# Catecholamines derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole: Characterization of chemical structure and fluorescence properties

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# Catecholamines derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole: characterization of chemical structure and fluorescence properties

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**Abstract**

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was evaluated as a fluorogenic derivatization reagent for the analysis of the catecholamines, dopamine, epinephrine, norepinephrine, and their naturally occurring metabolites, metanephrine and normetanephrine, homovanillic acid, 3,4-dihydroxyphenyl acetic acid. These compounds reacted rapidly with NBD-F under mild conditions to form stable derivatives. The optimal reaction conditions were found to be 12.5 mM borate buffer pH 8.0 in water:acetonitrile (1:1) at 50 °C for 5 min. New NBD derivatives of all the catecholamines and metabolites were prepared and purified and were shown by electrospray mass spectrometry to be fully reacted at all available catechol and amine sites, resulting in di- or tri-substituted derivatives. Homovanillic acid and 3,4-dihydroxyphenyl acetic acid reacted with NBD-F but gave non-fluorescent derivatives. The fluorescence excitation wavelength maximum demonstrated a red shift for the derivatives with increasing polarity of the solvent and the fluorescence intensity increased linearly with increasing organic ratio in the solvent–aqueous buffer complex. The presence of electrolyte in the solvent and the electrolyte concentration in the solvent–electrolyte complex had little effect on the fluorescent intensity. The fluorescence quantum yields in acetonitrile were also obtained. The separation behavior of the NBD-catecholamines was determined by high-performance liquid chromatography (HPLC). The studies demonstrated good potential for the application of NBD-F derivatization to the quantitative analysis of catecholamines and related compounds in biological matrices.


*Keywords:* NBD-F; Fluorescence; Norepinephrine; Epinephrine; Dopamine; Metanephrine; Normetanephrine; Chromatography; Quantum yield

## 1. Introduction

Catecholamines are signal messengers that have a number of important functions in the central and peripheral nervous system. The concentration of norepinephrine, epinephrine, dopamine and their 3-*O*-methylated metabolites normetanephrine and metanephrine (see Fig. 1 and Table 1 for structures) in blood, urine and extracellular fluid of brain tissue is of clinical and pharmacological relevance, for example, in the diagnosis of neurological disorders [1], altered function of catecholamine synthesizing tissues [2], suspected pheochromocytoma (tumor of chromaffin cells) and neuroblastoma [3,4].

Although underivatized catecholamines can be readily separated by either high-performance liquid

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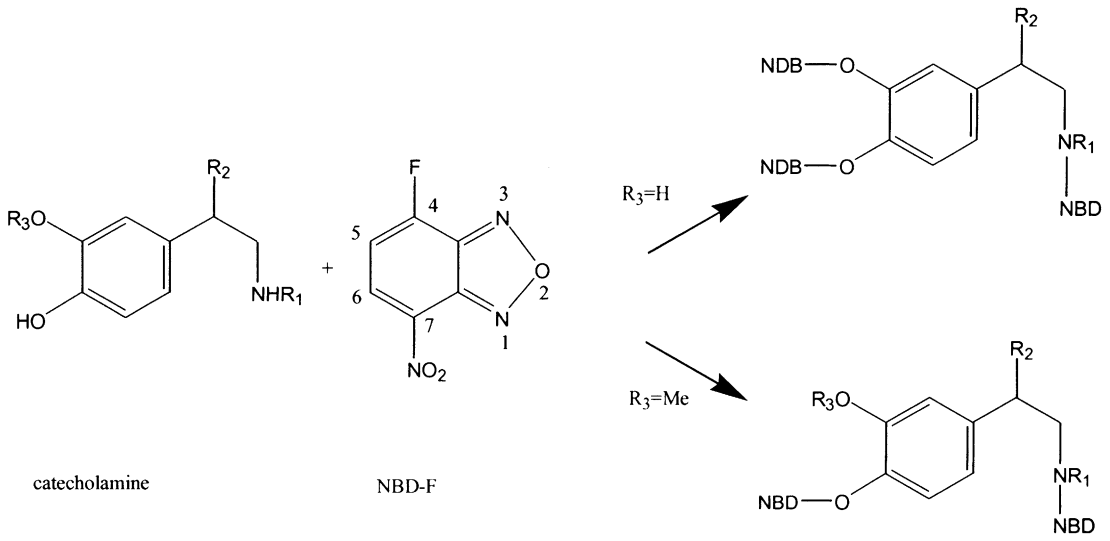


Fig. 1. Structures of catecholamines, *O*-methylated metabolites and NBD derivatives.

chromatography (HPLC) or by capillary electrophoresis (CE), detection by ultraviolet-visible (UV-Vis) absorbance does not have sufficient sensitivity or selectivity for quantitative analysis of catecholamines in biological matrices where concentrations are often of the order of 10 nM or less. Several approaches to catecholamine analysis involving fluorescence have been used to improve assay sensitivity: native fluorescence ($\lambda_{ex}$ = 280 nm) [5], peroxylate chemiluminescence [6,7], derivatization with naphthalene-2,3-dicarboxaldehyde (NDA) [8] or fluorescamine [9]. However, the native fluorescence approach does not have sufficient sensitivity for application to biofluids such as plasma or microdialysate samples and both the NDA and fluorescamine methods are unable to react with secondary amines such as epinephrine and metanephrine or with the acidic catecholamines metabolites, homovanillic acid and 3,4-dihydroxyphenyl acetic acid.

