Focussing Review

Development of Benzofurazan−bearing Fluorescence Labeling Reagents for Separation and Detection in High−performance Liquid Chromatography

Toshimasa Toyo'oka*

Laboratory of Analytical and Bio−Analytical Chemistry, Graduate School of Pharmaceutical Sciences, and Global COE Program, University of Shizuoka, 52−1 Yada, Suruga−ku, Shizuoka 422−8526, Japan

Abstract

This review summarizes the synthesis, features and application of benzofurazan (i.e., 2,1,3−benzoxadiazole)−bearing fluorescence labeling reagents for the determination of biologically important molecules, such as biogenic amines, amino acids, carboxylic acids, thiols and drugs. Ammonium 4−fluoro−2,1,3−benzoxadiazole−7−sulfonate (SBD−F), 4−fluoro−7−aminosulfonyl−2,1,3−benzoxadiazole (ABD−F) and 4−fluoro−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−F) were synthesized from the investigation of various substituted groups at the 7−position of the 4−fluoro−2,1,3−benzoxadiazole. The fluorescence property of these derivatives was almost the same, but the reactivity and solubility were different for each reagent. The synthesized reagents were applied to the sensitive determination of biological thiols and amines, such as cysteine, homocysteine and cysteinylglycine in human plasma, glutathione in human blood cells, α−lipoic acid in animal tissues, and histamine and polyamines in hair. Furthermore, the chiral derivatization reagents, e.g., 4−(3−aminopyrrolidin−1−yl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−APy), 4−(3−aminopyrrolidin−1−yl)−7−aminosulfonyl−2,1,3−benzoxadiazole (ABD−APy), 4−(3−aminopyrrolidin−1−yl)−7−nitro−2,1,3−benzoxadiazole (NBD−APy), 4−(3−isothiocyanatopyrrolidin−1−yl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−PyNCS), 4−(3−isothiocyanatopyrrolidin−1−yl)−7−nitro−2,1,3−benzoxadiazole (NBD−PyNCS), 4−(2−chloroformylpyrrolidin−1−yl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−Pro−COCl), and 4−(2−chloroformylpyrrolidin−1−yl)−7−nitro−2,1,3−benzoxadiazole (NBD−Pro−COCl), were developed for the resolution of various chiral molecules, in terms of reactivity, separatability, handling easiness, sensitivity and selectivity. The chiral separation of various racemates was efficiently performed by reversed−phase chromatography after labeling with the chiral reagents. Some applications utilizing these reagents for the analyses of bioactive chiral compounds and drugs are also described in this paper.

Keywords: Benzofurazan−bearing labeling reagent, Fluorescence detection, Mass spectrometry, Chiral reagent, Bioactive molecule, Enantioseparation, Reversed−phase chromatography.

Introduction

High−performance liquid chromatography (HPLC) coupled with various detection systems has been widely adopted for the analyses of various molecules in many research fields, such as biological, pharmaceutical, food, and environmental [1,2]. The widening use of HPLC in the last four decades seems to be due to the advances in instrumentation, column resins, detection systems, and data processing. However, trace analysis of a target molecule in a sample containing multiple components is not very easy. A selective and sensitive detection system is thus required for the analysis of small amounts of compounds in complex matrices such as biological samples. Among the various detection systems, fluorometry is one of the most reliable for the determination of trace quantities of biomolecules. However, almost all biomolecules do not fluoresce and require conversion to fluorescent molecules. For this purpose, many fluorescence tagging reagents have been developed and successively applied to the determination of various molecules, such as amines, amino acids, carboxylic acids, thiols, carbohydrates, lipids, and nucleic acids, in pre− and post−column derivatization methods. Several fluorogenic reagents having the benzofurazan (i.

* To whom correspondence should be addressed.
TEL: +81−54−264−5656, FAX: +81−54−264−5593,
E−mail: toyooka@u−shizuoka−ken.ac.jp

e., 2,1,3−benzoxadiazole) structure have also been developed in our laboratory and used for the sensitive determination of amines and thiols [3,4].

Macromolecules, such as proteins, maintain their chirality in living organisms. Large differences in the activities of a pair of enantiomers are usually observed in biological systems. The chiral protein environment is responsible for the different reactivities of the enantiomers. The difference derived from chirality equally applies to all bioactive substances, such as drugs and agrochemicals. The majority of naturally occurring drugs are chiral molecules and are marketed as the single enantiomer. In spite of the inherent differences between pairs of enantiomers, some synthetic drugs are sold as racemates, instead of a single enantiomer [5,6]. The Food and Drug Administrations (FDA) in the USA still accepts racemates as new chemical entities [7]. However, the risk and benefit of candidate chemicals must be examined in detail, if the racemates are developed as drugs [8]. Therefore, chiral separation is equally important in chiral synthesis as in the investigations of the differences in biological effects, such as the pharmacological and toxicological properties.

Two strategies have mainly evolved for the separation of a pair of enantiomers by HPLC. The first one is the “direct method” using a chiral stationary phase (CSP) [9]. The second one is the “indirect method” based upon the diastereomer formation with a suitable chiral derivatization reagent. This indirect method involving a derivatization step is suitable for the trace analysis of enantiomers in biological samples, such as blood and urine, because a highly sensitive detection can be performed with the option of coupling analytes with suitable chiral reagents which have a high molar ultraviolet−visible (UV−VIS) absorptivity (ε), and high fluorescence (FL) quantum yield (ϕ). During the course of our studies to develop fluorescence reagents having the benzofurazan structure, several chiral tagging reagents were synthesized and used for the sensitive determination of various racemates.

The present review focuses on the feature and application of the benzofurazan bearing reagents which were developed in our laboratory over the last three decades. The synthesized reagents involve fluorogenic and fluorescence tags for various functional groups, such as amines, carboxylic acid and thiols. The chiral tagging reagents for the highly sensitive and selective determination of various enantiomers are also described in this paper. Furthermore, some examples for the analyses of achiral and chiral molecules are also mentioned.

1. Benzofurazan−bearing reagents for the analysis of achiral molecules

The usefulness of the benzofurazan structure for the fluorometric analysis of compounds containing the amino group was first demonstrated by 4−chloro−7−nitro−2,1,3−benzoxadiazole (NBD−Cl) [10] which was used for the determination of hydroxyproline and proline in blood plasma [11]. NBD−Cl equally reacts with primary and secondary amines to produce highly fluorescent derivatives. Although the excitation and emission wavelengths (i.e., ex. 470 nm and em. 540 nm) are preferable for the determination of amines in the biological samples which contain many interfering substances having fluorescence of short wavelengths, the reaction rate is slow. To improve the reactivity, 4−fluoro−7−nitro−2,1,3−benzoxadiazole (NBD−F), in which the chloro moiety of NBD−Cl was replaced with fluorine, was proposed [12]. NBD−F was 50−100 times more reactive toward amines than NBD−Cl. NBD−F is currently used for the determination of various amines and amino acids in many samples.

NBD−F also reacts with thiol compounds under mild conditions. However, the fluorescence intensity of the derivatives with thiols was extremely low, although the reaction toward thiols is very fast. The reactivity of the electrophiles (e.g., NBD−F and 2,4−dinitrofluorobenzene (DNFB)) to nucleophiles (e.g., thiol and amine) is reflected by the intensities of the electron withdrawal at the 7−position of the 4−fluorobenzofurazan. The nucleophilic activity of thiol compounds is generally stronger than that of amines. Therefore, a higher intensity of the electron withdrawal at the 7−position than the nitro group is not requested for the selective tagging of thiol compounds. Thus, the development of new fluorogenic reagents for the thiol group was carried out based upon the Hammett constants. According to the literature [13, 14], the Hammett constants for nitro, sulfonamide and sulfonic acid substitutes are 0.78, 0.62 and 0.33, respectively. However, that for dimethylsulfonamide (SO_2NMe_2) has not been established. Based on the structure, the electron withdrawing activity of dimethylsulfonamide may be higher than that of the sulfonamide group and less than the nitro group [15]. Based on these observations, the syntheses of ammonium 4−fluoro−2,1,3−benzoxadiazole−7−sulfonate (SBD−F), 4−fluoro−7−aminosulfonyl−2,1,3−benzoxadiazole (ABD−F) and 4−fluoro−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−F), having the 4−fluorobenzofurazan structure were planned for the fluorogenic tagging reagent of the thiol group (Fig. 1). SBD−F was first synthesized from the reaction of fuming sulfuric acid with 4−fluorobenzofurazan which is obtained from 2,6−difluoroaniline in two steps [16]. ABD−F and DBD−F was also obtained from the reaction of ammonia and dimethylamine, respectively, with 4−fluoro−7−chlorosulfonylbenzofurazan, derived from the reaction of 4−fluorobenzofurazan with thionylchloride (SO_2Cl_2) [15, 17].

