unconverted BODIPY-aminozide compared to those reagents do not have this property.

UV–vis spectra of the reagent and its formaldehyde derivative were investigated in the scanning range of 400–600 nm. Maximum absorptions of both BODIPY-aminozide and its formaldehyde derivative appear at the wavelength of 495 nm. The molar absorption coefficients (ε) of BODIPY-aminozide and its formaldehyde derivative are 8.33×10^4 and 1.07×10^5 L mol^{-1} cm^{-1} (495 nm), respectively.

3.3. Separation of BODIPY-aminozide and its derivatives

A reversed-phase mode is usually used in HPLC for the separation of aldehyde derivatives for convenience of handling biological samples [40]. The separation of twelve aldehyde derivatives was tested on C_{18} column and the flow rate was set at 1.0 mL/min at room temperature.

Methanol–water, acetonitrile–water and acetonitrile–THF–water were initially tested as the mobile phase for the separation of twelve aliphatic aldehyde derivatives. All the aldehyde derivatives except formaldehyde and acetaldehyde can be easily separated by all above elution systems. Considering a relatively shorter time and a higher sensitivity, acetonitrile–THF–water system was used as the mobile phase.

A mixture of formic acid/ammonia buffer was chosen to control pH value, which can avoid salt precipitation when organic eluent at higher concentration was used. It was found that pH value of buffer in mobile phase had a little influence on the retention times of all aldehyde derivatives in the range of pH from 2.50 to 8.50. However, it could be seen from Fig. 5 that pH values lower than 6.50 resulted in obvious partly overlap for the reagent and formaldehyde (acetaldehyde) derivative because of the tailing of the reagent. Also, at low pH values, the interference from the unknown peaks which might be produced by the degradation of the reagent (Fig. 5, peaks b and c) was another problem. Therefore, pH 7.50 was used to obtain better chromatogram. Therefore, pH 7.50 was used to obtain better chromatogram.

Isocratic elution was tried but more than 3 h was needed to elute all the derivatives. To shorten separation time, gradient elution was employed and the procedure was given in Table 1. Under the optimum separation conditions, the reagent and its formaldehyde/acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonaldehyde, decanal, undecanal and lauraldehyde derivatives are baseline separated in less than 20 min (Fig. 6). Unfortunately, the derivatives of formaldehyde and acetaldehyde

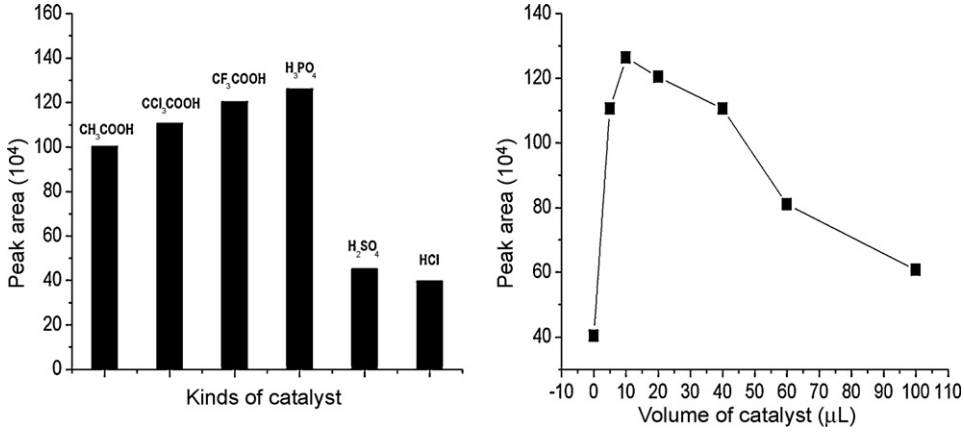

Fig. 7. Effect of catalyst on the peak area of aldehyde derivatives.