Table 1
Composition of catecholamines, *O*-methylated metabolites and NBD derivatives

| Catecholamine | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| Dopamine (DA) | H | H | H |
| Norepinephrine (NE) | H | OH | H |
| Epinephrine (E) | $CH_3$ | OH | H |
| Normetanephrine (NM) | H | OH | $CH_3$ |
| Metanephrine (MN) | $CH_3$ | OH | $CH_3$ |

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was introduced 20 years ago by Imai and Watanabe [10] as a fluorogenic reagent with convenient excitation/emission wavelengths and mild reaction conditions. Extensive studies have been carried out in applying NBD-F derivatization to the detection of biogenic amines such as amino acids [11,12], peptides [13] and to a range of proteins [14] and Uchiyama et al. [15] have made an in-depth review on this topic. High-sensitivity detection of NBD derivatives of catecholamines should be achievable using argon-ion laser-induced fluorescence (LIF) in combination with HPLC or CE since the excitation wavelengths of the NBD derivatives should match the output of the argon-ion emission at 488 nm. However, no detailed research has been reported in applying NBD-F derivatization to the analysis of catecholamines, possibly due to the potential complexity of the derivatization reaction [16] or the likely quenching of the fluorescence derivatives under the predominantly aqueous conditions used in HPLC or CE methods. We propose that NBD-F derivatization may provide a relatively simple alternative method to analyze sub nanomolar concentrations of catecholamines in biological samples.

In this study, we have synthesized and purified NBD derivatives of a number of catecholamines and

their 3-*O*-methylated metabolites in order to optimize the derivatization reaction and to identify the chemical structure of the derivatives. We have also investigated the influence of solvents, solvent–electrolytes and solvent–aqueous buffer conditions on the fluorescence properties of the derivatives.

## 2. Experimental

### 2.1. Chemicals

All reagents were of analytical grade or better. 4-Fluoro-7-nitrobenzo-2,1,3-oxadiazole, acetonitrile, formamide, *n*-methylformamide, dichloromethane (DCM), and glacial acetic acid were obtained from Fisher Scientific (Loughborough, UK). Dopamine, epinephrine, norepinephrine, normetanephrine, metanephrine (all as hydrochlorides), 3,4-dihydroxylbenzylamine hydrobromide, homovanillic acid, 3,4-dihydroxyphenyl acetic acid, fluorescein, sodium phosphate monobasic and dibasic were obtained from Sigma (Poole, UK). The chemical structures of the catecholamines and *O*-methylated metabolites are shown in Fig. 1 and Table 1. High purity water (USF Elga, High Wycombe, UK) was used throughout the experiments.

Stock solutions of the catecholamines (50 μM) were prepared by dissolving the compounds in water. These solutions were stored in the dark at −20 °C and were thawed and diluted to obtain the working solutions as required. NBD-F was dissolved in anhydrous acetonitrile and was stored in the dark at −20 °C.

### 2.2. Instrumentation

The HPLC system comprised a Gilson 305 pump, a Gilson 401C dilutor, a Gilson 231XL injector (Gilson Medical Electronics, Villers le Bel, France), a Perkin-Elmer LC240 fluorescence detector (Perkin-Elmer, Beaconsfield, UK), and a ABI 759A UV-Vis absorbance detector (Applied biosystems, Bedford, UK). The excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelengths were set at 480 and 530 nm, respectively. The absorbance wavelength was 350 nm. The system was controlled by Gilson 715 software. The HPLC column was a Hypersil ODS (150 mm × 4.6 mm i.d., 5 μm particle diameter) fitted with a guard column (4 mm × 3.0 mm i.d.) (Hypersil, Runcorn, UK). The HPLC mobile phase used for separation was acetonitrile:10 mM aqueous phosphate buffer pH 8 (60:40) and the flow rate was 1.0 ml $min^{-1}$. The injection volume was 20 ml.

### 2.3. Optimization of derivatization reaction conditions

Generally, 10 μl of a 50 μM catecholamine working solution was mixed with 10 μl aqueous buffer (phosphate or borate) and 10 μl of NBD-F dissolved in acetonitrile. The effects of reaction variables were investigated: buffer pH (pH 6.5–11), buffer concentration (3.1–12.5 mM) and NBD-F concentration (2.5–25 mM) in the reaction mixture, reaction temperature (20–60 °C) and reaction time (3–30 min). For certain experiments borate and phosphate buffers were used slightly outside their normal buffering range, but they were still found to be able to maintain the pH at the desired value.

### 2.4. Stability of NBD-catecholamine derivatives

The appropriate catecholamine solution (10 μl, 50 μM) was mixed with 10 μl of borate buffer (50 mM, pH 8), 10 μl acetonitrile and 10 μl NBD-F solution (50 mM), reacted for 5 min at 50 °C, and the reaction quenched by the addition of 200 μl of acetonitrile. The mixture was then kept at room temperature and was sampled at 0, 30, 60 min and 24 h after initiating the reaction.