1.1. Feature of DBD−F, ABD−F and SBD−F

DBD−F and ABD−F quantitatively reacted with thiols at 50℃

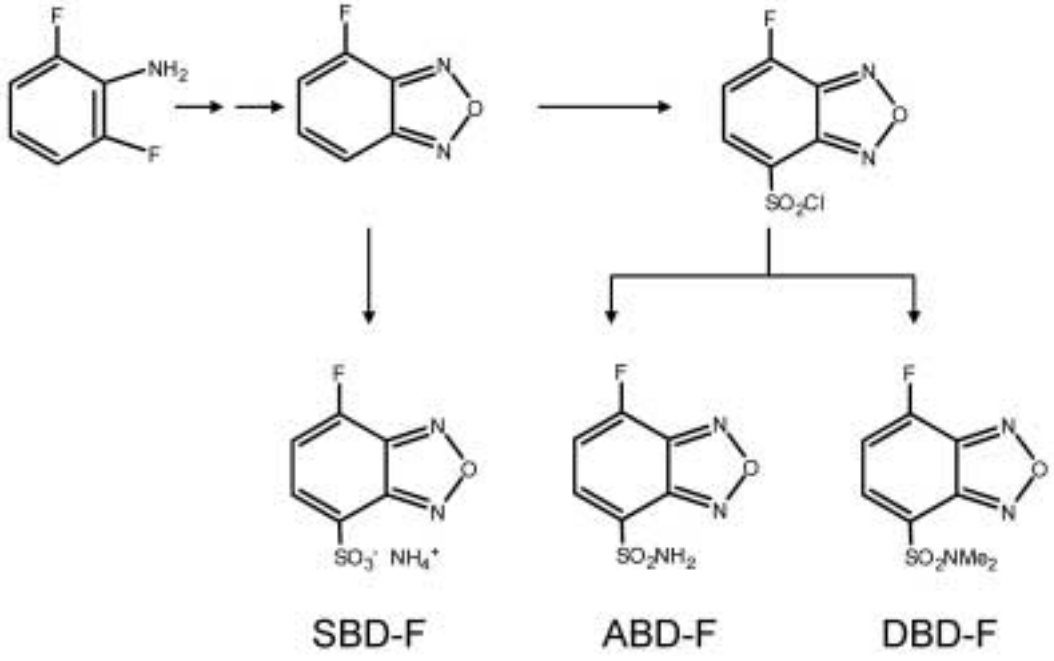

Fig 1. Syntheses and structures of SBD−F, ABD−F and DBD−F.

and pH 8.0 for 10 min. SBD−F required rather drastic conditions (at pH 9.5 and 60℃ for 1 h) for the quantitative derivatization. The pseudo−first−order reaction rate constants of homocysteine with excess DBD−F, ABD−F and SBD−F were larger in this order. The derivatives of thiols with DBD−F, ABD−F and SBD−F fluoresce intensely at 510−520 nm (excitation at 380−395 nm). The solvent composition and pH affected the fluorescence intensity and the wavelength of their derivatives. The fluorescence is high at pH 2−7 and in organic solvents rather than in water [18]. The hydrolysis products of these reagents have a negligible fluorescence. DBD−F, ABD−F and SBD−F dissolved in acetonitrile or water are stable at room temperature for at least 1 week.

Although thiol compounds are quantitatively labeled with DBD−F, ABD−F and SBD−F, a few percent of amines was also labeled with ABD−F and DBD−F under the conditions for the quantitative derivatization of thiols. The sensitivities of the ABD− and DBD−proline were extremely lower than that of the thiol adducts when the fluorescence was detected at 510 nm (excitation at 480 nm) for the ABD−derivative, and at 520 nm (excitation at 495 nm) for the DBD−derivatives. For the determinations of the amine, complete reaction was achieved after 30 min at pH 9.3 and 50℃. However, the reaction of amines with DBD−F was only 78% complete after 2 h under the same conditions [15]. The fluorescence intensity of the DBD−amine adducts was the highest at pH 2−3, and was 2.5 times higher than that of the corresponding ABD−amine adducts, but only 25% of the NBD−amine adducts. The optimum emission wavelengths of the DBD−amine and ABD−amine were 594−600 nm (excitation at 466−469 nm) and 600−613 nm (excitation at 466−469 nm), respectively. Although the reactivities of DBD−F and ABD−F to amines are limited, care should be taken in the determination of samples containing both amine and thiol compounds.

1.2. Application of DBD−F, ABD−F and SBD−F

1.2.1. Determination of thiols

DBD−F, ABD−F and SBD−F were successfully used for the selective and sensitive detection of low molecular weight thiols. An antihypertensive drug, captopril, was derivatized with SBD−F and determined by HPLC−FL [19]. Furthermore, biological thiols, such as cysteine, homocysteine and cysteinylglycine in human plasma [20, 21], glutathione in human blood cells [20], and α−lipoic acid in animal tissues [22, 23], were labeled with ABD−F, separated by reversed−phase LC and fluorometrically detected from 50 fmol to 1 pmol.

1.2.2. Simultaneous determination of thiols and disulfides

Biological thiols (reduced form) and disulfides (oxidized form) were simultaneously determined by HPLC with fluorescence detection using both ABD−F and SBD−F [24]. The strategy of the method is shown in Fig. 2. Initially, the thiols in the sample were labeled with ABD−F in an alkaline medium (pH 9.3) containing 5 mM ethylenediamine tetraacetic acid disodium salts (Na_2EDTA). After the extraction of both the unreacted and hydrolyzed ABD−F with ethyl acetate, the remaining disulfides were derivatized with SBD−F in the presence of a reducing agent, *n*−tributylphosphine

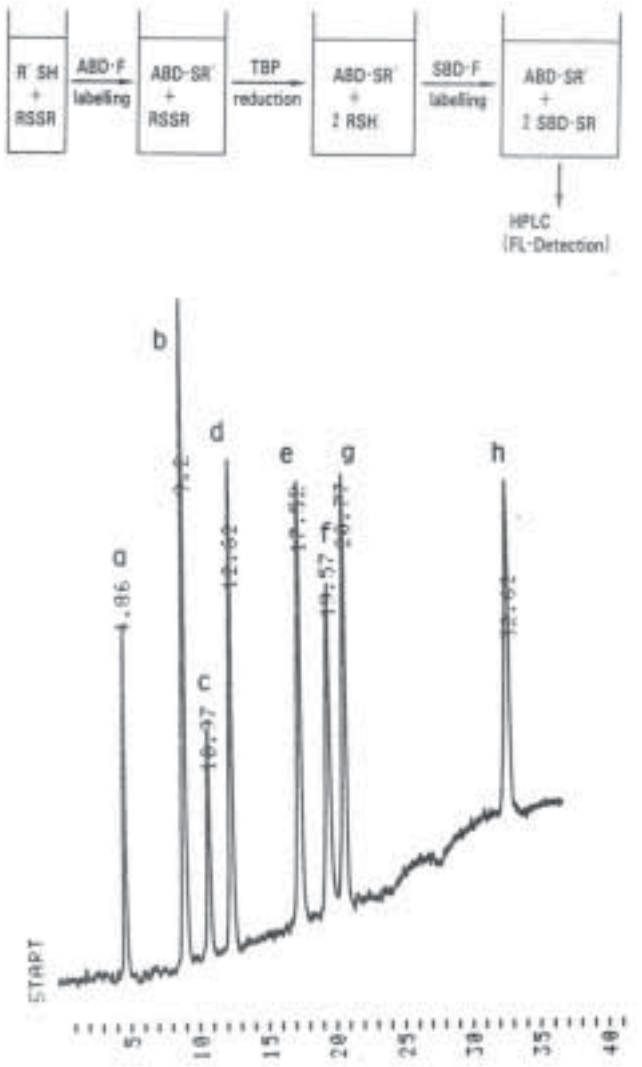

Fig 2. Strategy of simultaneous determination of thiols and disulfides, and the HPLC separation of ABD−thiols and SBD−thiols.

Peaks: a, SBD−cysteine; b, SBD−homocysteine; c, ABD−cysteine; d, SBD−glutathione, e, ABD−homocysteine; f, SBD−*N*−acetylcysteine; g, ABD−glutathione; h, ABD−*N*−acetylcysteine.

(TBP). The ABD−thiols derived from the reduced form and SBD−thiols derived from the oxidized form are separated during a single chromatographic run and fluorometrically detected (excitation at around 380 nm, emission at 510 nm), because both derivatives have almost the same fluorescence maximal wavelengths (Fig. 2). The chromatogram obtained from rat liver tissue demonstrates the capability of the simultaneous determination of the reduced and oxidized forms of glutathione by this method. The proposed procedures were applied to the simultaneous determination of thiols and disulfides in rat and hamster tissues [25].

1.2.3. Environmental analysis of cysteine residues in protein

Egg−white albumin has four cysteine residues. However, the micro−environment of each cysteine residue is not well characterized. Thus, egg−white albumin was reacted with ABD−F to determine how many cysteine residues are located on the surface of the molecule [26]. The protein was derivatized with ABD−F under mild conditions (pH 8.0 and 40℃ for 1 h) and then subjected to enzymatic hydrolysis using α−chymotrypsine. HPLC separation of the ABD−labeled fragments and amino acid sequence analysis of the peptides revealed that one cysteine residue (367 residues from the *N*−terminus) was located on the outer surface of the molecule. In the presence of 0.5 % sodium dodecylsulfate (SDS), all the cysteine residues (367, 382, 30, and 11 residues) were specifically labeled at pH 8.0 and 60℃ for 1 h, judging from the amino acid sequence analysis of the enzymic digested peptides by a gas−phase sequencer (Fig. 3). The results showed that the other three residues, except for the 367 residue, are located inside the molecule.

