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REVIEW

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The Application of 7-Chloro-4-Nitrobenzoxadiazole and 4-Fluoro-7-Nitro-2,1,3-Benzoxadiazole for The Analysis of Amines and Amino Acids Using High-Performance Liquid Chromatography

Abdalla A. ELBASHIR[1], Fakhr Eldin O. SULIMAN[2], Hassan Y. ABOUL-ENEIN[3]♠

[1]*Chemistry Department , Faculty of Science, University of Khartoum P. O. Box 321 Khartoum, Sudan,*
[2] *Department of Chemistry, College of Science, Sultan Qaboos University,Box 36, Alkhod 123, Oman*
[3]*Pharmaceutical and Medicinal Chemistry Department, National Research Centre, Cairo, Egypt*

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ABSTRACT

This paper reviews analytical methods, instrumental developments and applications for derivatization of amines and amino acids with 7-Chloro-4-nitrobenzoxadiazole (NBD-Cl) and 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) using high performance liquid chromatography (HPLC). Discussion of the literature related to the application from 2001 to 2010 is presented.

***Key Words**: NBD-Cl, NBD-F, HPLC, 2D-HPLC, Amines and Amino acids*

1. INTRODUCTION

Analysis of amines and amino acids usually requires a derivatization step to increase the detection limits by introducing chromophores and/or fluorophores for chromatographic and spectrophotometric detection. Several derivatizing reagents such as naphthalene-2,3-dicarboxaldehyde [1], 2,4-dinitro-1-fluorobenzene; Sanger reagent (FDNB) [2], ninhydrin [3], o-phtalaldehyde (OPA) [4], fluorescamine [5], 1,2-naphtoquinone-4-sulfonate (NQS) [6] and halogenobenzofurazan [7] have been employed for the determination of amines and amino acids in pharmaceutical and biomedical samples, and each has its advantages and disadvantages.

High Performance Liquid Chromatography (HPLC) has always been the method of choice for the determination of amines and amino acids in various matrices. Unfortunately, many compounds cannot be detected by HPLC because they do not contain the main chromophoric, or fluorophoric groups. Nevertheless, this problem can be handled by inducing derivatization reactions by adding one of the above groups to the molecule under investigation. These reactions should in principle, produce stable derivatives rapidly and can be achieved in the following modes:

- In pre-column mode (before the analytical separation), the reaction is generally performed manually in vials before HPLC injection but can also be automated. This mode allows more flexible working conditions (reaction time, solvent reaction, elimination of excess reagent, etc…). Separation of the molecules takes place after their modification by derivatization; derivative stability is necessary.
- In post-column mode, the reaction is performed automatically by adding a derivatization reagent after separation, before detection, by means of a second HPLC pump. This method typically requires less sample preparation and clean–up procedure than precolumn derivatization methods, have less

♠Corresponding author, e-mail: haboulenein@yahoo.com

interferences from the reagents employed, and the results are overall more reproducible.

This approach, however requires heavier and complicated instrumental setup but automation considerably reduces the number of manipulations. On the other hand the derivatization reaction has to be fast and compatible with the mobile phase.

Derivatization is also used for enantiomeric separation. Enantiomers quantification methods, such as chemical resolution or rotating power of polarized light, are not adequate to determine enantiomer traces in a biological environment.

HPLC has been found to be the most adequate method because many enantiomers present weak thermal stability and because gas phase chromatography requires high temperatures that can induce racemization.

In pre-column derivatization (indirect method), diastereoisomers are formed before injection by using a highly pure enantiomeric chiral reagent. Separation is then performed on an achiral column with an achiral mobile phase.

The reaction of the fluorogenic reagents 7-Chloro-4-nitrobenzoxadiazole

(NBD-Cl) and 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) with amines and amino acids produces a highly fluorescent NBD-product [8-10] (Fig. 1), that are relatively stable. This approach for labeling amines and amino acids has become a standard method for analysis of very low levels of amines and has widespread analytical applications. A large number of studies have been carried out with these fluorogenic dyes to develop methods for quantification of amines and amino acids in pharmaceutical and biological samples by labeling amine functional groups.

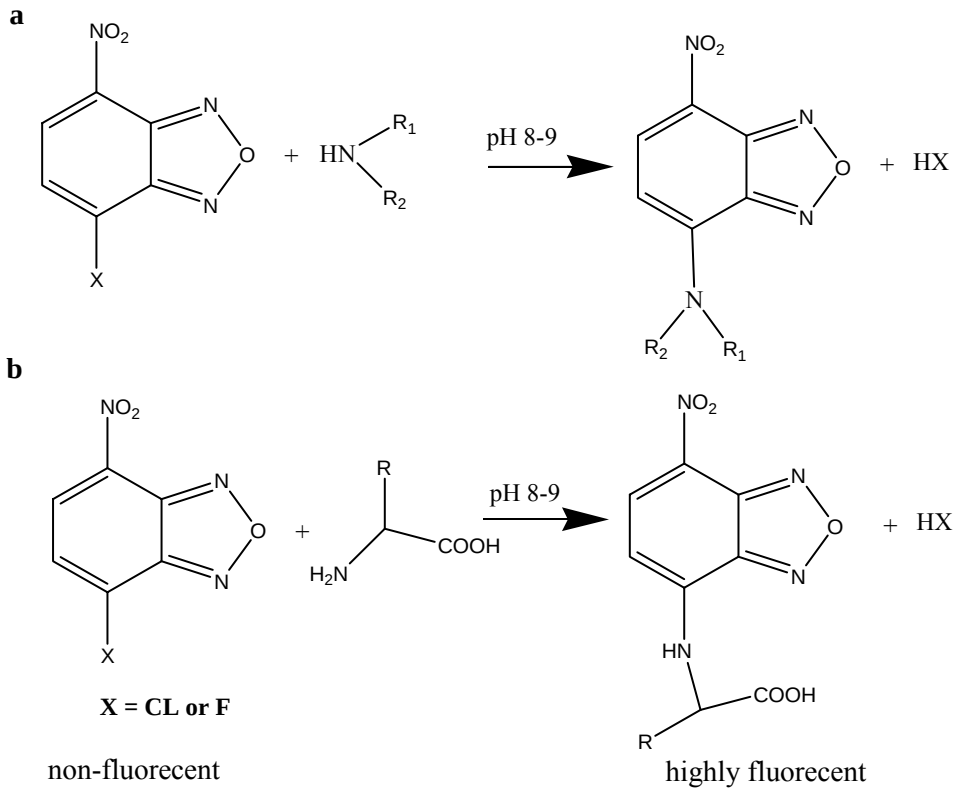

Figure 1. Schematic diagram for the reaction of amines and amino acids with NBD-Cl and NBD-F

Since highly sensitive procedures that lead to be formed derivatives of reasonable stability using low-cost reagents are very desirable when analysing small samples or samples with low concentration of the amine, by HPLC, NBD-Cl and NBD-F has become quite popular. Uchiyama et al. [11] have made a review on benzofurazan (2,1,3-benzoxadiazole) skeleton in which synthetic method, reactivity, fluorescence characteristics and some of the application up to 2000 were presented. In this review applications using NBD-Cl and NBD-F labeling in HPLC in the period of 2001- 2010 will be reviewed. The derivatization conditions, separation conditions and the detection systems will be presented.

The NBD-Cl reagent was synthesized by Ghosh and Whitehouse, [12, 13], by nitrating 4-chlorobenzofurazan obtained from 2,6-dichloroaniline via the dichloronitrosobenzene. The strong fluorescence of the NBD-amines is the best observed in solvents of low polarity. The optimum excitation wavelength for NBD-Cl derivatives is 464 nm. NBD chloride is more stable to moisture and more soluble in aqueous solutions than dansyl chloride (DNS), there are therefore some distinct

advantages in employing NBD chloride for detecting and determining small quantities of amines and amino acids as their fluorescent NBD derivatives. NBD-Cl is widely used for the analysis of compounds with primary or secondary amino groups owing to its low cost [14, 15] in spite of its lower reactivity towards amino acids compared to NBD-F and a longer reaction time for NBD-Cl is usually observed [16].