### 2.5. Synthesis and purification of NBD derivatives

The appropriate catecholamine solution (2 ml, 500 μM) was mixed with 2 ml borate buffer (50 mM, pH 8), 2 ml acetonitrile and 2 ml NBD-F (50 mM) and reacted for 5 min at 50 °C. The reaction mixture was allowed to cool down to room temperature and was then extracted with 4 ml dichloromethane. The extract was washed with 3 × 4 ml water and then dried quickly under $N_2$ flow and the residue obtained was dissolved in around 4 ml HPLC mobile phase (60:40 acetonitrile:10 mM phosphate buffer pH 8). The derivatized catecholamine was then purified by HPLC using the conditions described above.

The collected HPLC fractions were extracted with around 40 ml dichloromethane, the extract washed with 3 × 40 ml water and the final extract was dried quickly under $N_2$ flow. Around 0.5 mg of a yellowish product was obtained for each of the NBD derivatives of dopamine, epinephrine, norepinephrine, normetanephrine, and metanephrine. The purity of the derivatives was checked by HPLC with fluorescence and UV detection. No unreacted NBD-F or other non-fluorescent impurity was found in the yellowish residue. No purification was undertaken for the non-fluorescent NBD derivatives of homovanillic acid and 3,4-dihydroxyphenyl acetic acid.

### 2.6. Confirmation of chemical structure of the NBD derivatives

A 0.5 mg ml$^{-1}$ solution of the derivatives in acetonitrile was prepared, and directly injected for analysis by mass spectrometry. Atmospheric pressure electrospray ionization mass spectrometry was done in positive and negative ion modes by direct injection on a Micromass Platform system (Micromass UK, Altrincham, UK). The cone voltage of the electrospray MS system was adjusted to provide fragmentation of the NBD-catecholamines to aid structural identification of the derivatives.

### 2.7. Fluorescence properties of the NBD derivatives

Aliquots of the NBD derivatized catecholamines were prepared as follows for emission/excitation/absorbance scans: 5 μl of a 0.5 mg ml$^{-1}$ solution of the purified NBD derivative in acetonitrile was added to a small vial, dried quickly under $N_2$ flow and the yellowish residues were then dissolved in 400 μl of the appropriate solvent, solvent–electrolyte, or solvent–aqueous buffer complexes. The final concentration of the derivatives was 6.25 μg ml$^{-1}$ in all cases. Fluorescence excitation/emission spectra were obtained using a F-4500 scanning fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The absorption spectra were obtained by Beckman DU640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA).

The obtained emission spectra and absorbance spectra were compared with those of fluorescein in 0.1 M NaOH to obtain the quantum yield ($\phi_x$) according to the following equation [17]:

$$\phi_x = \frac{\phi_{st}(n^2/n_{st}^2)(S_x/S_{st})(1 - 10^{-A_{st}})}{(1 - 10^{-A_x})}$$

$\phi_x$ is the quantum yield of the testing compound solution; $\phi_{st}$ the quantum yield of the standard, i.e. 0.95 for fluorescein in 0.1 M NaOH [18]; $n$ the reflective index of the solvent for the compound; $n_{st}$ the reflective index of the solvent for the standards; $S_x$ the area under the corrected emission spectrum of the testing compound solution; $S_{st}$ the area under the corrected emission spectrum of the standard solution; $A_{st}$ the optical density of the standard solution at its excitation wavelength; $A_x$ the optical density of the testing solution at its excitation wavelength.

## 3. Results and discussion

### 3.1. Development of HPLC method for reaction optimization studies

A simple HPLC method with fluorescence detection was developed in order to characterize the effect of different reaction conditions on the formation of NBD derivatized catecholamines. The method was capable of separating all the potential fluorescent products of the reaction from the fluorescent hydrolysis products of the NBD-F reagent which eluted close to the solvent front. The NBD-catecholamines were substantially more lipophilic than the underivatized catecholamines as indicated by a much higher percentage of organic modifier required for elution by HPLC. The peak areas of the individual NBD derivatives were used to quantify the fluorescence yield. A typical HPLC chromatogram showing the separation of all the fluorescent NBD derivatized catecholamines and metabolites is shown in Fig. 2.

### 3.2. Effects reaction conditions on derivatization efficiency

As the derivatization reaction of catecholamines with NBD-F is a typical nucleophilic reaction, basic reaction conditions were expected to be beneficial. Borate and phosphate buffer ranges from pH 6.5 to 10 were studied. Borate buffers generally gave a higher

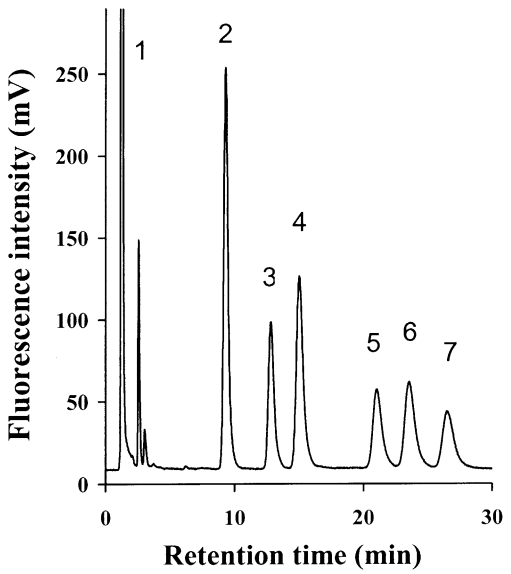


Fig. 2. HPLC separation of NBD-F derivatized catecholamines. Compounds: (1) NBD-OH, (2) normetanephrine, (3) norepinephrine, (4) metanephrine, (5) epinephrine, (6) dihydroxybenzylamine (internal standard), (7) dopamine. Conditions: mobile phase acetonitrile:10 mM aqueous phosphate buffer pH 8.0 (65:35), Hypersil ODS, 5 μM 150 mm × 4.6 mm, 1.0 ml $min^{-1}$, fluorescence detection $\lambda_{ex} = 480$ nm and $\lambda_{em} = 530$ nm.