Cysteine containing peptides of bovine high molecular weight kininogen were also detected by the labeling with SBD−F [27]. A peptide bridged with a disulfide bond was reduced and cleaved with TBP, and then labeled with SBD−F under alkaline conditions. The resulting fluorescent peptides were separated by reversed−phase chromatography and efficiently detected at 515 nm (excitation at 385 nm).

Consequently, ABD−F and SBD−F are useful not only for the

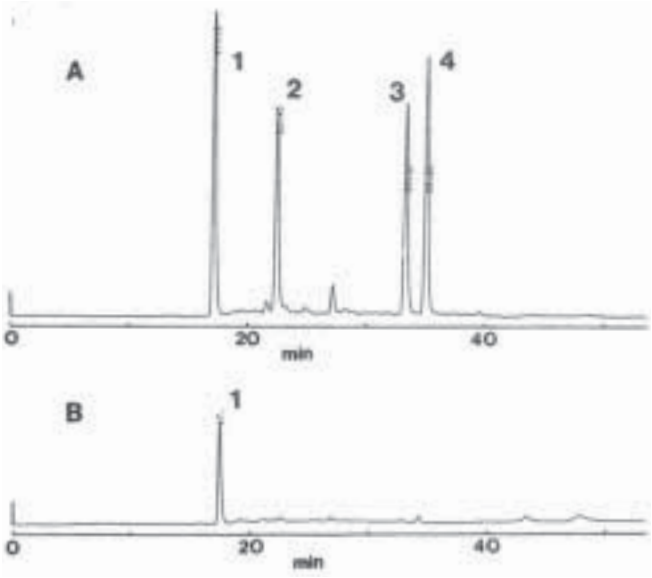

Fig 3. Chromatograms of ABD−labeled peptides obtained from α−chymotryptic digestion of ABD−egg albumins.

Chromatograms: A, ABD−peptides obtained from enzymic digestion of egg albumin labeled with ABD−F in the presence of SDS; B, ABD−peptides obtained from enzymic digestion of egg albumin labeled with ABD−F in the absence of SDS. Peaks: 1, Cys−Ile−Lys (residues 367−369); 2, Gly−Arg−Cys−Val−Ser−Pro (residues 380−385); 3, Cys−Pro−Ile−Ala−Ile−Met (residues 30−35); 4, Cys−Phe−Asp−Val−Phe (residues 11−15).

sensitive detection of low molecular mass thiols, but also for the specific and selective labeling and characterization of cysteine residues of proteins, and the isolation of trace levels cysteine containing peptides and proteins.

1.2.4. Determination of biological amines

Amines are labeled with DBD−F to produce fluorescence derivatives under mild conditions, although the conditions are relatively more severe than those for thiols. The biological amines, such as polyamines and histamine, were labeled with DBD−F at 60℃ for 30 min in a mixture of 0.1 M borax (pH 9.3) and acetonitrile (CH_3CN) [28−32]. The resulting derivatives of polyamines were perfectly separated using an ACQUITY UPLC™ BEH C_{18} column (1.7 μm, 100 mm × 2.1 mm i.d.) by a gradient elution with a mixture of water−acetonitrile containing 0.1% formic acid (HCOOH). The separated polyamine derivatives were sensitively detected by fluorometry. For the determination of polyamines in complex matrices, however, the determination of several polyamines by FL detection was interfered by endogenous substances. Therefore, the simultaneous determination of polyamines was carried out by the combination of ultra−performance liquid chromatography (UPLC) separation and electrospray−ionization time−of−flight mass spectrometry (ESI−TOF−MS) detection. The sensitivities of TOF−MS (2−5 fmol) were higher than those of the FL detection (11−86 fmol). The structures of the polyamines were identified from the protonated−molecular ions $[M+H]^+$ obtained from the TOF−MS measurement.

The rapid, sensitive and simultaneous determination of six polyamines, i.e., ornithine (ORN), 1,3−diaminopropane (DAP), putrescine (PUT), cadaverine (CAD), spermidine (SPD) and spermine (SPM), in human hairs was performed by UPLC with FL detection and ESI−TOF−MS. The proposed method was applied to the determination using the hairs of healthy volunteers (Fig. 4). The mean concentrations of ORN, DAP, PUT, CAD, SPD and SPM in 1 mg

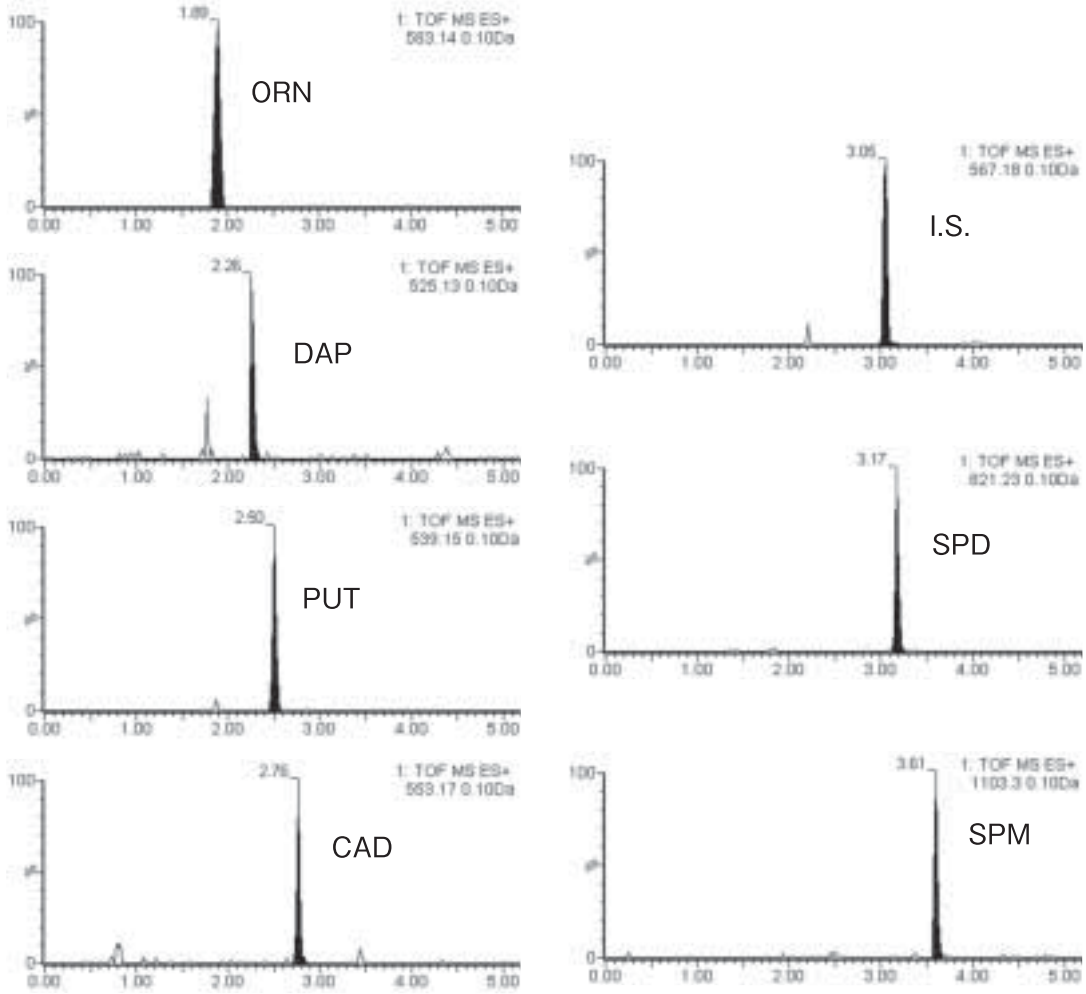

Fig 4. Selected−ion chromatograms of polyamine derivatives in human hair by ESI−TOF−MS.
Peaks: ornithine (ORN), 1,3−diaminopropane (DAP), putrescine (PUT), cadaverine (CAD), spermidine (SPD), spermine (SPM) and 1,6−diaminohexane (I.S.).

of human hairs were 1.46, 0.18, 1.18, 0.11, 1.97 and 0.98 pmol, respectively. Because the proposed method provides the trace detection of polyamines in hair, the determination of various hair samples from patients with disorders such as cancers, Alzheimer's disease and diabetes, may be possible.

The rapid determination of histamine (HA) and several metabolites, i.e., 1−methylhistamine (MHA), imidazole−4−acetic acid (IAA), and 1−methyl−4−imidazole−acetic acid (MIAA), in mice hairs was also performed by UPLC−ESI−TOF−MS [33]. HA and MHA, having a primary amino group in their structures, were labeled with DBD−F. On the other hand, 4−(*N,N*−dimethylaminosulfonyl)−7−piperazino−2,1,3−benzoxadiazole (DBD−PZ) was used for the labeling of a carboxylic acid group in IAA and MIAA in the presence of 2,2′−dipyridyl disulfide (DPDS) and triphenylphosphine (TPP). The reaction with DBD−PZ was completed at 50℃ after 2 h. The resulting derivatives of HA and the metabolites were perfectly separated using an ACQUITY UPLC™ BEH C_{18} column (1.7 μm, 100 × 2.1 mm, i.d.) with the mixture of 20 mM $HCOONH_4$ and CH_3CN (8:2). The detection limits of HA, MHA, IAA, and MIAA on the mass chromatograms were 0.21, 1.0, 0.17, and 0.11 pmol, respectively [33, 34].