Application of NBD-Cl in HPLC

Analysis of amines

Tatar and Atmaca [17] have developed a sensitive and specific HPLC method for the assay of amlodipine in human plasma. The method involves derivatization with NBD-Cl, solid-phase extraction on a silica column and isocratic reversed-phase chromatography with fluorescence detection. The method was found to be applicable in the linear range of 0.25-18.00 ng/ml. Within-day and day-to-day reproducibility and accuracy less than 11.80% and 12.00% were obtained respectively.

An isocratic reversed-phase liquid chromatographic RP-HPLC with UV detection for assay of tyramine in cheese was developed by Yigit, and Ersoy [18]. The method is based on coupling of tyramine with NBD-Cl and measurement of the absorbance at 458 nm after chromatographic separation on a C-18 column. The assay was linear over in the concentration range of 25-300 ng per 10 µl of tyramine. The mean recovery of tyramine from cheese 98.0% was obtained. The quantification limit is almost same with comparison method [19].

A simple and reliable HPLC method with fluorimetric detection for the determination of lisinopril in dosage forms as well as in spiked human plasma using solid phase extraction (SPE) procedures has been developed by El-Emam et.al. [20] .The method is based on measurement of the derivatized product using fluorescence detection at 540 nm (excitation at 470 nm). The method was applied successfully to the determination of the drug in pharmaceutical dosage forms either alone or co-formulated with hydrochlorothiazide. The percentage recoveries were satisfactorily accurate and precise. The method was also applied to spiked human plasma samples, with a percentage recovery of 101.6±3.35. A proposal for the reaction pathway was presented. The primary amino group of the drug reacts with the active chloride of NBD-Cl with formation of benzofurazon whereas the reaction of the secondary amino group is retarded by the steric hindrance effect.

A simple and rapid methods based on reversed-phase HPLC with fluorimetric detection is described for the analysis of amlodipine in human serum [21]. Amlodipine is extracted from serum by ethyl acetate converted to a sensitive fluorescent derivative with NBD-Cl. The mobile phase consisted of sodium phosphate buffer (pH 2.5) containing 1 mL/L triethylamine and methanol at flow rate of 2.8 mL/min and C18 column as stationary phase were used. The calibration graph was linear over the concentration ranges 0.25-16 ng mL^{-1} of amlodipine in human serum with minimum detectability of 0.25 ng mL^{-1}. The method has advantages over the previously reported method [17] such as short analysis time, better resolution, simple sample extraction and clean-up.

A selective, sensitive, and precise HPLC method for the simultaneous determination of fluoxetine (FL) and its N-demethylated metabolite norfluoxetine (NFL) in human plasma has been developed [22]. FL and NFL were extracted with n-hexane, followed by derivatization with NBD-Cl under weakly alkaline conditions. Calibration curves were linear over the range of 1.0-100.0 ng mL^{-1} and 0.1-50.0 ng mL^{-1} for FL and NFL, respectively, with inter- and intraassay precision given by a relative standard deviation (RSD%) of less than 9.2%. The lower limits of quantification were 1.0 ng mL^{-1} for FL and 0.1ng mL^{-1} for NFL. The method was successfully applied to pharmacokinetic study.

Tatar [23], has developed new and selective HPLC method with fluorimetric detection for the determination of tianeptine (TIA) in human plasma using solid phase extraction (SPE) procedures. The TIA is converted to a sensitive fluorescent derivative with NBD-Cl at pH 8.5, followed by reversed-phased chromatography and fluorescence measurement (ex = 458 nm, fl = 520 nm). The HPLC separation was performed on a Phenomenex C_{18} column (250 mm × 4.6 mm) using a mobile phase of acetonitrile-10 mM orthophosphoric acid (pH 2.5) (77:23, v/v) solvent system at 1 mL/min flow rate. The method was found to be applicable in the linear range of 5-300 ng mL^{-1} and a mean percentage recovery of 88.6 was obtained. The detection and quantification limits were found to be 2 and 5 ng mL^{-1}, respectively which was superior over the reported one using HPLC-UV [24].

A simple, accurate, and specific HPLC method for quantitation of the gabapentin in human serum using NBD-Cl as a fluorescent labeling agent was described by Bahrami, and Mohammadi, [25]. Chromatographic separation was performed on a Shimpack CLC-C18 (150 mm × 4.6 mm) column ***and*** mobile phase consisting of methanol and sodium phosphate buffer (0.05 M; pH 2.5) containing 1 mL triethylamine (65:35, v/v)**.** The fluorometric detector was operated at 470 nm (excitation) and 573 nm (emission). The calibration curve was linear over the concentration range of 0.002-15 µg mL^{-1}, with limit of quantification of 0.002 µg mL^{-1}. The method did not require an automated instrument and more simple procedure for derivatization is applied.

An HPLC method with fluorescent detection for the determination of insulin in spiked serum after precolumn derivatization with NBD-Cl was reported by Yang et al.[26] . The optimal derivatization conditions were as follows: temperature 50; time 2 h, in dark medium; 0.1 M phosphate buffer (pH 9.0). Analytical separation was carried out on a C18 column and the mobile phase

including acetonitrile-water containing 0.1% trifluoroacetic acid (TFA) (v/v: 30/70). The fluorescence derivative of the drug was monitored at excitation and emission wavelengths of 470 and 540 nm, respectively. The calibration curve was linear over the concentration range of 0.46 µM -16.10 µM, recovery of insulin in serum was 95.06% and the detection limit was 90 nM.

A novel, sensitive and simple HPLC method with precolumn derivatization and fluorescence detection for analysis of topiramate, an antiepileptic agent was reported [27]. The HPLC separation was achieved on a Shimpack CLC-C18 (150 × 4.6 mm) column using a mobile phase of a mixture of sodium phosphate buffer (0.05 M; pH 2.4): methanol (35:65 v/v). The method was validated with respect to specificity, sensitivity, linearity, precision, accuracy. The method was shown to be accurate, with intra-day and inter-day accuracy from 3.4 to 10% and precise, with intra-day and inter-day precision from 1.1 to 18%. The method was found to be linear over the concentration range of 0.01 to 12.8 µg mL^{-1}, with limit of quantification (LOQ) of 0.01µgmL^{-1} which is superior to their reported methods [28, 29].

Validation of an HPLC method for the determination of reboxetine (REB) in human plasma was described [30]. The method is based on the derivatization of REB with NBD-Cl. The HPLC separation was achieved on a reversed phase C_{18} column with isocratic elution using a mobile phase of acetonitrile and aqueous nitric acid (pH 3) solution. The procedure was validated over the concentration range of 2.0-200.0 ng mL^{-1} with inter- and intra-assay precision (RSD%) of less than 4% and mean recovery was about 94% for REB. The method was applied for analysis of plasma.

Bahrami and Mohammad, [31] have described fast and sensitive HPLC method for the analysis of fluvoxamine (FL) in human serum using NBD-Cl as pre-column derivatization agent. The drug and an internal standard (fluoxetine) were extracted from 0.25mL of serum using ethyl acetate followed by pre-column derivatization by the NBD-Cl. The calibration curve was linear over the concentration range of 0.5-240 ng mL^{-1} with a limit of quantification (LOQ) of 0.5 ng mL^{-1}. The method allows the determination of low blood FL levels, in only 6 min.

Darwish et al. [32] have developed and validated HPLC method with fluorescence detection for the trace measurement of trimetazidine (TMZ) in human plasma. The drug and an internal standard (fluoxetine) were extracted from plasma by protein precipitation with acetonitrile and derivatized by heating with NBD-Cl in borate buffer (pH 8) at 70°C for 30 min. A mobile phase consisting of acetonitrile-10 mM sodium acetate buffer (pH 3.5)-methanol (47 + 47 + 6, v/v/v) at a flow rate of 1.0 mL/min and chromatographic separation was performed in the isocratic mode on a Nucleosil CN column. The calibration curve was linear over the concentration range of 1-120 ng mL^{-1}. The proposed method has low limits of detection and quantitation for the determination of TMZ in plasma with values of 0.3 and 0.95 ng mL^{-1}, respectively.

Darwish et al. [33] have also described a new and simple offline precolumn derivatization HPLC procedure with fluorescence detection for the trace determination of paroxetine (PXT) in human plasma where a priorly extraction step is not required. The proposed method had high throughput, as the analysis involved a simple sample pretreatment procedure and short run time (<10 min). A linear relationship was found between the peak area and PXT concentrations in the range of 5-600 ng mL^{-1}. The LOD and LOQ were 1.37 and 4.14 ng mL^{-1}, respectively.