and more stable product yield than phosphate buffers (as indicated by the HPLC peak area of the derivative) and the best yields were obtained at pH 7.5 with 25 mM borate buffer (Fig. 3a). Borate and phosphate buffers had different effects on the formation of the hydrolysis product of NBD (NBD-OH) (Fig. 3b). However, borate buffers were found to give a reduced hydrolysis of the NBD-F reagent below pH 8 compared to the phosphate buffers. This is likely to assist in reducing potential interference from the highly fluorescent NBD-OH in bioanalytical applications.

For potential applications of the approach to quantitative analysis it is important to achieve a single fluorescent product from the NBD derivatization reaction with catecholamines. This is particularly important with the catecholamines and metabolites because their chemical structures reveal the potential for up to three different derivatization sites (Fig. 1 and Table 1). Above pH 7 and below pH 11 a single HPLC peak was observed from the derivatization reaction for all the catecholamines. However, reaction conditions below pH 7.0 or above pH 11.0 produced one or two additional fluorescent peaks in the HPLC chromatogram with shorter retention times than the main fluorescent derivative (data not shown). These unidentified peaks are likely to be incompletely derivatized catecholamines because the unsubstituted functional groups (phenols or amine groups) will be strongly hydrophilic and hence would explain the reduced retention observed in the reversed-phase HPLC separation. It is known that reduced pH can inhibit nucleophilic reactions, so it may be potentially difficult to completely derivatize all three active sites of catecholamines below pH 7.0. The additional peaks observed at pH 11 were linked to the instability of the NBD derivatives at high alkaline pH (see later). Hence, it was important to limit the pH of the reaction to between pH 8 and 10.

It has been reported that a higher organic percentage in the reaction matrix will increase the reaction speed and inhibit the hydrolysis of NBD-F [19]. To avoid excessive dilution of the sample, a maximum acetonitrile to water ratio of 50:50 was evaluated and proved to be advantageous because it gave a small (5.3%, $n = 3$) increase in fluorescence compared with 30:70 water:acetonitrile coupled with significant reduction (23.0%, $n = 3$) in the unwanted hydrolysis product of the NBD-F reagent.

Based on the optimization studies, the derivatization conditions for further studies was chosen as 12.5 mM borate buffer pH 8 in water: acetonitrile (1:1 (v/v)).

### 3.3. Effects of reaction time, temperature and NBD-F concentration

The derivatization reaction was found to be rapid even at 20 °C, readily observed as a color change to yellow of the reaction mixture within a few seconds of the addition of NBD-F, while the reagent blank color changed to yellow only slowly (NBD-F hydrolysis reaction). There was only a very small change on the yield of fluorescent derivatives (±5%) when the reaction temperature increased from 20 to 50 °C or reaction time increased from 3 to 30 min. However, at 60 °C there was a significant reduction (around 80%) in the product peak area. Neither reaction time nor temperature had a major effect on the production of the unwanted NBD-F hydrolysis product peak, whose area increased only slightly over time and with increasing temperature. Based on these data, a reaction