The proposed method was used for the determination in the hair shafts of C3H mice. The average concentrations of HA, MHA, IAA and MIAA in 1 mg of the hair shafts were 16.3 pmol, 21.6 pmol, 6.6 pmol and 7.1 pmol, respectively [33].

The effects of hair cycle and age on the concentration of HA and the metabolites (i.e., 1−MHA, IAA, and MIAA) in the hair shaft and hair root of C3H/HeNCrj mice were next investigated by the proposed method. The concentration of HA in the hair shaft was relatively higher in the telogen phase. In contrast, the HA content in the anagen phase increased only in the hair root of old mice [33]. Therefore, HA appears to have some effect on hair growth. However, the exact reason is currently not obvious. The HA metabolites, i.e., MHA, MIAA and IAA, were also determined the same as HA. However, the difference in the metabolite concentrations between the hair cycle and age was not clear in both the hair shaft and hair root. As shown in the examples described in this section, the proposed method provides for the trace detection of amines and carboxylic acids in hair, thus this analytical technique seems to be applicable not only for the determination of polyamines, histamine and its metabolites, but also for various biological compounds in hair.

1.2.5. Determination of illegal drugs

The abuse of designer drugs has become a serious social problem in the world [35−39]. "Designer drugs" are psychoactive substances that are especially designed to circumvent existing drug laws. This is achieved by modifying the molecular structures of existing drugs to varying degrees. Various new psychotropic compounds possessing phenethylamine and piperazine structures have appeared and been distributed on the streets. Their use, especially by young persons, is one of the most serious social problems. To avoid their widespread use, some of these compounds were listed as psychotropic substances controlled as "designated substances" (Shitei−Yakubutsu) by the Pharmaceutical Affairs Law in Japan in April 2007 [40−42]. Therefore, the qualitataive and quantitative analyses are an important subject for the screening of these drugs. Based on background information, a rapid and simultaneous determination method for eleven hallucinogenic phenethylamines by HPLC with FL detection after labeling with DBD−F was developed in our laboratory [43]. The qualitative and quantitative determinations of phenethylamine in products that appear on the Japan market have been successfully performed using this method (Fig. 5). However, the simultaneous determination of very low concentrations of designated substances in complex matrices, such as plasma and urine, seemed to be rather difficult, even with highly sensitive FL detection, due to the interference from endogenous substances in the samples. Therefore, a more sensitive and selective determination method is required for bioanalysis. Furthermore, this simultaneous determination method must be simple, selective and sensitive. Thus, the simultaneous determination of fourteen hallucinogenic phenethyamines and two piperazines was tried by UPLC separation coupled with both FL and ESI−TOF−MS detection [44]. The designated drugs were labeled with DBD−F and then the resulting derivatives were simultaneously separated within 8.5 min by UPLC and sensitively determined by both FL and ESI−TOF−MS. Although individual determinations were possible using the FL detection method, the determination of all of the designated substances in human plasma and urine using FL detection failed due to interference from endogenous substances. On the other hand, a sensitive and reliable determination was carried out by ESI−TOF−MS. Because the proposed method allows for the trace detection of the designated substances in human plasma and urine, it seems to be applicable to various biological specimens.

2. Benzofurazan−bearing reagents for the analysis of chiral molecules

Various chiral compounds have been determined by a direct method that employs CSPs containing immobilized chiral selectors [45]. This method requires no complex pre−treatment, such as derivatization, and possible racemization during the separation is negligible. Therefore, the direct resolution using a CSP may be preferable for the trace analysis of the antipode enantiomer in the major chiral component. However, the separation is highly influenced by the interaction between the CSP and the enantiomers. Consequently, a lot of experience is required for the choice of the best

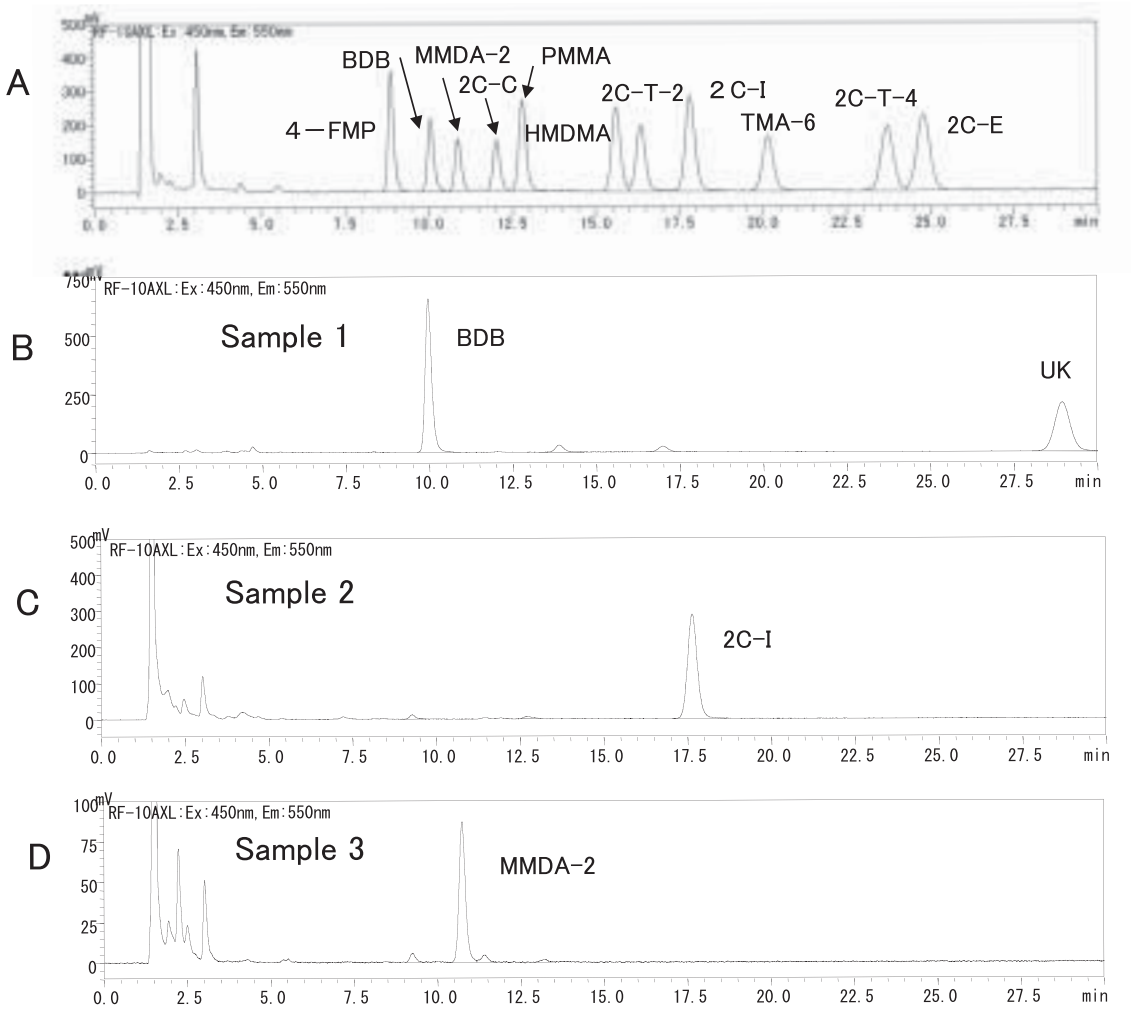

Fig 5. HPLC−FL Chromatograms of illegal drugs after derivatization with DBD−F.
Chromatograms: A, Authentic compounds; B−D, Real samples.

column for the separation of each racemate. To solve the separation difficulty, various types of CSP columns, which can be used under normal−phase and reversed−phase conditions, are now on the market. However, the high−throughput separation utilizing small particle porous silica−gel columns (less than 2 μm) in an ultra−high pressure chromatographic system is currently difficult. The elution order of a pair of enantiomers is also dependent upon the type of the CSP column and cannot be easily changed. Furthermore, the sensitivity of the direct methods is often not adequate for trace analyses of a biological specimen.

The indirect resolution of enantiomers involving a derivatization step with a chiral labeling reagent has been shown to be an efficient technique for the separation of many racemates [46−52]. A pair of enantiomers is labeled with a chiral derivatization reagent to produce a pair of diastereomers which are subsequently separated by reversed−phase chromatography utilizing conventional achiral stationary phase columns such as an ODS column.

The characteristics of the direct and indirect methods are summarized in Table 1. In spite of the many drawbacks associated with the indirect method, i.e., optical purity of the reagent, stability of the reagent, possible racemization during the labeling reaction, and commercial availability of the reagent, the good sensitivity and selectivity of the indirect method coupled with an efficient detection system are attractive for the determination of chiral molecules in real sample analyses.

The labeling with a chiral derivatization reagent is carried out by the reaction of reactive functional groups in the chiral molecules, e. g., amines (primary and secondary), carboxyl, carbonyl, hydroxyls (alcohol and phenol) and thiol. Thus, many optically active labeling reagents have been developed for each functional group in the chiral molecules [53−57].