Recently Khalil, et al. [34] have proposed non-extractive, highly sensitive and accurate HPLC method with automated online column-switching system and fluorescence detection for trace determination of sertraline in human plasma. The method was found to be applicable in the linear range of 5-5,000 ng mL^{-1} with recovery of 99.76-102.62 ± 2.19-5.63% and The LOD and LOQ were 1.41 and 4.28 ng mL^{-1}, respectively.

Analysis of amino acids

Vázquez-Ortíz, et al. [35] has developed sensitive and selective method for the assay of hydroxyproline for the determination of collagen in meat and meat products. The method is based on precolumn derivatization with NBD-Cl, followed by reversed-phased chromatography and fluorescence measurement (ex = 465 nm, fl = 535 nm). The limit of quantification for hydroxyproline and proline was 0.0027 ng mL^{-1}. The proposed method was applied for analysis of meat samples with different levels of collagen normally found in meat and meat products. The method represents a considerable improvement over existing methodologies [36, 37] for determination of proline and hydroxyproline, in terms of analysis time and chemical consumption.

A sensitive HPLC method for the analysis of free amino acids in islets of Langerhans is described using NBD-Cl as pre-column derivatization agent [38]. Several parameters influencing the derivatization reaction and chromatographic separation were optimized. Derivatized amino acids were separated on a C18 column with acetonitrile-acetate buffer as mobile phase and detected at 470 nm/540 nm (Ex/Em). The method was successfully applied for the determination of 15 free amino acids in islets of normal and obese rats. The absence of a suitable method for determining the chromatographic peak purity is a limiting factor of the method. Good linarites with correlation coefficients better than 0.9972 were obtained over a wide range of 0.42-42.11 µM for most of the amino acids.

A new, sensitive HPLC method was reported for the determination of the neurotoxin domoic acid (DA) using a reversed phase separation followed by post-column derivatization with NBD-Cl and subsequent fluorescence detection [39]. The parameters affecting the reversed phase separation of DA and those that affect the post-column derivatization of DA with NBD-Cl were identified and optimized in order to obtain the most favorable detection limits for the DA. The developed

post-column method provides the ability for a fully automated analysis, with LOD 25 ppb in real samples of mussel extracts. The author's claimed that the absence of the fluorescence of the product of the NBD-Cl with tryptophan is also one of the major advantages of the method. The method was successfully applied for the quantitative determination of DA in mussel muscle tissues at quantities as low as 75 µg/kg tissue.The post-column derivatization method developed provides cleaner chromatograms free of interferences than the reported pre-column method [40]. Comprehensive overviews of the applications of NBD-Cl as labeling reagent for determination of amines and amino acids using HPLC are summarized in Table 1.

Table 1 Application of NBD-Cl for the determination of amines and amino acids using HPLC

Derivatized compounds	Dearivatization conditions	HPLC	Detection	Application	References
Amlodipine	Sample + Borate buffer pH 8.5 + 0.1 mL NBD-CL, 70°C for 30 min	Bondapak C_{18} column (300 mm×3.9 mm, I.D.) Elution: isocratic, Eluent: methanol–water (80:20)	Ex:459nm, Em:528nm	Human plasma	[17]
Tyramine	Sample + borate buffer + 0.2 ml + NBD-Cl (18 mg/ml) 60 °C for 40 min	Phenomenex Luna (150×4.6 mm) packed with RP-18, 5 µ, Elution: isocratic, Eluent: MeOH–H_2O (70:30)	UV-458 nm	Cheese	[18]
Lisinopril	Sample + borate buffer pH 9 + 1.6±0.2ml NBDCl (0.3%) 70 °C for 30 min	reversed-phase ODS column Elution: isocratic, Eluent: methanol–0.02M sodium dihydrogen phosphate, pH 3.0 (55:45, v/v)	Ex: 470 nm, Em:540nm	Dosage form, spiked human plasma human serum	[20] [21]
Amlodipine	Sample + borate buffer (pH 7.8) + 100 µl NBD-Cl 80 °C for 15 min	Shimpack–CLC-ODS 150 mm × 6 mm i.d.) Elution: isocratic, Eluent: 0.05 M sodium phosphate buffer (pH 2.5) 1 ml/l triethylamine (solvent A) methanol (solvent B).	Ex: 470nm, Em:537nm	Plasma	[22]
Fluoxetine (FL) Norfluoxetine (NFL)	Sample + buffer pH pH 8.0 + NBD-Cl 5 mg/mL 8.0, 60 ˚Cfor 5 min	a reversed-phase column, elution gradient, Eluent: acetonitrile and 0.1 mol/L nitric acid (pH 3) solution	Ex:456nm, Em:535nm 1.0 ng mL^{-1} for FL and 0.1 ng mL^{-1} for NFL	Human plasma	[23]
Tianeptine	Sample + borate buffer pH 8.5 + 100 µL NBD-CI 80 °C for 20 min	Shimpack CLC-C18 (150 mm × 4.6 mm) column, Eluation:isocratic eluent: methanol sodium phosphate buffer (0.05 M; pH 2.5) containing 1 ml/l triethylamine (65:35, v/v)	EX:458 nm,Em:520 LOD) 2 $ngmL^{-1}$	Human serum	[25]
Gabapentin	Sample+phosphate buffer (25 µl),+ 100 µl NBD-Cl 60 °C for 10 min	Phenomenex C_{18} column (250 mm × 4.6 mm) elution: isocratic Eluent: acetonitrile–10 mM orthophosphoric (pH 2.5) (77:23, v/v	Ex:470 nm, Em:537nm LOD) 0.002 µg mL^{-1}	Serum	[26]
Insulin	Sample + borate buffer 0.05 M; pH 10.6 100 µL NBD-Cl,dichloromethane 60 °C for 10 min	C18 column (5 mm, 300 A ° 4.6 250 mm, Hanbon Science and Technology. Co., Ltd., Jiangsu, China)., elution:isocratic, eluent: acetonitrile-water containing 0.1% trifluoroacetic acid (TFA) (v/v: 30/70)	Ex: 476 nm, Em:533nm, LOQ, 2.0 ng mL^{-1}	Plasma	[27]
Topiramate	Sample + 300 mL 0.1 M phosphate buffer (pH 9.0), 50 mL + 150 mL 3 mM NBD-Cl 50 °C for 2 h	Shimpack CLC-C18 (150 mm × 4.6 mm) column, Eluation:isocratic eluent: methanol sodium phosphate buffer (0.05 M; pH 2.5) containing 1 ml/l triethylamine (65:35, v/v)	Ex:470 nm, Em:537nm LOQ, 0.01 µg mL^{-1}	Human serum	[30]
Reboxetine	Sample + 0.2 M borate buffer pH 8.5 + 0.2 mL NBD-Cl 70 °C for 5 min	Shimpack CLC-C18 (150 mm × 4.6 mm) column, Elution: isocratic Eluent sodium phosphate buffer (0.05 M; pH 2.4): methanol (35:65 v/v)	Ex: 470 nm, Em: 537nm LOQ 0.5 ng mL^{-1}	human plasma	[31 32]
Fluvoxamine	Sample + borate buffer 0.05 M; pH 10.6 100 µL NBD-Cl,dichloromethane 60 °C for 10 min	Shimpack CLC-C18 (150 mm × 4.6 mm) column, Eluation:isocratic eluent: methanol sodium phosphate buffer (0.05 M; pH 2.5) containing 1 ml/l triethylamine (65:35, v/v)	Ex:470 nm, Em:537nm LOQ, 0.01 µg mL^{-1}	human plasma	[32]
Trimetazidine	Sample + borate buffer 0.05 M; pH 10.6 100 µL NBD-Cl,dichloromethane 60 °C for 10 min	C18 column (5 mm, 300 A ° 4.6 250 mm, Hanbon Science and Technology. Co., Ltd., Jiangsu, China)., elution:isocratic, eluent: acetonitrile-water containing 0.1% trifluoroacetic acid (TFA) (v/v: 30/70)	Ex: 476 nm, Em:533nm, LOQ, 2.0 ng mL^{-1}	Plasma	[33]
Paroxetine	Sample + 0.2 M borate buffer pH 8.5 + 0.2 mL NBD-Cl 70 °C for 5 min	Shimpack CLC-C18 (150 mm × 4.6 mm) column, Elution:isocratic eluent: methanol sodium phosphate buffer (0.05 M; pH 2.5) containing 1 ml/l triethylamine (65:35, v/v)	Ex:470 nm, Em:537nm LOQ, 0.01 µg mL^{-1}	human plasma	[34]
Sertraline	Sample + 0.2 M borate buffer pH 8.5 + 0.2 mL NBD-Cl 70 °C for 5 min	C18 column (5 mm, 300 A ° 4.6 250 mm, Hanbon Science and Technology. Co., Ltd., Jiangsu, China)., elution:isocratic, eluent: acetonitrile-water containing 0.1% trifluoroacetic acid (TFA) (v/v: 30/70)	Ex: 470 nm, Em: 537nm LOQ 0.5 ng mL^{-1}	Human plasma	[35]
Hydroxyproline	Sample + . buffer pH 8.0 + NBD-Cl 5 mg/mL, , 60 °C for 5 min	Shimpack CLC-C18 (150 × 4.6 mm) column, Elution: isocratic Eluent sodium phosphate buffer (0.05 M; pH 2.4): methanol (35:65 v/v)	EX:470 nm, Em: 530nm 0.95 ng mL^{-1}	meat	[38]
Amino acids	Sample + borate buffer, pH 8+ NBD-Cl 70°C for 30 min	Phenomenex C18-column; 5 lm (250 mm · 4.6 mm i.d.) Elution;	Ex: 470 nm, Em:530nm 4.14 ng/Ml	islets	[39]
Domoic acid	Sample + borate buffer			mussels	