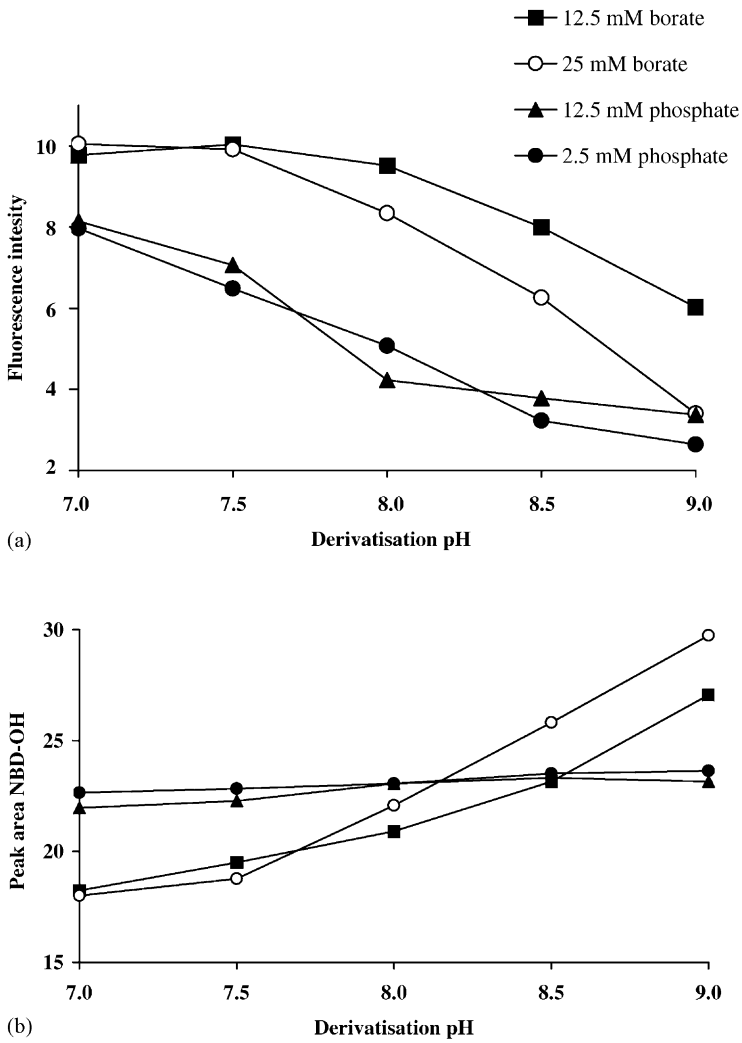


Fig. 3. Effects of buffer type, concentration and pH on the NBD-F derivatization of norepinephrine. (a) Formation of NBD-NE; and (b) formation of NBD-OH. Reaction conditions: 12.5 μM norepinephrine, 3 mM NBD-F in water:acetonitrile (1:1), 5 min at 50 °C. Analysis by HPLC with fluorescence detection.

time of 5 min and reaction temperature of 50 °C were chosen for further studies.

NBD-F has been used at a typical concentration of around 12.5 mM for the derivatization of amino acids and peptides [13]. We observed that, for simple aqueous solutions of the catecholamines, concentrations of NBD-F as low as 1 mM could be used without sacrificing the yield. Considering that the method may be applied to a biological matrix which will contain other compounds capable of reacting with NBD-F we chose 3 mM as the optimal reagent concentration in the reaction mixture, but this has to be evaluated in any further application of the method to biological samples.

### 3.4. Stability of the NBD derivatives

The NBD-catecholamines were found to be stable at room temperature (20 °C) in the reaction mixture (pH 8.0) up to 24 h after derivatization. A minor increase in fluorescence intensity was observed between 5 and

24 h and this was attributed to a small loss of sample volume due to evaporation. However, under more alkaline conditions (>pH 10) all the NBD derivatives were unstable. Tri-NBD substituted catecholamines were found to degrade into bi-substituted derivatives and NBD-OH (hydrolysis product of NBD-F) and this observation was confirmed by electrospray mass spectrometry (data not shown). This finding explains why two or more product peaks appeared in the HPLC chromatograms at pH 11 or higher. Hence, care was taken not to expose the derivatives to alkaline conditions during reaction or during storage prior to analysis.

### 3.5. Structural characterization of purified NBD derivatives

Purified NBD derivatives of norepinephrine, epinephrine, dopamine, normetanephrine, metanephrine and 3,4-dihydroxybenzylamine (DHBA, internal standard) were prepared as described and subjected to analysis by negative or positive ion electrospray mass spectrometry. Negative ion mode gave the most informative structural information for most of the derivatives apart from NBD-E (which gave highly fragmented and weak spectrum) for which positive ion electrospray was used. Clear evidence was obtained that both the catechol sites and the amine site were completely derivatized by NBD-F, hence all the active functional groups of the catecholamines were derivatized, so forming di- or tri-labeled products (Fig. 4). Molecular ions of the multi-substituted compounds were obtained, as well as fragmentation patterns consistent with NBD derivatization. When one phenol group of the catecholamines was methylated (as in normetanephrine or metanephrine) di-substituted NBD products were formed instead of tri-substituted. These data also confirm that NBD-F reacts with both the primary amine group (as in dopamine, norepinephrine, normetanephrine) and the secondary amine group (as in epinephrine and metanephrine).

The di- or tri-NBD substituted catecholamines are extremely hydrophobic in nature and hence are better suited to analysis by reverse-phase chromatography compared with the very hydrophilic underivatized catecholamines.

### 3.6. Fluorescence properties of NBD-catecholamines

The fluorescence properties of the purified NBD derivatives of norepinephrine, epinephrine, and dopamine were studied in a range of solvents, solvent–electrolyte and solvent–buffer conditions. The highest fluorescence intensity was observed for all the NBD derivatives in dichloromethane and there was an approximate inverse relationship between fluorescence intensity and the polarity of the solvent (Table 2). Ward and Khaledi [20] also showed that the polarity and viscosity of the solvent had a direct effect on the fluorescence intensity of maltotriose derivatized with 8-aminonaphthalene-1,3,6-trisulfonic acid