Although various FL labeling reagents have been used for the resolution of chiral molecules, this review focused on the benzofurazan−type reagents. In the next section, the synthesis, characteris-

Table 1. Characteristics of direct and indirect methods for chiral separation

Chiral Separation Method	
Direct	Indirect
Advantages	Advantages
1. No derivatization required	1. Possible sensitive and selective detection
2. Negligible racemization	2. Possible use of conventional achiral columns
3. Easy recovery of enantiomer	3. Variable choices of chiral derivatization reagent
4. Same detector response of enantiomers	4. Easy control of elution order of the derivatives
5. Possible separation of racemate without reactive functional group	5. Increase retention time of the derivatives
6. Relative short total analytical time	6. Good chromatographic property
7. No critical chiral selector purity in CSP column	7. Variable choices of detectors (UV, FL, MS, etc.)
Drawbacks	Drawbacks
1. No universal CSP column for all racemates	1. Requires reactive funtional group in chiral molecules
2. Relatively low theoretical plates of CSP columns	2. Requires high optical purity of chiral reagents
3. Difficult control of elution order	3. Difficult recovery of original enantiomers
4. High cost of CSP column	4. Possible different detector response by a pair of diastereomers
5. Difficult speculation of elution order and retention time	5. Requires stability of both chiral reagent and the derivatives
6. Difficult high throughput separation	6. Difficult preparative application
7. No small particle column less than 3 μm	7. Time−consuming total analysis

tics and some applications of chiral derivatization reagents having the benzofurazan structure for the resolution of optical isomers by HPLC are described. The reagents include 4−(3−aminopyrrolidin−1−yl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−APy), 4−(3−aminopyrrolidin−1−yl)−7−nitro−2,1,3−benzoxadiazole (NBD−APy), 4−(3−aminopyrrolidin−1−yl)−7−aminosulfonyl−2,1,3−benzoxadiazole (ABD−APy), 4−(3−isothiocyanatopyrrolidin−1−yl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−PyNCS), 4−(3−isothiocyanatopyrrolidin−1−yl)−7−nitro−2,1,3−benzoxadiazole (NBD−PyNCS), 4−(2−chloroformylpyrrolidin−1−yl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−Pro−COCl), 4−(2−chloroformylpyrrolidin−1−yl)−7−nitro−2,1,3−benzoxadiazole (NBD−Pro−COCl), 4−(2−carbazoylpyrrolidin−1−yl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−ProCZ), 4−(2−carbazoylpyrrolidin−1−yl)−7−nitro−2,1,3−benzoxadiazole (NBD−ProCZ), 4−(*N*−prolyl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−Pro), and 4−(*N*−β−prolyl)−7−(*N*,*N*−dimethylaminosulfonyl)−2,1,3−benzoxadiazole (DBD−β−Pro), etc. (Fig. 6).

2.1. Analysis of carboxylic acid enantiomers

DBD−APy, NBD−APy and ABD−APy (*R*(−)− and *S*(+)−enantiomers) have been developed for the resolution of carboxylic acid enantiomers in HPLC [58]. The chiral derivatization reagents were quantitatively synthesized from the one−pot reaction of the optically active 3−aminopyrrolidine (APy) with the corresponding 4−fluoro−2,1,3−benzoxadiazoles (DBD−F, NBD−F and ABD−F). The reactions proceeded under mild conditions at room temperature. The optical purity of the synthesized reagents, which were determined using a CSP column, was higher than 99.5 % [59]. The high optical purity is an important factor for the trace analysis of the antipode enantiomer in a large amount of the opposite enantiomer.

Carboxylic acids, such as anti−inflammatory drugs (e.g., naproxen and ibuprofen), react with DBD−APy (*S*(+)− and *R*(−)) or NBD−APy (*S*(+)− and *R*(−)) at room temperature in the presence of DPDS and TPP as the activation agents [59]. Although the reaction time to obtain a quantitative yield is different for each carboxylic acid, a 1 hr reaction at room temperature is usually sufficient. The reaction time could not be reduced by heating. The chiral carboxylic acid was converted to the corresponding amide diastereomer. The maximal fluorescence excitation and emission wavelengths were around 470 nm and 580 nm (DBD−APy derivative), 470 nm and 550 nm (NBD−APy derivative), and 470 nm and 580 nm (ABD−APy derivative). The emission maxima shifted to a shorter wavelength region together with an increase in the organic solvent in the medium. The fluorescence intensities also elevated with the changes.

The diastereomers derived from DBD−APy were completely separated by both reversed−phase and normal−phase chromatographies [59]. The Rs values for the reversed−phase and normal−phase chromatographies using a conventional column were 1.62−

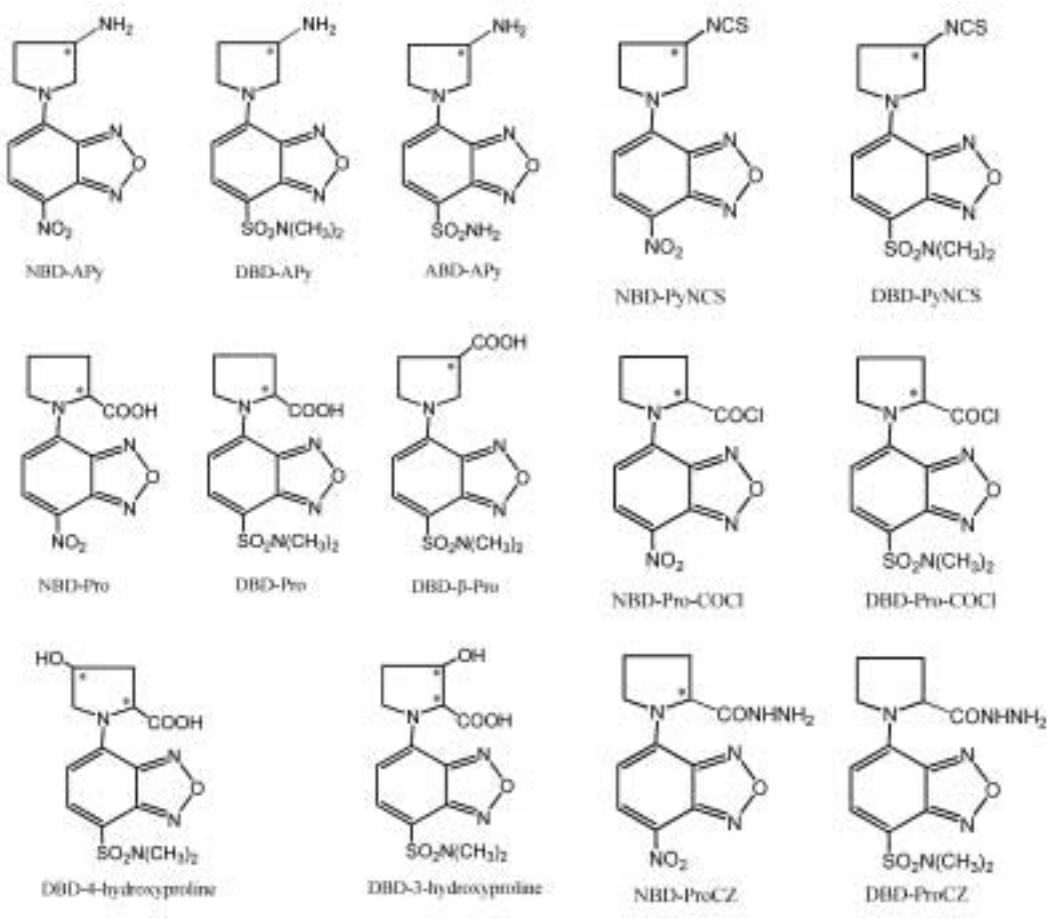

Fig 6. FL chiral labeling reagents having benzofurazan structure.

6.96 and 2.58−7.60, respectively. The elution order of the derivatives obtained from a pair of enantiomers was possible to change using the opposite enantiomer of the reagents. The detection limits of the chiral carboxylic acids were 10−30 fmol levels using a conventional FL detector. Although the complete resolutions of the enantiomers of the carboxylic acids were achieved by the normal−phase chromatography, the separation is not recommended because biological chiral molecules generally exist in an aqueous matrix.

The excitation wavelength of the NBD−APy derivative was around 480 nm and applicable for the wavelength of an argon−ion laser source. Thus, the diastereomers derived from NBD−APy provided a sensitive detection not only with conventional FL detection, but also with argon−ion (488 nm) LIF detection [59, 60]. The detection limits of the naproxen derivatives of DBD−APy, NBD−APy and ABD−APy by the LIF using reversed−phase chromatography were 10, 15 and 30 fmol, respectively. Furthermore, the use of a semi−micro column instead of the conventional column increased the sensitivity to the 1 fmol level. The laser power was another important factor to obtain a high sensitivity.

DBD−APy derivatives were also amenable to the peroxyoxalate chemiluminescence (CL) [61]. Hydrogen peroxide (H_2O_2) and an oxalate ester, i.e., bis(2,4,6−trichlorophenyl)oxalate (TCPO) or bis[4−nitro−2−(3,6,9−trioxadecyloxy)phenyl]oxalate (TDPO), were employed for the excitation of the carboxylic acid derivatives. The CL reaction depends upon the reaction pH. The optimum pH was different for each oxalate. The CL intensity obtained from TCPO−H_2O_2 was the strongest at pH 7.0, whereas the highest intensity with TDPO−H_2O_2 was at pH 6.5. As the catalytic effect of the imidazole in the CL reaction was stronger than that of phosphate buffer, which is commonly used as an eluent, the imidazole buffer was selected for the separation of the derivatives. The purity of the imidazole has a significant influence on both the level of the background emission and the variation in the baseline noise. Thorough mixing of the effluent from the column outlet and CL reagents provides a stable baseline and reproducible peaks. Thus, a rotating mixing device is recommended instead of the usual T−type mixer. The order of the detection sensitivity of the derivatives was DBD−APy>ABD−APy>NBD−APy. The detection limits of the naproxen derivatives of DBD−APy, ABD−APy, and NBD−APy were 0.5, 1.9 and 15 fmol, respectively. The determinations of *RS*−ibuprofen in rat urine and plasma were carried out as one of the applications. The resulting diastereomers derived from DL−ibuprofen were com-

pletely separated without interference by the endogenous substances [61].