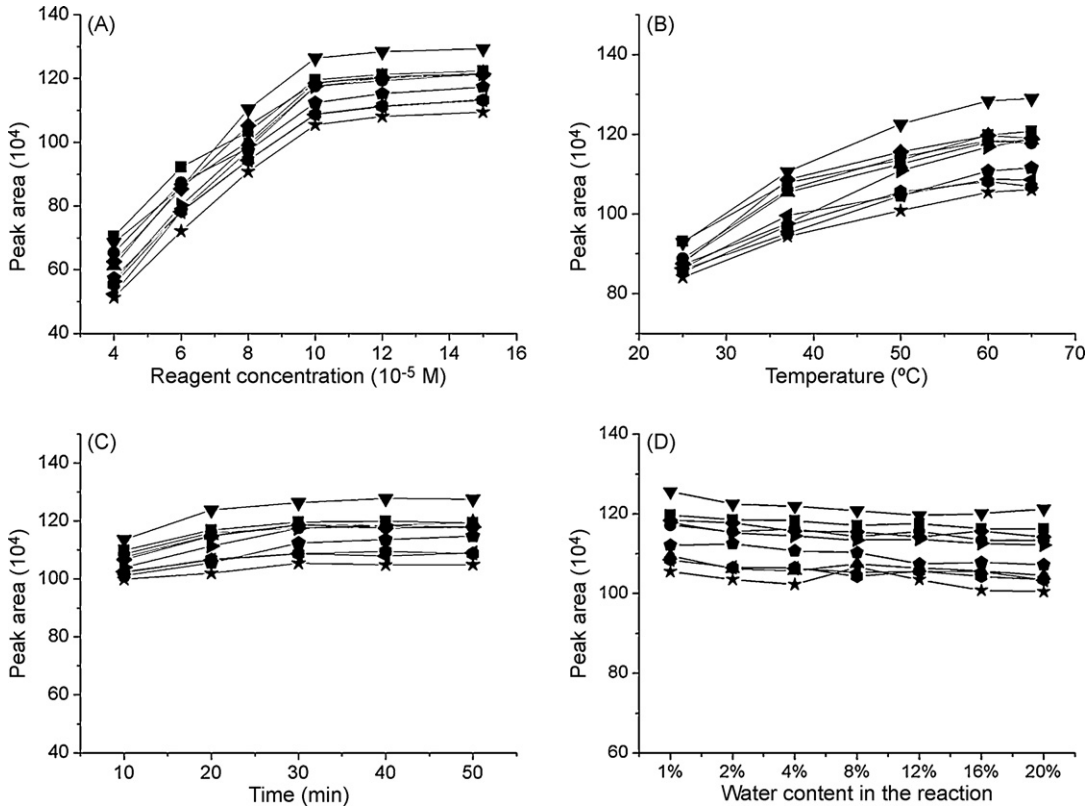

Fig. 8. Optimization of derivatization conditions. (A) BODIPY-aminozide concentration; (B) reaction temperature; (C) reaction time; (D) water content in the reaction. Symbol assignation: (■) propanal; (●) butanal; (▲) pentanal; (▼) hexanal; (◄) heptanal; (►) octanal; (♦) nonaldehyde; (⬟) decanal; (⬢) undecanal; (★) lauraldehyde.

with BODIPY-aminozide are co-eluted throughout in our optimization experiments.

3.4. Optimization of derivatization conditions

The derivatization efficiency is of great importance in procolumn derivatization strategy, which is determined by many factors such as the catalyst, the amount of labeling reagent, reaction time and temperature.

The reactions of carbonyl compounds with hydrazine are usually accelerated in the presence of acidic catalyst to give the corresponding hydrazones. Some acidic catalysts were evaluated in this study for optimal derivatization using 0.1 M aqueous solution of them, including hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, trichloroacetic acid and trifluoroacetic acid. The catalyst amounts are tested from 0 to 100 μL. The results indicated that phosphoric acid was the best choice and the preferred volumes were 10 μL, as shown in Fig. 7. Therefore, 10 μL 0.1 M phosphoric acid solution was adopted here.

The effect of the amount of BODIPY-aminozide used in the derivatization on the derivatization yields was studied at deferent concentration. The highest and most constant detector response was observed with the concentration of BODIPY-aminoide in the range $(1.0–1.5) \times 10^{-4}$ M and increasing the amount of BODIPY-aminozide beyond this level has no significant effect on the derivatization yields (Fig. 8A). The amount 1.0×10^{-4} M of the reagent was thus appropriate.

The effect of the reaction temperature from 25 to 65 °C on the derivatization was tested in acetonitrile solvent containing 10 μL 0.1 M phosphoric acid solution. By fixing the concentration of the reactants and reaction time (30 min), the peak areas of all the derivatives increased along with the temperature (Fig. 8B). High

Table 2
Liner calibration range, regression equation and detection limits of aldehydes.