Table 2
Fluorescence properties of purified NBD derivatives of catecholamines in different solvents/solvent–electrolyte mixtures

| Medium | $\varepsilon$ [25] | $\eta$ (cP) [26] | NBD-norepinephrine | | | NBD-epinephrine | | | NBD-dopamine | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | FI[a] | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | FI[a] | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | FI[a] |
| DCM[b] | 9.08 | 0.44 | 450 | 514 | 749 | 475 | 525 | 633 | 450 | 513 | 336 |
| MeOH | 32.7 | 0.545 | 467 | 534 | 287 | 480 | 537 | 132 | 466 | 530 | 115 |
| MeCN | 37.5 | 0.341 | 462 | 528 | 371 | 478 | 532 | 128 | 462 | 524 | 108 |
| FA | 111.0 | 3.30 | 479 | 539 | 159 | 480 | 537 | 208 | 480 | 537 | 122 |
| FA+ | | | 475 | 537 | 233 | 472 | 532 | 254 | 479 | 536 | 148 |
| NMF | 182.4 | 1.65 | 477 | 537 | 197 | 480 | 538 | 166 | 476 | 534 | 139 |
| NMF+ | | | 474 | 536 | 285 | 480 | 538 | 161 | 475 | 534 | 173 |

$\varepsilon$: dielectric constant (relative permitivity); $\eta$: viscosity; FA+: formamide containing 20 mM ammonium acetate and 1% (v/v) acetic acid; NMF+: *N*-methylformamide containing 20 mM ammonium acetate and 1% (v/v) acetic acid; $\lambda_{ex}$: excitation wavelength, $\lambda_{em}$: emission wavelength; concentration of all NBD-catecholamines tested here was 6.25 μg ml$^{-1}$.

[a] FI: fluorescence intensity (arbitrary output units).

[b] All physical properties at 25 °C except DCM at 20 °C.

and that the presence of electrolyte in the solvent had a strong quenching effect. Our data (Table 3) are in agreement with the observed decreasing trend of fluorescence intensity with increasing polarity of the solvent. However, we did not observe any quenching effect when electrolytes in the form of ammonium acetate (Table 3) or 20–100 mM sodium phosphate (data not shown) were added into the solvents, rather we observed a minor increase in the fluorescence intensity suggesting that electrolyte had little or no fluorescence quenching effect on the NBD derivatives of catecholamines.

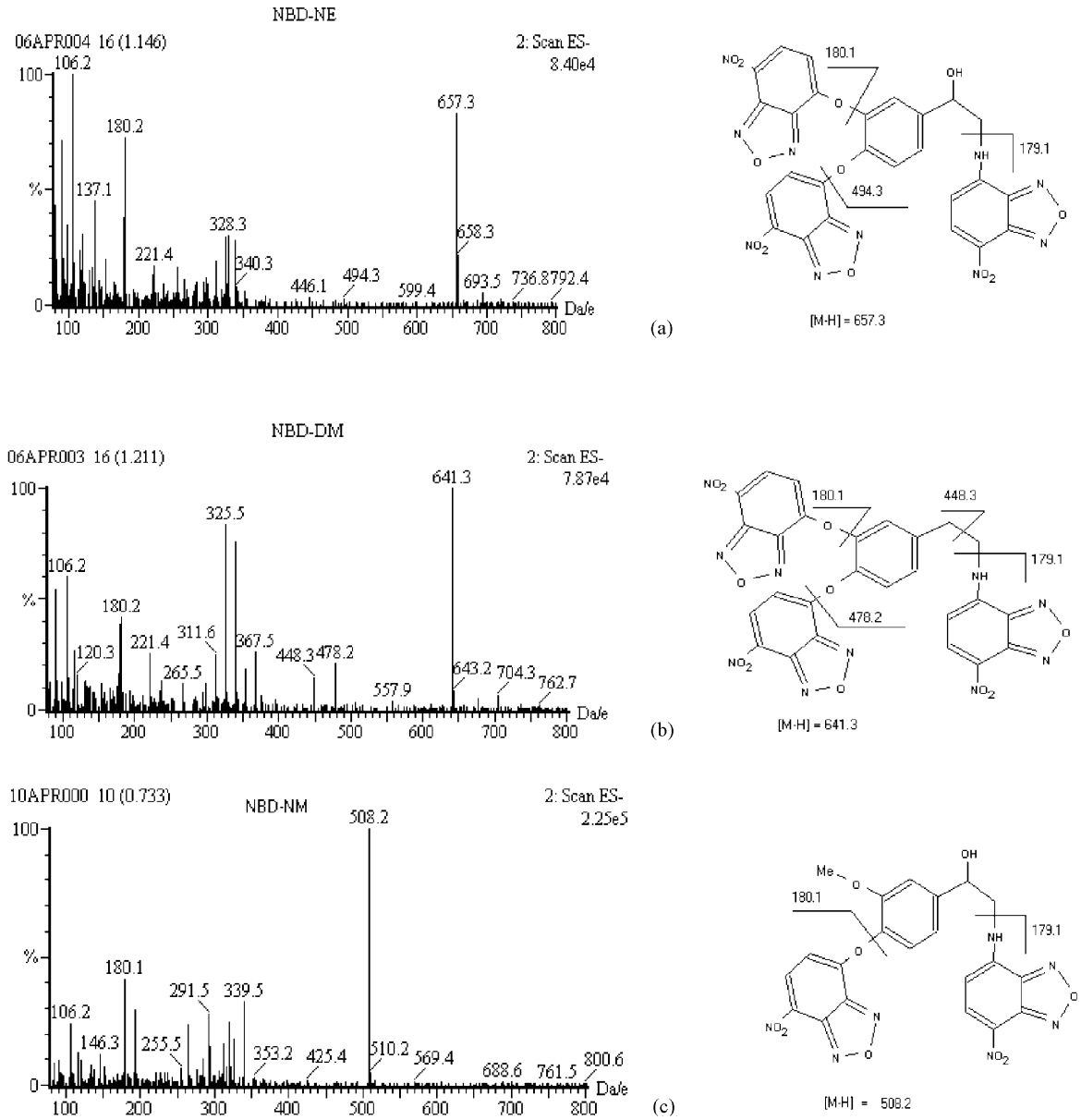


Fig. 4. Electrospray mass spectra of HPLC purified NBD-catecholamines. (a) NBD-norepinephrine, (b) NBD-dopamine, (c) NBD-normetanephrine, (d) NBD-metanephrine, (e) NBD-epinephrine.