	pH 8 + NBD-Cl 70°C for 30 min Sample + 4 mL borate buffer pH 7.9 + 300 lL NBD-Cl (0.05%, w/v) 70 °C for 30 min Sample+ borate buffer, pH 10.4 + 250 mL NBD-C, 60 (+1) °C, 5.0 min Sample + 0.1 M borate buffer pH 8.2 + 100 µL of NBD-Cl 60 °C for 60 min Sample + borate buffer pH 10.0. + 9.0 mM NBD-Cl 90 °C	isocratic elutient: acetonitrile nitric acid (pH 3) solution Shimpack CLC-ODS 150 mm × 4.6 mm ID., 5 µm particle size, elution: isocratic Eluent: methanol , sodium phosphate buffer (0.05 M; pH 2.8) containing 1 mL/L triethylamine (72:28 v/v) Nucleosil CN column, Elution: isocratic Eluent; acetonitrile-10 mM sodium acetate buffer (pH 3.5)-methanol (47 + 47 + 6, v/v/v) Nucleosil CN column eluetuion: isocratic eluent: mobile phase acetonitrile-10 mM sodium acetate buffer (pH 3.5)-methanol (47 + 47 + 6, v/v) Nucleosil C8 analytical column (150 mm 9 4.6 mm i.d., 5 lm particle diameter) elution: isocratic Eluent: acetonitrile—10 mmol L-1 sodium acetate buffer (pH 3.5)—tetrahydrofuran (40:40:20,v/v) Varian/Rainin C-18 column (89-200-E3) 10 cm x 4.6mm ID, elution gradient Eluent: (0.1M, pH 7.2) methanol tetrahydrofuran (900 :95 : 5 v/v/v) A Phenomenex C18(2) Luna (25064.6 mm, 5 lm elution: gradient, Elutent: phase A: 25 mM acetate buffer, pH 5.5; mobile phase B: acetonitrile. Nucleosil C18, 250 mm × 4.6 mm, 5 µm, 100 Å column elution: isocratic eluent: 0.1% trifluoroacetic acid (TFA) in 87:13 water: acetonitrile	Ex:470 nm, Em:531 nm LOD 4.28 ngmL^{-1} Ex:456nm, Em:535 nm LOQ 0.0027 ng mL^{-1} Ex:470nm, Em:540nm ,LOQ 6.1–51 nM. Ex: 469 nm, Em: 529nm LOD 25 ppb		

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)

NBD-F was synthesized as a fluorescent reagent by Nunno et al. in 1970 (41), and introduced 30 years ago by Imai and Watanabe (8) 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 [42, 43] peptides [44] a range of proteins [45] and simultaneous determination of amino acids as well as amino glycoside [46-48]. The reactivity of NBD-F with amines was reported to be higher than that of NBD-Cl and recommended for the sensitive determination of amines [9)].

Application of NBD-F in HPLC

Analysis of amines

A new HPLC method with pre-column derivatization of the sample with a fluorescent tagging reagent, NBD-F is described for the determination of agmatine, an endogenous neuromodulator [49].The resultant derivative has high photostability and favorable excitation maximum (480 nm, close to an argon ion laser line at 488 nm), which allows the use of laser-induced fluorescence (LIF) detection. The method was validated and applied for the determination of agmatine in biological samples including human plasma, rat brain and stomach tissues.

The application of NBD-F derivatization for the quantitative analysis of catecholamines and related compounds in biological matrices was demonstrated [50]. The reaction conditions were optimized and were found to be 12.5 mM borate buffer pH 8.0 in water:acetonitrile (1:1) at 50°C for 5 min.

HPLC method for the determination of Fluoxetine (FLX) and the N-desmethyl metabolite, norfluoxetine (NFLX) in rat brain microdialysis samples with fluorescence detection using pre-column derivatization with NBD-F was demonstrated [51]. The detection limits for FLX and NFLX were approximately 17 and 5 nM (23 and 7 fmol), respectively. The relative recovery of FLX across microdialysis membrane was enhanced by adding β-

cyclodextrin (β-CD) or β-CD polymer to microdialysis perfusion fluid. [52-54].

Another HPLC method using a Cholester column and fluorescence detection for the determination of Fluoxetine (FLX), norfluoxetine (NFLX) in human serum and urine was reported by Higashi et al. [55]. The samples were extracted in basic medium with pentane and then derivatized with NBD-F in borate buffer (pH 8.5) at 70 ºC for 2 min. The linear range of the method for FLX and NFLX were determined to be of 0.01-0.5 μg mL^{-1} with the detection limit of 0.005 μg mL^{-1}, and in the range of 0.005-0.5 μg mL^{-1} with the detection limit of 0.002 μg mL^{-1}, respectively. The sensitivity of the method was less than has been obtained with other NBD related labeling agents [22, 57], whereas the derivatization procedure was much quicker.

Higashi et al. [56] investigated the quantitative determination of fluvoxamine (FLU) in 100 μL of rat plasma by isocratic HPLC coupled with fluorescent detection using NBD-F as a labeling reagent. In this method extracted plasma samples were mixed with NBD-F at 60°C for 5 min and injected into HPLC. The calibration curve was linear in the range of 0.015-1.5 μgmL^{-1} and the lower limits of detection and quantification of FLU were 0.008 and 0.015 μgmL^{-1}, respectively, in 100 μL of plasma. The method has advantages over previously derivatization methods using dansyl chloride [57] and 4- (*N*-chloroformylmethyl-*N*-methyl)-amino-7-nitro-2,1,3-benzoxadiazole [58] in terms of sample volume and speed.

HPLC method for assay of amantadine and its four related compounds [2-adamantanamine hydrochloride (2-ADA), 1-adamantanmethylamine (ADAMA), 1-(1-adamantyl)ethylamine hydrochloride (rimantadine) and 3,5-dimethyl-1-adamantanamine hydrochloride (memantine)] in phosphate-buffered saline using NBD-F as a fluorescent derivatization reagent was developed [59]. Five derivatives were well separated from each other. The lower limits of detection of amantadine, 2-ADA, ADAMA, rimantadine and memantine were 0.008, 0.001, 0.0008, 0.0015 and 0.01μg mL^{-1}, respectively.

The results obtained demonstrate that NBD-F is a good candidate as a fluorescent reagent for simultaneous HPLC assay of AMA and its four related compounds compared to previously derivitizing agent o-phthalaldehyde [60] and dansyl chloride [61].

Development of a new automated highly sensitive and accurate HPLC method with fluorescence detection for the determination of reboxetine (RBX) in plasma was described [62]. The method involved a pre-column derivatization with NBD-F, and an automated highly efficient on-line pre-treatment procedure by using a bio-sample analysis column-switching system (co-sense for BA) equipped with a Shim-pack MAYI-ODS bio-sample pre-treatment column. The linear range was determined to be 2-500 ng $mL^{-1}$with limits of detection and quantification of 0.5 and 1.7 ng mL^{-1} respectively. The method was successfully applied to the determination of RBX in spiked human plasma samples. The outstanding feature of the method is the no need for the extraction of RBX from the plasma and no need to use a guard column to protect the analytical column against the huge amount of plasma proteins. As well, the run-time was short (<12 min).