2.2. Analysis of amine and amino acid enantiomers

2.2.1. Reagents of proline analogues

The derivatization of chiral amines are easily performed by the coupling reaction of a carboxylic acid in the presence of activation agents. For the resolution of chiral amines, the enantiomers of DBD−Pro and NBD−Pro were synthesized from the reaction of D− or L−proline with DBD−F or NBD−F [62, 63]. The diastereomers, derived from chiral amines and DBD−Pro or NBD−Pro, were separated by reversed−phase chromatography. However, the separation performance was not good enough for real sample analyses. To increase the separatability, several DBD−proline analogues (i.e., DBD−β−Pro, *cis*−4−hydroxyl−D(or L)−proline, *trans*−4−hydroxy −L−proline, and *trans*−3−hydroxy−L−proline) were developed [64, 65]. The separation efficiency of the derivatives varied in the chiral amines tested. DBD−*cis*−4−hydroxyl−D(or L)−proline was recommended for the separation of *RS*−1−(1−naphtyl) ethylamine; while DBD−*trans*−3−hydroxy−L−proline and DBD−*trans*−4−hydroxy−L−proline were suitable for the resolution of the DL− phenylalanine methyl ester [65]. The results demonstrated that the stereostructure, steric hindrance, hydrophobicity and hydrogen bonding of the diastereomer, etc., affected the separation. However, the detailed separation mechanism is not obvious because the interaction among the diastereomer, mobile phase and stationary phase in flow system is very complicated.

2.2.2. Reagents of the isothiocyanate type

The isothiocyanate−type reagents, such as phenylisothiocyanate (PITC), react with primary and secondary amines to produce thiocarbamoyl derivatives. PITC is a famous reagent for the amino acid sequence analysis of peptide residues. DBD−PyNCS and NBD−PyNCS, which contain the same reactive group (−NCS), were synthesized from the reaction of $CSCl_2$ with DBD−APy or NBD−APy [66]. The reaction essentially required a heat treatment, and thus, the racemization possibly occurred during the reaction. Judging from the direct resolution using a CSP column, however, the racemization was negligible, and the optical purities were greater than 99.5% for all the reagent enantiomers. These reagents exhibited an excellent stability, not only as the solid, but also in solution. No significant degradation was observed in an acetonitrile solution after storage for 2 weeks at room temperature and 1 month at 5℃ in a refrigerator.

The chiral reagents reacted with primary and secondary amino functional groups in the presence of triethylamine (TEA) to produce the corresponding fluorescent thioureas [67]. Instead of TEA, 0.5% quinuclidine, 0.5% 1,8−diazabicyclo[5.4.0] undecene (DBU), and 0.05 M borate buffer (pH 10) can also be used as the activation reagent. The derivatization reaction of these type reagents and chiral amines effectively proceeded at 55℃ for 20 min in the presence of TEA. The resulting diastereomers were well separated by reversed−phase chromatography.

The procedures using DBD−PyNCS and NBD−PyNCS were applicable for the resolution of a pair of enantiomers of primary and secondary amines, including amino acids [68], peptides [69, 70], and some drugs (e.g., β−blockers and illegal drugs) [71, 72]. For instance, the pairs of enantiomers of α,α−diphenyl−2−pyrrolidinemethanol (D2PM) and *threo*−methyl α−phenyl−α−(2−piperidyl) acetate (*threo*−MPH) were labeled with DBD−PyNCS, separated by reversed−phase chromatography and detected by fluorometry (Fig. 7). Furthermore, β−blockers were clearly separated and detected by reversed−phase HPLC−FL. The β−blockers with the *iso*−propylamino moiety (i.e., oxoprenolol, propranolol, alprenolol,

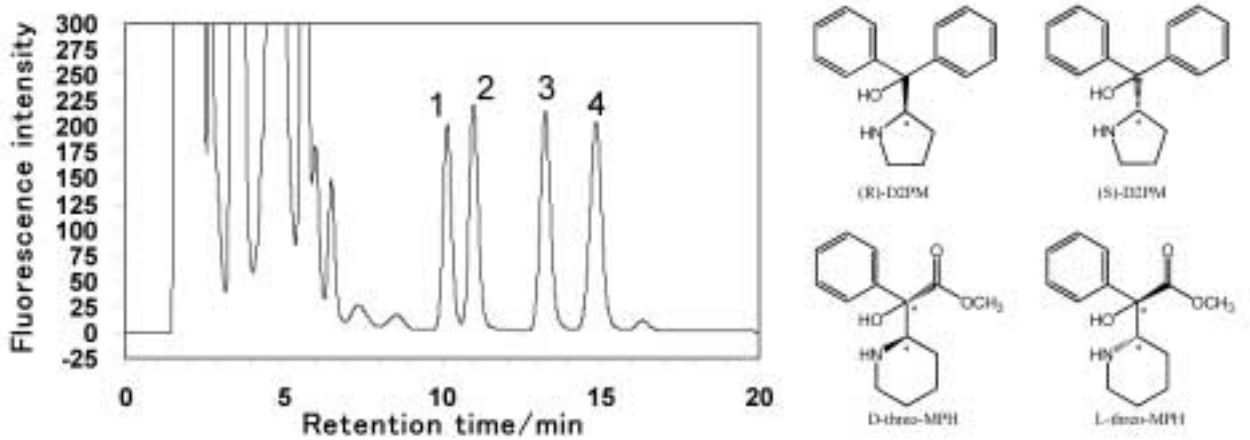

Fig 7. Structures of *RS*−D2PM and DL−*threo*−MPH, and the enantioseparations after derivatization with *R*(−)−DBD−PyNCS. Peaks: a, D−*threo*−MPH; 2, L−*threo*−MPH; 3, (R)−D2PM; 4, (S)−D2PM.

atenolol, indenolol, and pindolol) were detected at lower amounts (16−320 fmol) than the β−blockers with the *tert*.−butylamino moiety (i.e., bupranolol, bucmolol, carteolol, timolol) (1.25−8.0 pmol). The limited sensitivity may be due to the lower chemical yields based on the steric hindrance around the reaction site of the β−blockers. Consequently, care should be taken when tagging to such hindered compounds.

The determination of *RS*−propranolol in rat plasma and saliva after its oral administration (10 mg/kg) were performed as one such application [71]. Only *R*−propranolol was detected from the samples, obtained from the *R*−isomer administration. On the other hand, not only *S*−propranolol, but also *R*−propranolol appeared on the chromatograms of the samples of the *S*−propranolol administration. Based on this observation, *S*−propranolol might be enzymatically converted to the *R*−isomer in a biological system.

R(−)−DBD−PyNCS was used for the separation and detection of DL−amino acids [68, 69, 73]. The Rs values of the amino acids were in the range of 0.55−3.57 for the diastereomers obtained from NBD−PyNCS, and 0.68−2.57 for those from DBD−PyNCS. The Rs values obtained from the neutral and aromatic amino acids were higher than those of the basic and acidic amino acids. The diastereomers corresponding to the *R*−configuration eluted faster than those of the *S*−configuration with the *R*−enantiomer of the reagent. The opposite elution order was obtained from the use of the *S*−enantiomer of the reagents. Because the total separation of 18 DL−amino acids in a single chromatographic run was fairly difficult, the separation was carried out after classifying into hydrophilic and hydrophobic DL−amino acids (Fig. 8). β−Ala and 6−amino−*n*−caproic acid were used as the IS for the hydrophilic (i.e., His, Arg, Ser, Thr, Gly, Glu, Asp, Ala, and Pro) and hydrophobic (Tyr, Val, Met, Ile, Leu, Phe, Trp, and Lys) amino acids, respectively. The best elution profile for the separation of the hydrophilic amino acids was by an isocratic elution with water−methanol−acetonitrile (180:21:49) containing 0.1 % TFA as the eluent. For the hydrophobic amino acids, linear gradient elutions using 25 mM sodium acetate (pH 5.2) and acetonitrile were the best [68].