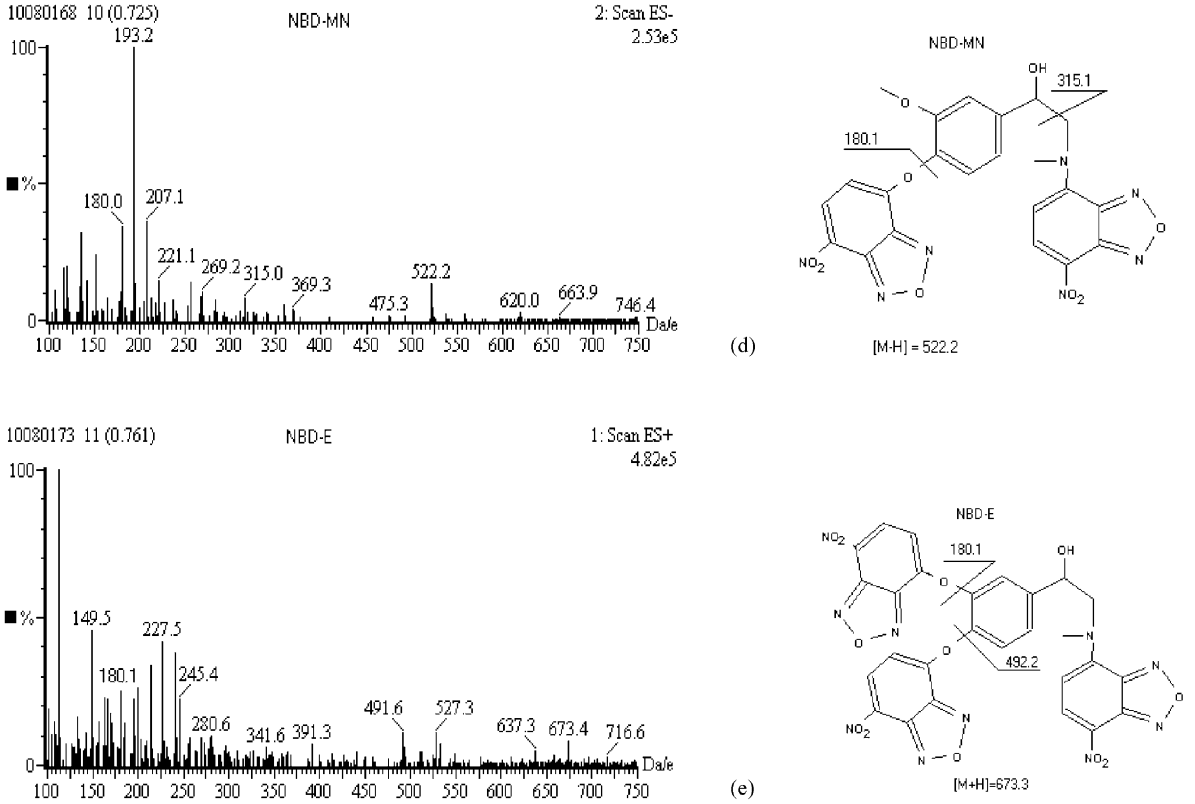


Fig. 4. (*Continued*).

In addition, a shift in both $\lambda_{ex}$ and $\lambda_{em}$ to higher wavelengths (red shift) was observed for all the catecholamine NBD derivatives as the solvent increased in polarity (e.g. for NBD-norepinephrine, $\lambda_{ex}$ in dichloromethane was 450 nm; in formamide it was 479 nm). The inverse relationship between solvent polarity and fluorescence and the red shift at increasing polarity was also observed in a range of solutions containing an increasing proportion of acetonitrile in 10 mM phosphate buffer pH 8 (Table 3). Al-Dirbashi et al. [21] studied the fluorescence properties of NBD-F derivatives of sympathomimetic amines

Table 3
Fluorescence properties of purified NBD derivatives of catecholamines in acetonitrile 10 mM phosphate pH 8 buffer mixture

| Acetonitrile (%) | NBD-norepinephrine | | | NBD-epinephrine | | | NBD-dopamine | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | FI[a] | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | FI[a] | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | FI[a] |
| 100 | 462 | 528 | 371 | 478 | 532 | 128 | 462 | 524 | 108 |
| 80 | 469 | 535 | 200 | 480 | 537 | 94 | 472 | 531 | 73 |
| 60 | 472 | 535 | 141 | 482 | 538 | 77 | 472 | 534 | 63 |
| 40 | 474 | 538 | 92 | 482 | 538 | 69 | 477 | 535 | 45 |
| 20 | 478 | 538 | 45 | 484 | 539 | 30 | 475 | 535 | 30 |

$\lambda_{ex}$: excitation wavelength, $\lambda_{em}$: emission wavelength; FI: fluorescence intensity; concentration of all NBD-catecholamines tested here was 6.25 μg ml$^{-1}$.