Analysis of amino acids

Fluorometric detection combined with fluorescence derivatization has been the most popular method for amino acid analysis. This is due to its simplicity and convenience and the higher sensitivity than MS.NBD-F reacts easily with amino group and gives highly fluorescent derivatives in short time. In this section the application of NBD-F as derivatizing reagent for analysis of amino acids will be presented.

A new HPLC method assay for plasma arginine-vasotocin (AVT) and isotocin (IT) based on fluorescence detection was established [63]. After solid-phase extraction (SPE) the sample was derivatized with NBD-F. The separation was carried out on a reversed-phase column with solvent gradient system. The assay was linear in the range of 15-220 pmol for AVT and 10-220 pmol for IT and detection limits of AVT and IT were 0.8 and 0.5 pmol respectively. It has been claimed that the assay can also be applied to plasma and tissue samples from other animals with only minor modification.

Aoyama et al. [64] has developed a fully automated amino acid analyzer using NBD-F as a fluorescent derivatization reagent. The derivatization reaction conditions were optimized and were found to be 20°C when using pH 9.5 borate buffer, 30% of acetonitrile, 3 mm of NBD-F with 40 min of reaction time. Automated derivatization reaction under these conditions provided good linearity and reproducibility. The calibration curves were linear in the range of 20 fmol to 20 pmol. The method expects to have contribution for the studies related to amino acids in wide areas of science.

A new, simple and highly sensitive HPLC method for measuring the quantity of hydroxyproline (Hyp) in small quantities of skin obtained in routine clinical procedures was developed [65]. The method was based on derivatization of Hyp with, NBD-F, and with the isocratic HPLC enables to detect Hyp in less than 1 mg of skin tissue and is clinically useful for ensuring accurate diagnosis and for monitoring specific skin conditions using small human skin samples collected in biopsies. The method has great clinical application in the diagnosis and assessment of disease states and the formulation of treatments for patients with scleroderma, keloid and Ehlers-Danlos syndrome, where the etiology of the diseases is related to the up-regulation of collagen synthesis in the skin. This method was also useful for measuring Hyp levels in other organs with results similar to previously reported values [66].

A column-switching HPLC method for a highly sensitive determination of three methylated arginines in rat plasma (30 μL) was developed [67]. A good linearity for calibration curves for each methylated arginine was observed within the range of 50-5000 fmol using homoarginine as an internal standard. The method did not need sample pre-treatment using solid phase extraction, which provided us with high precision and accuracy. The method has advantages over the previously reported method (68) in that did not need sample pre-treatment

using solid phase extraction, which was time consuming and complicated.

Higashi et al. [69] also reported a sensitive method for the determination of 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP) a metabolite of haloperidol, in a rat biological sample. After basic extraction of the samples with benzene, the derivatization with NBD-F was conducted in borate buffer (pH 8.0) at 60°C for 3 min. Mexiletine was used in the procedure as an internal standard. The regression equation for CPHP showed a good linearity in the range of 0.03-1 μg mL^{-1} with a detection limit of 0.008 μg mL^{-1}. The author's anticipate that the method will be utilized for therapeutic drug monitoring of CPHP in plasma from haloperidol treated patients with schizophrenia.

A simple and rapid column-switching HPLC method for the determination of N^G,N^G-dimethyl-l-arginine (ADMA) in rat plasma using a monolithic silica column instead of conventional particulate columns was developed [70]. The NBD-derivatized ADMA was trapped on a cation-exchange column and separated within 15 min on a monolithic silica column. The use of monolithic silica columns enables high flow rate (6 mL/min) analysis holding competent chromatographic characteristics. A good linearity for calibration curve for ADMA was observed within the range of 140 nM (1.0 pmol per injection) – 140 μM (1.0 nmol per injection) using *N*G-monomethyl-l-arginine (l-NMMA) as an internal standard. The proposed method was successfully used for the quantitative determination of ADMA in rat plasma. The concentrations of ADMA in four different rat plasma were 0.82±0.045 μM, in consistent with the previous reports [67, 68].

The HPLC method for determination the level of 4-(4-bromophenyl)-4-hydroxypiperidine (BPHP), a bromperidol (BRO) metabolite, in rat plasma after pre-column derivatization with NBD-F was described [71]. The samples were extracted with benzene; in borate buffer (pH 8.0) at 60°C for 3 min. Mexiletine was used as an internal standard (IS). The linear range of the method was determined to be 0.01-1 mg mL^{-1}. The detection limit of BPHP was 3.3 to 6.7- fold improved compared with previous data [72] and about 3 times better to that of CPHP [69]. The method was applied for a pharmacokinetic study of BPHP in comparison with 4-(4-chlorophenyl)-4-hydroxypiperidine (CPHP), the corresponding haloperidol (HAL) metabolite, in rats.

HPLC with fluorescence detection have been developed to quantify the N-dealkylated basic metabolites, such as 1,3-dihydro-1-(1,2,3,6-tetrahydro-4- pyridinyl) -2H-benzimidazole-2-one, 1-phenyl-1,3,8-triazaspiro[4.5]decan-4-one, 4-(4-chlorophenyl)-4hydroxypiperidine, and 4-(4-bromophenyl)-4-hydroxypiperidine, of droperidol, spiperone, haloperidol, and bromperidol, respectively, in rat plasma. The method is based on precolumn derivatization of the analytes with NBD-F (73). The derivatives of four basic metabolites and the internal standard were well separated from each other in less than 48 min. The author claims that the method will be useful for toxicological analysis and monitoring the levels of the metabolites in patients and in experimental animals.

Zhang et al. [74] has described RP-HPLC method for the determination of 3-nitrotyrosine and tyrosine in plasma. The method is based on NBD-F pre-column derivatization and a post-reaction SPE cleaning. Limits of detection of 0.5 and 1.1 nM for NO-Tyr and tyrosine (Tyr), respectively close to that achieved by LS-MS/MS were obtained [75]. The method was utilized for analysis of samples obtained from smokers and non-smoking subjects.

Simple and rapid method solid phase microextraction (SPME) coupled with HPLC with fluorescence for the analysis of anatoxin-a was reported [76]. Four kinds of fiber (100 lm polydimethylsiloxane, 60 lm polydimethylsiloxane/divinylbenzene, 50 lm Carbowax/template resin-100, and 85 lm polyacrylate) were evaluated for an efficient extraction of thetoxin. Variables affecting extraction such us ionic strength, temperature, and time have been also optimized. The linear concentration range was determined to be in range of 10-2000 ng and a limit of detection of 0.29 ng mL^{-1} in river water. The design of the SPME method developed offers advantages over reported method [77].

A rapid and simple method to determine taurine in energy drinks by pre-column HPLC was developed using a derivative of NBD-F [78]. The reaction of taurine with NBD-F finished in 10 min at 60°C. Percent recovery in the range of 98.2-99.9%, the precision as standard deviation in the range of 0.3-0.5%, were obtained.

A simple and sensitive determination method for selenomethionine (Se-Met) using an HPLC-fluorescence detection system coupled with an on-line electrochemical reactor has been developed [79]. The fluorescence intensity of NBD-Se-Met was enhanced by oxidation reaction. The method was found to be applicable in the linear range of 300 fmol to 30 pmol. Detection limit (S/N = 3) was calculated to be 50 fmol, which is comparable to that of inductively coupled plasma mass spectrometry [80-83]. The method should be useful for the determination of Se-Met in physiological samples, such as serum or urine.

HPLC-based fluorometric method for assaying hydroxymethyltransferase (SHMT) activity toward formation of serine was reported [84]. Serine formed by SHMT activity is reacted with (NBD-F) to form the fluorescent adduct NBD-serine. The fluorescent assay components are then separated by reversed-phase chromatography, and NBD–serine is quantified by comparison with standards.