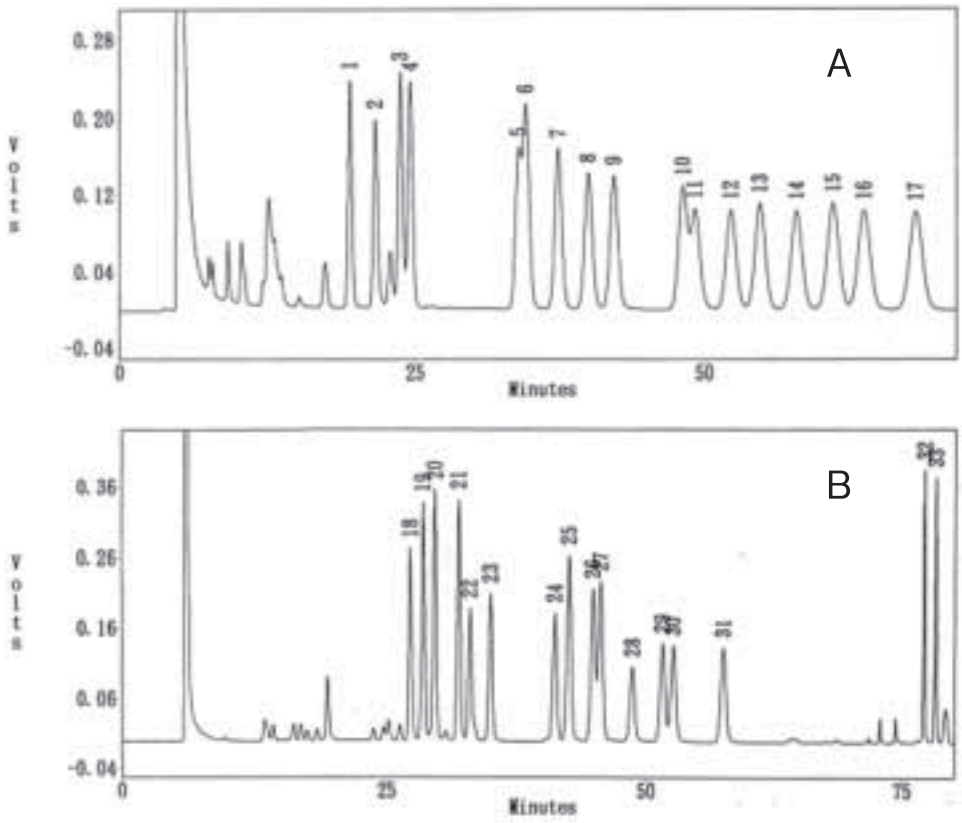

Fig 8. Reversed−phase HPLC separation of DL−amino acids after derivatization with *R*(−)−DBD−PyNCS. Chromatograms: A, Separation of hydrophilic amino acids by an isocratic elution; B, Separation of hydrophobic amino acids. Peaks: 1, L−His; 2, D−His; 3, L−Arg; 4, D−Arg; 5, D−Ser; 6, L−Ser; 7, Gly; 8, D−Glu; 9, L−Glu; 10, D−Thr; 11, D−Asp; 12, L−Asp; 13, L−Thr; 14, D−Ala; 15, D−Pro; 16, L−Ala; 17, L−Pro; 18, D−Tyr; 19, L−Tyr; 20, D−Val; 21, L−Val; 22, D−Met; 23, L−Met; 24, D−Ile; 25, D−Leu; 26, L−Ile; 27, L−Leu; 28, D−Phe; 29, D−Trp; 30, L−Phe; 31, L−Trp; 32, D−Lys (disubstituted derivative); 33, L−Lys (disubstituted derivative).

D−amino acids in human urine and some foodstuffs were determined using the conventional reversed−phase HPLC−FL system [73]. A relatively high concentration of D−serine was detected in human urine, and this result was similar to a previous report. A couple of D−amino acids, i.e., D−aspartic acid, D−glutamic acid and D−alanine, also appeared on the chromatograms of fermented foodstuffs. Although a long run time was required for the separation of the amino acid mixture by conventional HPLC, the high−throughput separation is currently carried out by using an ultra−high pressure HPLC system with a sub−2 μm column [74, 75].

A nail analysis provides an important means for determining the individual past history of long−term chemical exposures [76, 77]. The determination of drug abuse, such as cocaine and amphetamine, in the human nail have been performed the same as hair analyses. The determination of DL−amino acids in the human nail of diabetes patients (age: 40−82; 3 men and age: 58−76; 3 women) and healthy persons (age: 69−83; 3 men and age: 47−78; 2 women) was tried by UPLC−FL and UPLC−ESI−TOF−MS after derivatization with *R*(−)−DBD−PyNCS [78, 79]. Although a long time was required for the separation of the amino acid mixture by the conventional HPLC system, the high−throughput separation is currently carried out using an ultra−high pressure HPLC system. Therefore, the UPLC system was used for the simultaneous separation of DL amino acid derivatives. The highly sensitive detection of the derivatives was carried out by the FL detection after separation by UPLC. However, the determination of several DL−amino acids in human nails by FL detection was interfered by the peaks corresponding to endogenous substances. Thus, the simultaneous determination of DL−amino acid derivatives was attempted by UPLC−ESI−TOF−MS. Fifteen kinds of L−amino acids and 5 kinds of D−amino acids, which were D−Leu, D−Ile, D−Ala, D−Pro and D−Val, were detected in human nails. When the amounts of the DL−amino acids were compared with healthy volunteers and diabetes patients, there was no significant difference in the L−amino acids in the nail. However, the quantity of the D−amino acids in diabetes patients obviously increased when compared to those in healthy volunteers (Fig. 9).

In the case of the diastereomers obtained from NBD−PyNCS, the maximal excitation and emission wavelengths of the diastereomers were 490 nm and 530 nm, respectively. Therefore, the diastereomers were also determined by capillary electrophoresis (CE)−LIF detection [80]. The DL−amino acid derivatives were sensitively detected at around 50 nM. The method was applied to the determination of D−proline and D−aspartic acid in rabbit plasma.

2.3. Analysis of peptides

Peptide mixtures were analyzed by DBD−PyNCS and NBD−PyNCS, the same as the DL−amino acids [69]. The DL−amino acid composition derived from the hydrolysate of the peptide seemed to be possible by a method using the isothiocyanate reagents. However, the analysis was difficult because racemization occurred during both vapor−phase hydrolyses using 7 M HCl and 10% TFA and liquid−phase hydrolysis using constant−boiling HCl. The racemization ratio of the vapor−phase procedure was higher than the liquid−phase hydrolysis. Furthermore, the racemization depended

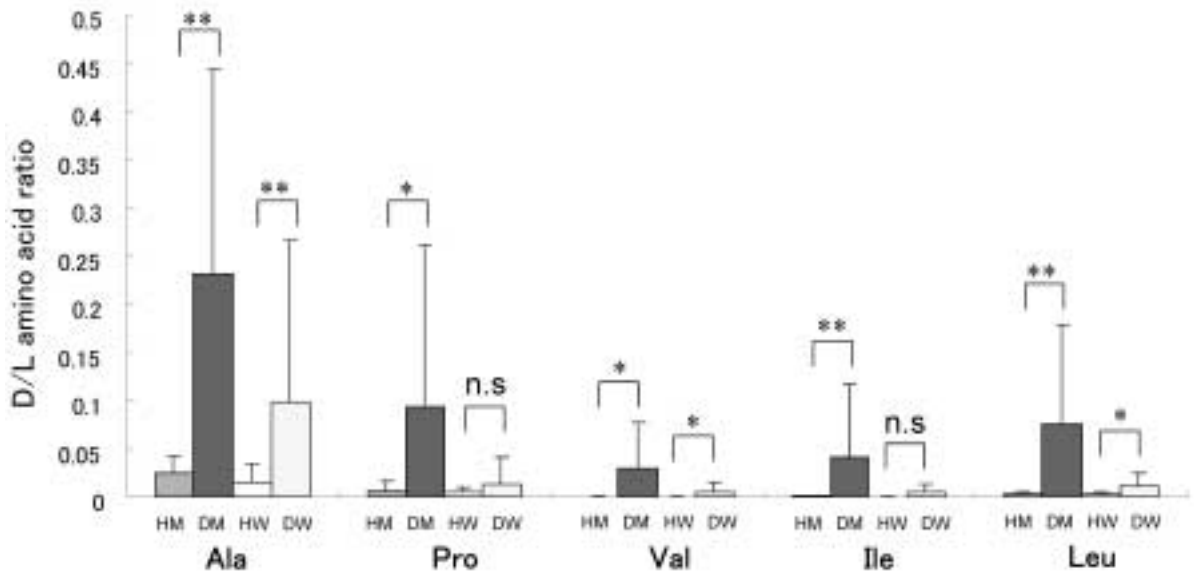

Fig 9. Analysis of DL−amino acid ratios in the nails of healthy volunteers and diabetic patients.
Samples (n = 10 each): HM, healthy men; DM, diabetic men; HW, healthy women; DW, diabetic women. (*P < 0.05; **P < 0.01); ns: not significant.

upon the amino acid sequence in the peptides. Since the content of the D−amino acids is generally very low, the determination of the D−amino acids in the peptide sequences was difficult by the method.

The Edman degradation method utilizing PITC is well known as the sequential analysis of *N*−terminal amino acids in peptides. Since PITC is an achiral reagent, the resolution of the DL−amino acids in a peptide sequence is essentially impossible. In contrast, DBD−PyNCS and NBD−PyNCS are chiral molecules, and thus the amino acid configuration in a peptide sequence seemed to be resolved by the Edman degradation method [81−84].