[a]FI: fluorescence intensity (arbitrary output units).

including as amphetamine, norephedrine and ephedrine in detail, and in agreement with our results demonstrated that all the derivatives showed an excitation ($\lambda_{ex}$) red shift with increasing polarity of the solvent.

The fluorescence intensity of the secondary amine derivative, NBD-epinephrine, was observed to be consistently higher than that of the primary amine derivative NBD-dopamine under identical solution conditions. A previous study [21] of NBD derivatives of sympathomimic amines noted the opposite effect: that primary amine derivatives had more intense fluorescence than secondary amines. However, previous studies of NBD reactions with a wide range of amines [22–24] have noted that the fluorescence intensity is determined by the overall electronic effects (resonance plus field) of the substituents at the 4- and 7-position of NBD structure (see Fig. 1 and Table 1) and is not simply a function of the reaction with a primary or secondary amine.

All the NBD-catecholamines showed an increase in fluorescence intensity as the acetonitrile percentage increased. For NBD-norepinephrine the slope of the line was steeper than for either NBD-epinephrine or NBD-dopamine and showed a non-linear increase from 80 to 100% acetonitrile. The effect of solvent on fluorescence intensity is likely to be important when optimizing the mobile phase of an HPLC or CE method involving NBD derivatives. We also noted that the NBD-catecholamines and metabolites were essentially insoluble at an acetonitrile concentration of less than 20% in pH 8 phosphate buffer.

We showed evidence above that the phenolic groups of the catecholamines reacted with NBD-F and were therefore encouraged to assess whether the acidic catecholamines metabolites homovanillic acid and 3,4-dihydroxyphenyl acetic acid (which have no amine group) would also react to give fluorescent derivatives. Since these acid catecholamines metabolites do not react with other fluorescent reagents such as NDA or fluorescamine there was potential for NBD derivatization for the simultaneous fluorescence analysis of all catecholamines and metabolites. Although the phenolic groups of homovanillic acid and 3,4-dihydroxyphenyl acetic acid could be derivatized by NBD-F (shown by HPLC-UV analysis) these NBD derivatives were found to have no fluorescence. This is because the oxygen covalently bonded to the NBD group does not have electron pairs parallel to the main $\pi$-orbit of the NBD group, resulting in difficulty in fluorescence excitation. The lack of fluorescence of other phenolic NBD derivatives has also been reported previously [22–24].

The fluorescence quantum yields of NBD-catecholamines solution in acetonitrile were also studied. The values obtained were 0.4116, 0.2016, and 0.1475 for NBD-norepinephrine, NBD-epinephrine and NBD-dopamine, respectively. The relative magnitude of fluorescence quantum yield was consistent with HPLC fluorescence peak area obtained with equimolar solutions of the NBD.

### *3.7. Chromatographic behavior of NBD-catecholamines*

To be suitable for application to the quantitative HPLC measurement of catecholamines in biological matrices there is a need for high fluorescent yield and a good resolution between main peaks and the fluorescent hydrolysis products of NBD-F.

Reversed-phase HPLC provided effective resolution of all the NBD-catecholamines using a simple isocratic mobile phase (Fig. 2). The separation mechanism was indicative of reversed-phase HPLC and showed a linear plot of $\ln k$ versus percentage acetonitrile in the mobile phase. Dihydroxybenzylamine showed suitable retention characteristics for use as an internal standard. Preliminary sensitivity data using capillary chromatography linked to LIF detection has demonstrated a limit of detection (S/N = 3) of less than 0.5 nM for all the NBD-catecholamines (data not shown) suggesting good potential for application to quantitative analysis in biological matrices. The high percentage organic in the HPLC mobile phase was advantageous for enhancing the fluorescent yield and hence the ultimate sensitivity of the method. Under the chromatographic conditions used non-derivatized catecholamines and other compounds with ionizable groups would be unretained on the column suggesting that the NBD derivatives would be well separated from potential interference in biological samples.

## 4. Conclusions

Catecholamines and their *O*-methylated metabolites are easily derivatized in a quantitative manner

by the fluorescent reagent NBD-F. The derivatization results in multiple NBD substitution of the compounds at the phenolic and amine sites forming stable fluorescent derivatives with a $\lambda_{ex}$ suitable for use with LIF detection using the argon-ion emission of 488 nm. The fluorescence intensities and $\lambda_{ex}$ of the NBD derivatives are strongly dependent on the presence of organic solvent, the nature of the solvent and, to a lesser extent, whether a primary or secondary amine was reacted. Evidence is presented to suggest that only the amine groups produce a fluorescent derivative with NBD and that, although the phenolic hydroxy (catechol) groups react with NBD they form non fluorescent derivatives. The NBD derivatization procedure for catecholamines shows good potential, using HPLC with fluorescence detection, to apply the method to the analysis of these compounds in biological samples such as plasma, urine and brain microdialysate samples. It is evident that this approach may have a more general application to the profiling of biological matrices for compounds containing amine groups capable of reacting with NBD-F.

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