Analysis of amines and amino acids enantiomers

Long et al. [85] has developed a new column-switching HPLC system with fluorometric detection for the analysis of D-amino acids in biological samples. The system consisted of the fluorogenic derivatization of amino acids with NBD-F and two chromatographic steps, one that separates individual amino acids in reverse phase mode and another that separates the chiral forms of each amino acid in normal-phase mode. The method was applied to determine D- and L-Aspartate levels in cell culture medium, and within cells of mouse proximal tubule

(MPT1) cell line. The method was found to be applicable in the linear range of 12.5 to 250 nM for both D- and L-Asp, with detection limit for D-Asp in culturing medium was 5 nM. HPLC system described can also be employed for the analysis of other amino acids after minor modification(s). The automated column-switching system introduced minimized the analysis time by omitting the tedious manual operations involved in sample preparation. The reliability and sensitivity of the system was compared with previously reported method [86].

Highly sensitive and automated column-switching HPLC system for determination N-methyl-D- and -L-aspartate (NMA) was reported by Sekine et al. [87]. The system comprises fluorescent derivatization of NMA with NBD-F and two chromatographic stepslinked by an automated column-switching system. The sample was treated with o-phthalaldehyde to remove primary amino acids that can interfere with sensitivity. The method was employed to determine the levels of NMDA in tissues from bivalves and the results obtained were consistent with the values reported previously. The detection limit of the method was found to be 5 fmol. The automated column-switching HPLC system described was reliable and highly sensitive compared to other reported methods [88, 89] and is suitable for detailed studies of NMA in living organisms.

Selective HPLC method with a column switching for the determination of S-D-lactoylglutathione (SLG), an intermediate metabolite of the glyoxalase system, in rat blood was described [90]. A single ODS column was not able to separate the peak of SLG from endogenous compounds, thus column-switching HPLC system was adopted NBD-SLG was efficiently separated by two different types of columns, a reversed-phase and an anion-exchange column.Calibration curves for the determination of SLG showed a good linearity) over the range of 20-100pmol SLG spiked in rat blood samples.The LOD and the LOQ were 8.12 and 27.07 fmol, respectively.The concentration of SLG in 30 μL of blood samples was successfully determined.By adopting the column-switching HPLC system, tedious procedures such as concentration steps were avoided.The method developed successfully examines the change of the SLG concentration in rat blood with satisfactory precision and accuracy.

A sensitive and selective method for the determination of four threonine (Thr) isomers (L-Thr, D-Thr, L-allo-Thr and D-allo-Thr) in mammalian tissues has been reported using two-step HPLC system [91]. The method involves the pre-column derivatization of amino acids with the highly fluorescent derivatizing reagent, NBD-F, and the NBD derivatives are separated by a combination of a micro-ODS column and a chiral column. The method was used for the first time to demonstrate the tissue distributions of D-Thr, D-allo-Thr and L-allo-Thr in rat mammals. The results obtained indicate significant amounts of D-Thr and D-allo-Thr are present in the frontal brain areas and urine.

A column-switching HPLC method with fluorimetric detection has been established for the simultaneous determination of D- and L-serine in rat brain microdialysis [92]. In this method an achiral fluorogenic reagent, NBD-F was used for the fluorescence derivatization of D- and L-serine in microdialysis samples from rat brain. The sample was directly derivatized with NBD-F without deproteinization procedure. A column-switching HPLC system consisting of an octadecylsilica (ODS) column and a tandem series of two chiral columns, Sumichiral OA-2500(*S*), was employed for the enantiomeric separation of the fluorescence derivatives, NBD–D- and -L-serine. The method was successfully applied and validated for the determination of D- and L-serine in the brain microdialysis sample from awake freely moving rats.

A multi-loop 2D column-switching HPLC system combining on-line as first dimension a reversed-phase system with an enantioselective column in the second dimension for the simultaneous determination of D-Val, D-*allo*-Ile, D-Ile, and D-Leu have been established [93]. Total analysis time for the reversed-phase separation of the four target NBD-amino acids is 60 min, and the integrated enantiomer separation of each of the four analytes is completed in approximately 5 min. The separation factor (1.22–1.37) and resolution (2.72–4.29) obtained were sufficient or better than those reported in the literature [94, 95].

Sensitive and selective two-dimensional HPLC system combining a micro-ODS column and an enantioselective column after fluorescence derivatization with NBD-F was described for the determination of D-alanine (D-Ala) in rat plasma [96].

Micro-2D-HPLC procedures for the determination of d-serine (D-Ser) and D-alanine (D-Ala) have been established [97]. The method was successfully applied for the determination of D-amino acids in 6 brain tissues, 4 peripheral tissues, serum and urine of mice having various D-amino-acid oxidase (DAO) activities. The lower limits of quantitation of D-Ser and D-Ala were 500 amol, and the within-day and day-to-day precisions were less than 6.8%. The micro-2D-HPLC method established should be powerful tools for evaluating the intrinsic levels of these d-amino acids in mammals.

Recently 2D-HPLC method for the simultaneous and sensitive determination of hydrophilic amino acid enantiomers, in mammalian tissues and physiological fluids was established [98]. The amino acids were first tagged with NBD-F to the respective fluorescent NBD derivatives which were separated in the first dimension by a micro-reversed-phase column. The automatically collected fractions of the target peaks were then transferred to the second dimension consisting of a Pirkle type enantioselective column generating separation factors higher than 1.13 for all the enantiomeric target analytes. The system was successfully validated using a rat plasma sample.

The 2D-HPLC method established was a fully automated procedure enabling the complete enantioseparation of 10 hydrophilic amino acids including allo-Thr as NBD derivatives in a single two-dimensional run. Comprehensive overviews of the applications of NBD-F as labeling for determination of amines, amino acids and enantiomers by HPLC are summarized in Table 2.

Table 2 Application of NBD-F for the determination of amines, amino acids and amino acids enantiomers using HPLC

Derivatized compounds	Dearivatization conditions	HPLC	Detection	Application	References
Agmatine	Sample + borate buffer (0.1 M, pH 8.5, 100 µl)+ NBD-F (10 mM, 60 µl), 60 °C for 40 min	ZORBAX C_8, 250 mm×4.6 mm i.d. HPLC column. Elution: gradient c, eluent: 30% A (phosphate buffer of pH 6.8) 70% B (6:2:2 mixture of methanol, acetonitrile, and water)	Ex=480 nm, Em=555 nm LOD 5×10^{-9} M	human plasma	[49]
Catecholamines	Sample + 10 µl aqueous buffer (phosphate or borate) + 10 µl NBD-F in acetonitrile, 50 °C for 5 min	Hypersil ODS (150 mm×4.6 mm i.d., 5 µm particle diameter) column: elution: isocratic eluent: acetonitrile:10 mM aqueous phosphate buffer pH 8 (60:40)	Ex=480 nm, Em=530 nm	Biological samples	[50]
fluoxetine and norfluoxetine	Sample + borate buffer (pH 8.0 20 µL 0.1 M) +30 µL 50 mM NBD-F in CH_3CN, 60 °C for 1 min.	C_{18} column, Elution gradient Eluent: CH_3CN/0.1% TFA in H_2O (10/90):CH_3CN/0.1% TFA in H_2O (90/10) (40:60 to 25:75), then CH_3CN/0.1% TFA in H_2O (90/10) from 15 to 20 min, and finally, CH_3CN/0.1% TFA in H_2O (10/90):CH_3CN/0.1% TFA in H_2O (90/10) (40:60)	Ex=470 nm, Em=540 nm	microdialysis sample serum and urine	[51]
Fluoxetine and Norfluoxetine	Sample + borate buffer (300 mL) pH 8.5 + NBD-F acetonitrile (10mM, 100 mL) 70 ˚C for 2 min	Cholester and C18-MS-II columns (Nacalai tesque, Kyoto) were 150X4.6mm i.d. with 5 µm particles, Elution; isocratic, eluent: acetonitrile (450mL) and ethanol (200 mL), water (350mL) trifluoroacetic acid (0.1 v=v%)	Ex=470 nm, Em=540 nm 0.005-0.002µgmL^{-1}, 0.40-0.06 µgmL^{-1}	rat plasma phosphate-buffered saline (pH 7.4)	[55]
Fluvoxamine	Sample + (300 µL) borate buffer, pH 8.0 + NBD-F in acetonitrile (25 mm, 100 µL) 60 °C for 5 min	Column 150X4.6 mm i.d. with 5 µm particles of C18 packing material. (Kanto Chemical,Tokyo, Japan), elution:isocratic, eluent: acetonitrile 600 mL:400 mL water containing trifluoroacetic acid (0.1% v/v)	Ex=470 nm, Em=540 nm, LOQ,0.015 µg mL^{-1}	human plasma	[56]
Amantadine related compounds	Sample + (300 µL) borate buffer, pH 8.0 + NBD-F in acetonitrile (20 mm, 100 µL) 60°C for 5 min	Column 150X4.6 mm i.d. with 5 µm particles of C18 packing material. (Kanto Chemical,Tokyo, Japan), elution:isocratic, eluent: acetonitrile (400 mL) : ethanol (200 mL) 400 mL water containing trifluoroacetic acid (0.1 v/v%)	Ex=470 nm, Em=540 nm LOD 0.0015-0.01 µg mL^{-1}	Fish Plasma	[59]
Reboxetine	Sample + 1000 µL of the borate buffer pH 8.0 + 200 µL NBD-F solution 70 °C for 30 m	pre-treatment column Co-sense Shim-pack MAY1-ODS (10mm length×4.6mm i.d., 12nm pore diameter, and 50 µm particle: diameter). Elution: isocratic elutent; acetonitrile:2% acetic acid (40:60, v/v)	Ex=470 nm, Em=530 nm LOD 0.5 ng mL^{-1} LOQ 1.7 ng mL^{-1}	rat plasma skin tissue rat plasma	[62] [63]
Arginine-vasotocin and isotocin		analytical column Hypersil 120A (250mm length×4.6mm i.d., 5µm particle diameter) manufactured by Phenomenex (USA).Eluent: isocratic, elutent: sodium acetate buffer (pH 3.5): tetrahydrofuran:acetonitrile (55:35:10, v/v/v)	Ex=470 nm, Em=530 nm LOD 0.8 and 0.5 pmol mL^{-1}	rat plasma rat plasma	[64]