The Edman degradation method for the sequential analysis of peptides with the isothiocyanate reagents is divided into the following four stages; (i) labeling of *N*−terminal amino group, (ii) cleavage of labeled *N*−terminal amino acid, (iii) cyclization of liberated amino acid into thiazolinone derivative, and (iv) recyclization to thiohydantoin derivative. Since the conversion of the unstable thiazolinone to thiohydantoin is very fast, the (ii)−(iv) reactions simultaneously proceed in a short time. The *N*−terminal amide bond of the peptide is cleaved in step (ii), and the thiocarbamoyl derivative of the *N*−terminal amino acid is liberated by the reaction. After these steps, the residual peptide that is a *N*−terminal amino acid shorter than the former peptide is produced. The yield of the next cycle depends upon the recovery ratio of the peptide. The yields at every step (e.g., yield of labeling, efficiency of the thiohydantoin extractions, and peptide loss during the extraction) dictate the detection limit and measurable cycle numbers of the analyte peptide. The thiocarbamoyl−amino acid is converted to the corresponding thiohydantoin derivative of the *N*−terminal amino acid via the thiazolinone. A long heating time not only decreases the production, but also increases the racemization. The degree of racemization is dependent on the stereostructure of the analyzed peptide. Thus, it is not easy to predict the racemization ratios of the amino acids in every peptide.

The simultaneous separation of all pairs of the thiohydantoin derivatives of the DL−amino acids was difficult by a single chromatographic run, even though using the gradient elutions. Therefore, the optimal isocratic elutions toward each pair were used for the resolution of the DL−amino acids.

A strong acid (e.g., TFA) treatment is essential for the cleavage of the derivative to the thiocarbamoyl amino acid, the cyclization from the thiocarbamoyl to thiazolinone derivatives, and conversion to the thiohydantoin derivative. The racemization during the cyclization and the conversion reactions were inevitable. Therefore, a pair of peaks corresponding to the DL−amino acid derivatives appeared on the chromatograms. Since the racemization ratio was usually less than 30%, the analysis of the DL−amino acids was possible. When the proposed procedure was applied to the peptides including the D−amino acid, the resolution of the peptide having up to 10 residues was successfully performed [83].

The proposed degradation method using DBD−PyNCS was also adopted for auto−analysis by a gas−phase sequencer. The resolution of the derivatives was possible without any change in the method using PITC as the tagging reagent [84]. However, the sensitivity was not very good due to the detection at UV 254 nm. Thus, FL and LIF detections are recommended for the resolution of trace amounts of DL−amino acid residues in a peptide.

2.4. Analysis of thiol enantiomers

In spite of the development of many fluorescence labeling reagents, the fluorescence reagent for the separation and detection of chiral thiols is very rare. Chiral thiols labeled with orthophthalaldehyde (OPA) and L−valine were separated and detected by reversed−phase chromatography [85].

A pair of thiol enantiomers also reacted with DBD−PyNCS in the presence of pyridine to produce the corresponding dithiocarbamate diastereomers. Several thiols, e.g., DL−cysteine, *RS*−thiopronin and (+)(−)−2−mercaptopropionic acid, were efficiently separated by a reversed−phase column with water−acetonitrile containing 0.1% TFA [86, 87]. The Rs values were in the range of 1.05−3.33 for the diastereomers obtained with *R*−DBD−PyNCS. The detection limits were 0.4−2.4 pmol. Since the chiral reagents label not only the thiol, but also amines under similar reaction conditions, care should be taken in the derivatization of analytes containing both thiol and amino functional groups in the structure because it is possible to transfer the FL moiety from *S* to *N*.

2.5. Analysis of hydroxyl enantiomers

The labeling of an alcoholic OH group is generally very difficult because alcohols are neutral compounds. The derivatization of alcohols is possible by the coupling reaction with carboxylic acid. However, drastic conditions, e.g., long time at reflux, are generally required for the ester formation. Therefore, the carboxylic acid for the labeling of an alcohol is usually converted to its acid chloride (activation form) and then used for the labeling. Based on these reasons, DBD−Pro−COCl and NBD−Pro−COCl were synthesized from the reaction of DBD−Pro and NBD−Pro with PCl_5 [88−92].

Although the acyl chloride group exhibits an excellent reactivity with alcohols, the reagents are easily decomposed by moisture. Thus, the reagent solution must be prepared just prior to use. The derivatization in a hydrophobic solvent produced a higher yield than in a hydrophilic solvent (benzene>THF>acetonitrile). The benzene used in the reaction has to be thoroughly dried with suitable materials, such as molecular sieves and sodium wire. The derivatization reaction proceeded even in the absence of pyridine in the medium. However, the reaction rate was accelerated by the addi-

tion of pyridine.

The resulting derivatives were stable for at least 240 min at 80 ℃. The reactivities of a pair of chiral reagent enantiomers were essentially the same for both enantiomers, judging from the reaction of heptan−2−ol, a representative chiral alcohol. The resulting ester diastereomers of heptan−2−ol were well separated by normal−phase chromatography, whereas the separation was insufficient by reversed−phase chromatography.

The Rs values of the derivatives, obtained from NBD−Pro−COCl and DBD−Pro−COCl, by normal−phase chromatography were 3.0−4.1 and 3.3−4.5, respectively. NBD−Pro−COCl was more suitable than DBD−Pro−COCl by reversed−phase chromatography. However, the derivatives obtained from alcohols and NBD−Pro−COCl can be separated by reversed−phase chromatography. When the *S*−enantiomer was used as the chiral derivatization reagent, the peaks corresponding to the *S*−enantiomers of the alcohols eluted more rapidly than the *R*−enantiomers.

These reagents reacted with not only alcohols but also with amines to produce the corresponding amides [89, 90]. The reaction proceeded under rather milder conditions than that of the alcohols. A good separation of the amide diastereomers was observed during normal−phase chromatography. The separation in reversed−phase chromatography was less than that of the ester diastereomers derived from alcohols. Thus, DBD−Pro−COCl and NBD−Pro−COCl are not recommended as reagents for the resolution of chiral amines. The derivatives were detected by not only conventional FL detector, but also by LIF detection. The detection limits with the FL detection were 10−500 fmol, while those with the LIF using argon−ion at 488 nm reached 2−10 fmol.

2.6. Analysis of carbonyl enantiomers

The reagent resolving chiral carbonyl compounds is very rare. (+)−2,2,2−Trifluoro−1−phenylethylhydrazine was reported for the resolution of chiral ketones, which was the derivatization reagent for GC analysis. Based on the reactive functional group in the reagent structure, DBD−ProCZ and NBD−ProCZ were synthesized from the reaction of hydrazine and DBD−Pro−COCl or NBD−Pro−COCl, respectively [93]. The reagents were reacted with chiral carbonyl compounds at 65℃ for 10 min in the presence of trichloroacetic acid (TCA) to produce the hydrazone derivatives. The separation of a pair of diastereomers was insufficient for the resolution of chiral ketones during both reversed−phase and normal−phase chromatographies. Of course, the reagents labeled the achiral ketones and aldehydes [94, 95]. As an application of DBD−ProCZ, aldehydes in perfumes were determined without any interference.

Conclusion

Although there are many fluorescent derivatization reagents for the determination of various achiral and chiral molecules, this review focused on benzofurazan−bearing reagents which were developed in our laboratory over the past last three decades. The synthesis, characteristics and application of the fluorescence labeling reagents, which have been developed for the analysis of various molecules having different functional groups using HPLC, are outlined. The benzofurazan fluorescent reagents react with target compounds to produce the corresponding derivatives under mild conditions. The resulting derivatives are stable and emit light in the long wavelength region. The handling of the reagents is relatively easy, and many of the reagents are now on the market. Although the FL detection by HPLC provides an excellent sensitivity and selectivity, the sensitivity is often insufficient for the trace determination in real samples. LIF detection is adopted in such cases. Some fluorophores emit light during the chemical reaction without the need of optical excitation by lamps, such as a xenon arc. As the flicker noise based upon the lamp is negligible, an extremely high sensitivity is theoretically obtained from the CL derived from the chemical reaction. Indeed, trace analyses at attomole levels have been achieved by this technique for the reaction of CL reagents (oxalates and H_2O_2). The LIF and CL methods are also possible to allow the sensitive detection of distereomers derived from FL chiral tagging reagents.

The FL properties of the resulting derivatives tend to be significantly affected by temperature, viscosity of the solvent, pH of the medium, etc. It should be also noted that undesirable FL materials can contaminate the tested samples, especially in biological specimens. Thus, the pretreatment of real samples is another important issue in trace analysis. In the analysis of real samples such as biological, environmental, and food, most the significant and major part of the procedure involves how effectively to obtain the trace analytes from a complicated matrix. Sample pretreatment, i.e., clean−up and concentration of the analytes, is inevitable for the HPLC measurement with derivatization.

As fluorometry is both sensitive and selective, many FL tagging reagents are used for the analysis of real samples. Even if the FL detection is very highly sensitive, trace determination is sometimes disturbed by endogenous substances in real samples. In such a case, MS is very useful, because the interference is avoidable by the selective *m/z* detection of the analyte. However, the determination of hydophilic and/or basic compounds, such as biomolecules, is generally very difficult due to the fast elution and/or adsorption on the column. The derivatization method described here seems to be suitable for the analysis of such molecules, because the hydrophobicity of the target molecule is increased by the derivatization. As the results, a longer retention time is observed and a highly sensitive detection is thus obtained by the MS because of avoiding the interference of endogenous materials in the samples, which are eluted near

the void volume during the HPLC. Consequently, the combination of derivatization and MS detection may be accelerated during the analysis of biological molecules.

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