Aldehyde	Calibration range (μM)	$Y = A + BX$[a] $X = 10^6 \times C$	R^2	RSD (%), $n = 6$, within-day	RSD (%), $n = 6$, between-day	Detection limit[b] (nM)
FA and AA	0.01–1.00	$Y = 7804 + 0.912X$	0.9990	3.5	3.8	0.55
Propanal	0.01–1.00	$Y = 2456 + 1.145X$	0.9987	3.1	4.7	0.44
Butanal	0.01–1.00	$Y = 273 + 1.002X$	0.9986	1.1	2.4	0.69
Pentanal	0.01–1.00	$Y = 2937 + 1.035X$	0.9993	0.7	1.9	0.53
Hexanal	0.01–1.00	$Y = -1596 + 1.286X$	0.9987	0.8	2.1	0.43
Heptanal	0.01–1.00	$Y = -933 + 1.104X$	0.9999	1.5	1.5	0.54
Octanal	0.01–1.00	$Y = 7267 + 0.748X$	0.9986	2.3	2.7	0.69
Nonaldehyde	0.01–1.00	$Y = -1491 + 1.047X$	0.9986	1.7	1.3	0.45
Decanal	0.01–1.00	$Y = 2583 + 0.822X$	0.9986	1.2	2.1	0.62
Undecanal	0.01–1.00	$Y = 1106 + 0.765X$	0.9987	1.5	1.7	0.65
Lauraldehyde	0.01–1.00	$Y = 619 + 1.091X$	0.9987	2.1	3.4	0.44

[a] Y: peak area; C: aldehyde concentration (μM).
[b] Signal-to-noise ratio = 3.

temperatures could accelerate the derivatization reaction. When the reaction temperature was higher than 60 °C, the peak areas had no obvious change. Therefore, 60 °C was chosen as the experiment temperature. Fig. 8C shows the complete derivatization can be achieved in 30 min with reaction temperature at 60 °C.

Stabilities of the reagent and its derivatives have also been studied. Although the BODIPY-aminozide is reactive to aldehyde and ketone, it has excellent stability in solid and solution form. Its acetonitrile solution remains unchanged at least one month at 4 °C in a refrigerator. Under the same storage conditions, the corresponding derivatives are stable at least 1 week with the change of the peak areas less than 2.5%.

Since the derivatization reaction is performed with 10 μL 0.1 M phosphoric acid as a catalyst, anhydrous reaction conditions are not required. But water content in the reaction system has considerable influence on the hydrazone formation. Water content from 1 to 20% was tested in the reaction and the results are shown in Fig. 8D. Water content less than 20% has no significant effect on the derivatization yields in terms of the detection responses.

3.5. Analytical calibration

A test mixture of 12 aliphatic aldehydes with different concentrations (0.01–1 μM) was analyzed under the optimized derivatization procedure and separation conditions. The linear calibration ranges, regression equations, and detection limits of these aldehydes were calculated. The results are listed in Table 2. Their correlation coefficients are from 0.9986 to 0.9999, and the RSDs are from 0.7 to 3.5% for within-day determination ($n = 6$) and from 0.7 to 4.7% for between-day determination ($n = 6$). The detection limits for the labeled aldehydes ranges from 0.43 nM for hexanal to 0.69 nM

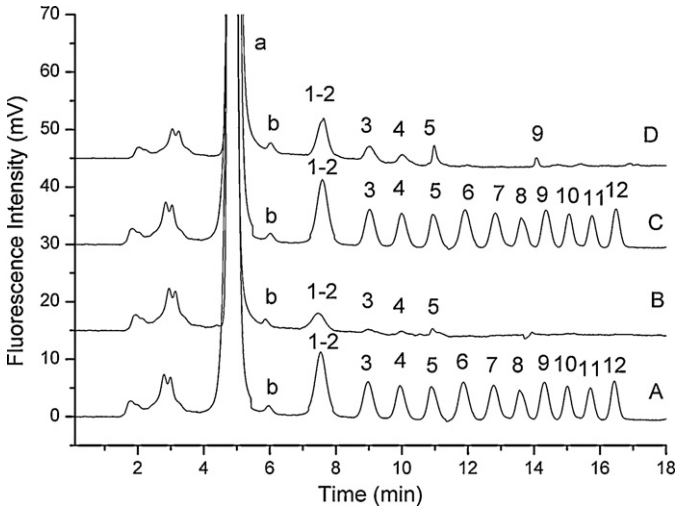

Fig. 9. Representative chromatograms for the determination of aldehydes using the proposed method. Chromatographic conditions and peaks 1–12 as in Fig. 6: serum of health (A) and with 0.2 μM of standard aldehydes (B); serum of patients with hepatitis B (C) and with 0.2 μM of standard aldehydes (D).