			Ex=470 nm, Em=540 nm, LOD 2.8–20 fmol	rat plasma	[65]
Amino acids	Sample + borate buffer (450 µl, pH 9.5) + 50µL NBD-F in acetonitrile, 20 ˚C for 20 min	Ultrasphere ODS column (250 mm×4.6 mm i.d., 5 µm particles diameter) preceded by a precolumn (45 mm×4.6 mm i.d.) Elution gradient, Eluent: (A) 0.1% TFA in water , solvent B, 0.1% TFA in acetonitrile–water (3:1). A linear gradient was 48–80% of eluent B	Ex=470 nm, Em=540 nm	rat plasma plasma	[67] [69]
Hydroxyproline	Sample + 25 µL 200 mm borate buffer (pH 9.5), + 30 µL 10 mM NBD-F in acetonitrile room temperature, for 40 min	Cadenza CD-C18 (250 □4.6 mm i.d., 3 µm) elution; gradient eluent A, water–acetonitrile–2-propanol–TFA (90:10:0.8:0.08, v/v/v/v); eluent B, water–acetonitrile–2-propanol–TFA (90:10:5:0.08, v/v/v/v); eluent C, water–acetonitrile (90:10, v/v); eluent D, water–acetonitrile–TFA (10:90:0.08, v/v/v)	Ex=470 nm, Em=530 nm LOD 10 fmol for L-NMMA and 20 fmol for ADMA and SDMA	Water sample energy drinks rat plasma human plasma	[70] [71] [73]
Methylated Arginines	Sample + borate buffer 20 mM EDTA-2Na (pH 8.0) + 10 µl NBD-F (10 mM in acetonitrile, freshly prepared) 60 °C for 1 min	column of SC-5ODS (200 mm × 4 mm, 10 µm, EICOM Corp., Kyoto, Japan) elution: isocratic, elutent: The phosphate buffers (pH 5.0) containing 2–16% acetonitrile	Ex=470 nm, Em=540 nm LOD 0.008 µg mL^{-1}	Biological sample	[74] [76]
Chlorophenyl)-4-4)-4-hydroxypiperidine	Sample + borate buffer, 200 mmol (105 µL) (pH 9.0) + 30 ml40 mmol /LNBD-F in acetonitrile, 60 °C for 4 min	column Unison UK-C18 (150 6 4.6 mm I.D., Imtakt, Kyoto, Japan) eltion:gradient, eluent: A 50 mmol l^{-1} sodium phosphate buffer (pH 3.2) : acetonitrile (91 : 9, v/v) and B acetonitrile. 100% A; A : B 5 90 : 10 Pre-column,CAPCELLPAK MF-SCX cartridge (20 6 4.0 mm I.D., SHISEIDO). Separation column, Unison UK-C18 (150 6 4.6 mm I.D., Imtakt	Ex=470 nm, Em=530 nm LOD 36 nM Ex=470 nm, Em=540 nm	bivalve tissues	[78] [79] [84]
NG,NG-dimethyl-larginine and dimethylarginine dimethylaminohydrolase	Sample + (300 µL) borate buffer, pH 8.0+ NBD-F in acetonitrile (20 mm, 100 µL) 60°C for 3 min	Column 150X4.6 mm i.d. with 5 µm particles of C18 packing material. (Kanto Chemical,Tokyo, Japan), elution:isocratic, eluent: acetonitrile (400 mL) : ethanol (200 mL) 400 mL water containing trifluoroacetic acid (0.1 v/v%)	LOD 0.003 $mgmL^{-1}$ Ex=470 nm, Em=540 nm LOD 0.002 to 0.03 mg mL^{-1}	Rat blood	[85] [87]
Bromophenyl)-4-4)-4-hydroxypiperidine	Sample + 100 µL 200 mM borate buffer (pH 9.0), + 15 µl of 20 µM L-NMMA solution as internal standard+ ,30 µl of 120 mM NBD-F in acetonitrile 60 °C for 4 min	Chromolith™ Performance RP-18e (100 mm × 4.6 mm I.D., Merck, Darmstadt, Germany) elution: isocratic, eluent: 50 mM sodium phosphate buffer (pH 3.2)–acetonitrile (96:4, v/v)	Ex=470 nm, Em=540 nm LOD 0.5 -1.1 nM	Mammals	[90] [91]
N-dealkylated metabolites	Sample + borate buffer (300µl) + NBD-F solution in acetonitrile (20mM, 100µL) 60 °C for 3 min	Mightysil RP-18GP ODS column (150X4.6 mm i.d., 5m m, Kanto Chemical, Tokyo), elution:isocratic, eluent: methanol-water-trifluoroacetic acid (600:400:0.4, v/v/v)	Ex=470 nm, Em=530 nm 0.29 ng mL^{-1}	rat brain microdialysis	[92]
Nitrotyrosine and 3-tyrosine		Mightysil RP-18GP ODS column (150X4.6 mm i.d., 5m m, Kanto Chemical, Tokyo),	UV-Visible detector (470 nm)	mammalian tissues and physiological fluids	[93]

anatoxin-a	Sample + borate buffer pH 8.5 ,(300 mL) + NBD-F solution in methanol 20 mM, 100 µL) 60 °C for 3 min	elution:isocratic, eluent: acetonitrile (400 ml) : ethanol (200 ml) : 400 ml water trifluoroacetic acid solution (0.1 v/v%)	Ex = 470 nm, Em =540 nm LOD 50fmol	rat plasma	[94]
Taurine	Sample + potassium borate buffer 10 µL 0.02 M (PBB;pH 9.5), + 5 µL 100 mM NBD-F 55°C for 10 min	A Nova-Pak C18 column (3.9 X150 mm; 4 µm particle size; 60 Å pore size), elution: gradient, eluent: A: acetonitrile 0.02 M phosphate buffer (pH 6.5; 90:10 v/v) plus 375 µL/L trifluoroacetic acid (TFA) 5 mL/L 2-propanol, pH 4.5, B: acetonitrile and 0.02 M phosphate buffer (pH 6.5; 10:90 v/v) plus 500 µL/L TFA, pH 3.5	Ex=470 nm, Em=530 nm	human plasma	[97]
Selenomethionine (Se-Met)	Sample + buffer pH 10 + 5 µL of NBD-F. 60 °C for 15 min	Luna C18 column (5µl, 2506X4.6 mm id) from Phenomenex at room temperature, elution; isocratic, eluent: acetonitrile–water (50:50)	Ex= 470, Em=530, LOD5 nM	mammalian tissues and physiological	[98]
Serine hydroxymethyltransferase	Sample + 2 mL 0.1 mol/L phosphate buffer (pH 9.0) +1mL 1 mol/L NBD-F, 60°C for 10 min	Octadecyl silane (ODS) column. Elution:isocratic, eluent: disodium hydrogenphosphate-citric acid buffer solution (pH 5.4) containing 10mmol/l tetrabutylammonium bromide and acetonitrile (7:3)	Ex=470 nm, Em=530 nm LOD 5 fmol		
Amino acids	Sample + 25 mL of 200 mM borate buffer (pH 8.5) + 30 mL 10 mM NBD-F in acetonitrile 60˚C for 3 min	column, a Coulochem III (ESA Bioscience, Chelmsford, MA) elution: isocratic, elutent: water–acetonitrile–2-propanol–TFA (70:30:2:0.02, v/v/v/v)	Ex=459 nm, Em=532 nm, LOQ 27.07 fmol		
N-methyl-D- and -L-aspartate	Sample + 20 µl water, +25 µl 0.2 M borate buffer (pH 9.5) + 30 µl of 0.05 M NBD-F in acetonitrile 60 °C for 5 min	C18 column (4.6X100 mm, 5 µm) (a Waters Alliance 2695 separation module equipped with an XTerra): eltion: gradient, eluent: 100 mM sodium phosphate (pH 6.2), 0–3 min; methanol/ 100 mM sodium phosphate (pH 6.2, 50:50), 3–4 min; 100 mM sodium phosphate (pH 6.2), 4–6 min	Ex=470 nm, Em=530 nm		
S-D-lactoylglutathione	Sample + 40 ml 50 borate buffer, pH 9.5 + NBD-F (30 ml 50 m*M*) in acetonitrile 60°C for 5 min	an octyl silica column (RPC), eluent:isocratic, eluent: 50 m*M* sodium acetate, pH5.1 Pirkle-type chiral columns (CSPC), OA3100 or OA2500S,elution:isocratic, eluent: 7 mM citric acid in methanol.	Ex=470 nm, Em=540 nm		
D- and L-enantiomers of threonine and allo-threonine	Sample + 20 µL of 50 mM sodium borate buffer pH 9.5 + NBD-F (10 µL 10 mM) in dry acetonitrlie, 60 °C for 3 min	octadecyl silica column (TSK gel ODS 80Ts, 250×4.6 mm i.d., 5 µm. Tosoh) elution: gradient eluent: solvent A : 1% tetrahydrofuran (THF) 0.02% TFA in 10% acetonitrile, solvent B 1% THF and 0.02% TFA in acetonitrile a Pirkle-type chiral stationary-phase column (OA4600, CS 10 mM citric acid in methanol:acetonitrile (40:60, v/v)	Ex=470 nm, Em=530 nm		
	Sample + 20 µl of				

D- and L-serine	100 mM phosphate buffer (pH 7.5) + 40 µl (20 mM), NBD-F in acetonitrile 40 °C for 3 min	Cadenza CD-C18 (150 × 4.6 mm i.d, Imtakt, Tokyo, Japan) elution; gradient, Eluent: (A) 0.5% CH_3COOH aq/MeOH/CH_3CN=92.5/5/2.5 (B) CH_3CN, 0.8 ml/min. and Inertsil NH_2 (150 × 4.6 mm i.d, GL Sciences, Tokyo, Japan) 2% HCOOH aq/CH_3CN=25/5 (B) 4% HCOOH aq/CH_3CN=50/50	Ex= 470nm, Em=530 nm		
aliphatic D-amino acids	Sample + 20 µl of 200 mM sodium-borate buffer (pH 8.0) + 10 µl 20 mM NBD-F in dry MeCN 60 °C for 2 min	Capcell pak C18-AQ (250 mm × 2.0 mm i.d., Shiseido, Tokyo, Japan) MeCN-TFA-water (12/0.08/88, v/v) a Sumichiral OA-2500S (250 mm × 4.6 mm i.d., Sumika Analytical Center, Osaka, Japan) eluent:1 mM citric acid in the mixed solution of MeOH–MeCN (60/40, v/v),	Ex=470 nm, Em=530 nm LOD 0.5 ng mL^{-1} LOQ 1.7 ng mL^{-1}		
D-alanine (D-Ala)	Sample + 20 µL 0.1 m borate buffer (pH 8.0), +20 µL 10 µm l-cysteinic acid as an internal standard (I.S.) in H2O, + 60 µL 50 mm NBD-F in CH3CN 60°C for 2 min.	Analytical column was a monolithic-ODS column (750mm×0.53mm I.D., prepare in a fused silica capillary, provided from Shiseido) elution:isocratic, eluent: for NBD-Ser was MeCN–TFA–water (5:0.05:95, v/v/v), and that for NBD-Ala was MeCN–TFA–water (15:0.05:85, v/v/v).	Ex=470 nm, Em=530 nm		
D-serine and D-alanine	Sample + 20 µl of 200 mM sodium borate buffer (pH 8.0) +10 µl of 20 mM NBD-F in dry MeCN 60 °C for 2 min	Sumichiral OA-2500 (*S*) and (*R*) (250 □4.6 mm i.d.), Elution; isocratic Eluent: 20 mm citric acid in MeOH' analytical column for the reversed-phase separation was a Capcell pak C18 MG II (150 mm × 1.0 mm I.D., Shiseido) elution:isocratic, elutent: THF–TFA–water (25/0.05/75, v/v/v) For enantiomer separations, Chiralpak QN-AX and Chiralpak QD-AX (150 mm × 4.0 mm I.D., Chiral Technologies Europe, Illkirch, France): 10 mM citric acid in the mixed solution of MeOH–MeCN (50/50, v/v)			
Hydrophilic amino acid enantiomers	Sample + 20 µl of 200 mM sodium-borate buffer (pH 8.0) + 10 µl of 20 mM NBD-F in dry MeCN 60 °C for 2 min.	analytical column for the reversed-phase separation was a Mightysil RP-18 GP (100 mm × 1.0 mm I.D., Kanto Chemical, Tokyo, Japan), elution:isocratic, eluent: MeCN–THF–TFA–water (10:1:0.02:89, v/v)			
	Sample + 20 µL of 200 mM sodium-borate buffer (pH 8.0) + 5 µL 40 mM NBD-F in dry MeCN 60 °C for 2 min	enantioselective column was a Sumichiral OA-2500S (250 mm × 4.6 mm I.D., Sumika Chemical Analysis Service, Osaka, Japan) 5 mM citric acid in MeOH microbore-monolithic-ODS column (750 mm × 0.53 mm I.D.) elution: isocratic, eluent:			

	Sample + 20 µl of 200 mM sodium borate buffer (pH 8.0) + 5 µl 40 mM NBD-F dry MeCN 60 °C for 2 min.	NBD-Ser was MeCN–TFA–water (5:0.05:95, v/v/v) a self-packed narrowbore-Pirkle type enantioselective column (Sumichiral OA-2500S, 250 mm × 1.5 mm I.D.), 5 mM citric acid in MeOH–MeCN (50:50, v/v), analytical column a microbore-monolithic ODS column (1000 mm × 0.53 mm I.D., prepared in a fused silica capillary, provided from Shiseido) elution: isocratic, eluent: % (v/v) MeCN and 0.06% (v/v) TFA in water enantiomer separations, a narrowbore-Sumichiral OA-2500S enantioselective column (250 mm × 1.5 mm I.D., self-packed, material was from Sumika Chemical Analysis Service, Osaka, Japan), eluent: 5 mM citric acid MeOH–MeCN (25:75, v/v)			

CONCLUSIONS

The applications of NBD-Cl and NBD-F with amines and amino acids determinations have undergone and continue to undergo major developments over the course of last 10 years. In most derivatization reactions with NBD-Cl or NBD-F, the derivatization buffer commonly used is borate buffer with pH in range 8-9. In many cases, the 2 D-HPLC with fluorescence detection combining a micro-ODS column for sample enrichment and/or clean up and an enantioselective column for chiral separation, coupled with pre-column fluorescence derivatization of the amino acids. Such system provides the optimum efficiency and selectivity for the evaluation intrinsic levels of d-amino acids in complicated tissue samples and physiological fluids, and the obtained results should be useful for designing novel drugs.

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