Table 3
Analytical results of serum samples.

Samples	Healthy human's serum				Serum of a patient with hepatitis B			
	Added (μM)	Found (μM)	RSD (%, $n = 6$)	Recovery (%)	Added (μM)	Found (μM)	RSD (%, $n = 6$)	Recovery (%)
Total FA and AA	0	0.110	3.5		0	0.217	2.8	
	0.4	0.537	2.9	107	0.4	0.635	2.1	105
	0.8	0.934	2.6	103	0.8	1.008	1.4	99
Propanal	0	0.034	2.5		0	0.075	2.9	
	0.2	0.241	1.7	104	0.2	0.273	3.5	99
	0.4	0.453	0.9	105	0.4	0.488	1.5	103
Butanal	0	0.035	2.7		0	0.076	3.5	
	0.2	0.237	4.5	101	0.2	0.272	4.1	98
	0.4	0.447	2.6	103	0.4	0.474	1.3	100
Pentanal	0	0.037	2.6		0	0.058	1.7	
	0.2	0.239	1.5	101	0.2	0.260	2.9	101
	0.4	0.430	2.1	98	0.4	0.461	3.4	101
Hexanal	0	0			0	0		
	0.2	0.206	1.9	103	0.2	0.205	1.8	103
	0.4	0.405	4.1	101	0.4	0.404	1.4	101
Heptanal	0	0			0	0		
	0.2	0.196	2.7	98	0.2	0.210	2.7	105
	0.4	0.404	1.8	101	0.4	0.409	4.5	102
Octanal	0	0			0	0		
	0.2	0.189	4.6	95	0.2	0.198	2.6	99
	0.4	0.393	3.5	98	0.4	0.410	2.4	102
Nonaldehyde	0	0.025	3.2	95	0	0.020	3.6	
	0.2	0.210	3.1	105	0.2	0.208	2.4	104
	0.4	0.407	1.6	102	0.4	0.419	4.3	95
Decanal	0	0			0	0		
	0.2	0.194	2.4	97	0.2	0.198	1.8	99
	0.4	0.395	1.3	99	0.4	0.4	1.4	100
Undecanal	0	0			0	0		
	0.2	0.202	0.9	101	0.2	0.198	1.7	99
	0.4	0.405	1.7	101	0.4	0.406	3.1	102
Lauraldehyde	0	0			0	0		
	0.2	0.201	1.9	100	0.2	0.203	1.4	102
	0.4	0.407	2.5	102	0.4	0.397	3.1	99

for octanal, which are sufficiently sensitive for the determination of trace amount aliphatic aldehydes in serum [40] and better than or comparable with those obtained by the existing methods based on aldehyde labeling [15–20].

Although formaldehyde and acetaldehyde derivatives are co-eluted under the chromatographic conditions, they are well separated from the reagent and other aldehyde derivatives. Therefore, the total amount of formaldehyde and acetaldehyde could be quantified using the proposed method.

3.6. Sample analysis

As an application of the proposed method, the determination of free aliphatic aldehydes in the serum of healthy people and patients with hepatitis B has been carried out. The chromatograms of the samples unspiked and spiked with the standard solutions are shown in Fig. 9. The analytical results are summarized in Table 3. The recoveries range from 95 to 107% and the RSDs vary from 0.9 to 4.6%. From the results, the contents of total formaldehyde and acetaldehyde, propanal, butanal and pentanal in the serum of the patient with hepatitis B are nearly two times higher than those of the healthy. Nonaldehyde is detected in the patient serum but not in the healthy. Hexanal, heptanal, octanal, undecanal and lauraldehyde are not found in both sera.

4. Conclusions

A new symmetric BODIPY hydrazine reagent with a *meso* hydrazide, 1,3,5,7-tetramethyl-8-aminozide-difluoroboradiaza-*s*-indacene (BODIPY-aminozide), has been developed for the trace determination of aliphatic aldehydes in biological samples using HPLC. The reagent generates bright green fluorescence with maximum emission at 505 nm and is highly reactive to aldehydes. The fluorescence quantum yields of its aldehyde derivatives (up to 0.94) are nearly 2.5-fold higher than BODIPY-aminozide, which are much favorable for the following determination. Anhydrous conditions are not necessary for the derivatization procedure or the storage of the reagent and its derivatives. The detection limits is low enough for the direct determination of biological samples. The further utilization of this reagent is currently under investigation.

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