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Review

Recent progress in derivatization methods for LC and CE analysis

Takeshi Fukushima, Noriko Usui, Tomofumi Santa, Kazuhiro Imai *

Laboratory of Bio-Analytical Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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Dedicated to Professor Terumichi Nakagawa on the occasion of his retirement and 63rd birthday.

Abstract

The derivatization procedure with a suitable fluorescence or chemiluminescence reagent is performed for the purpose of increasing the detection sensitivity and selectivity, in high-performance liquid chromatography (HPLC) and/or capillary electrophoresis (CE). In this article, recent derivatization methods and their applications to biosamples are described. In HPLC, femto mol order of mass detection limits are obtained by derivatization. Regarding the fluorescence reagents, the use of water-soluble reagents has been effective to avoid an undesired adsorption in the process of determination of peptides. In CE, the advantages of having extremely low mass detection limits (ranging from atto to yocto mol level) and requiring only a very short analysis time (less than a few minutes) are made possible by using laser-induced fluorescence or near infra-red detections.

Keywords: HPLC; CE; Fluorescence; Chemiluminescence; Derivatizing reagent

1. Introduction

In order to promote the research in the field of biosciences, selective and sensitive detection of analytes is critical. At the present time, high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have mostly been used to enhance the selectivity of analytical methods because of their high efficiencies on the separation of analyte from various interfering substances. A recent technology enabled the separation of 5.0×10^4–2.0×10^5 and 1.0×10^5–1.0×10^6 theoretical plates/m for HPLC and CE, respectively. In comparison, the sensitivity of the method can be highly enhanced by the use of fluorescence or chemiluminescence detection in place of UV and visible absorption of the analytes. In particular, when an analyte exists in the concentration range of sub μM to pM, a highly sensitive detection is essential in order to accomplish a precise and accurate determination.

* Corresponding author. Tel.: +81-3-5841-4760; fax: +81-3-5841-4885
E-mail address: kimai@mol.f.u-tokyo.ac.jp (K. Imai).

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Because few compounds emit a strong fluorescence, a derivatization procedure with a fluorescence reagent to produce a fluorescent adduct is necessary. An ideal derivatizing reagent should have characteristics to react rapidly and quantitatively with the analytes while the resultant derivative emits a fluorescence at long excitation and emission wavelengths to avoid background fluorescence derived from the substances present in biosamples. To date, there have been a number of fluorescence reagents which fulfill these criteria. Most of them can react selectively with each functional group such as amino, thiol, carboxyl, and so on. At present, there have been several books and reviews describing the derivatizing reagents for the HPLC with fluorescence detection [1–8].

Because of its highly efficient peak separation and short analysis time, CE has become a popular tool for the determination of a variety of compounds in the last decade. CE certainly has an advantage over HPLC because of the separation power. However, because the injection volume of a sample is quite small as compared to HPLC, it also inherently has a high concentration detection limit. To overcome this, a highly sensitive detection system can be employed. The adoption of laser-induced fluorescence (LIF), which can focus the excitation light with a high energy into the internal diameter of the capillary, affords a remarkable increase of the detection sensitivity. By using CE–LIF system, a number of endogenous compounds have been determined following a suitable derivatization with a fluorescence reagent [9–12].

In this article, we will overview the HPLC methods with fluorescence and chemiluminescence detections and CE methods with LIF detection published in recent years. The details of the separation and the detection methods, and their applications to biosamples together with the derivatizing reagents employed will be reviewed.

2. HPLC with fluorescence detection

A fluorescence detection has been extensively employed for the determination of trace amount of compounds. The fluorescence derivatization methods can be mainly divided into two categories: pre- and post-column derivatization methods. Most fluorescence reagents have been used for the pre-column derivatization method, i.e. the derivatization is carried out before the chromatographic separation. In practice, care should be taken to ensure the stability of the resultant derivatives during their storage before injection into HPLC.

As regard with the pre-column fluorescence reagents developed to date, most reagents have been synthesized on the basis of fundamental skeletons such as coumarin, quinoxaline, benzofurazan, etc. Additionally, the substituent groups attached to the aromatic rings or the side chains in the reagent have been modified in order to improve their fluorescence characteristics [13,14] or reactivities towards each functional group in the analyte [15]. For example, there have been a number of fluorescence reagents synthesized on the basis of coumarin skeleton: 4-bromomethyl-7-acetoxycoumarin [16], 3-bromoacetyl-7-methoxycoumarin [17], 3-bromoacetyl-6,7-methylenedioxycoumarin [18] and 3-bromo-6,7-[2′,3′-(1′,4′,7′,10′,13′-pentaoxacyclopentadeca)-2′-ene]-coumarin [19–21], and so on. On quinoxaline skeleton, 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone [22–25], 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonyl azide [26] and 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone-3-propionylcarboxylic acid hydrazine (DMEQ-Hz) [27–29], and so on, have been developed.

As for the benzofurazan reagents, approximately 40 kinds of benzofurazan reagents have been developed to date. Recent benzofurazan reagents are theoretically designed and synthesized based on the relationships between the fluorescence characteristics of the benzofurazan reagents and the Hammett substituent constants of the substituent groups at 4- and 7-positions [30]. In addition, total electron densities on the benzofurazan skeleton and the dipole moment from 4- to 7-position obtained by PM3 calculations have also been helpful for the design of ideal benzofurazan reagents [31–33]. A list of benzofurazan reagents developed prior to 2000, along with their historical background to their application to biosamples, can be found in a recent review [8].

On the other hand, the post-column derivatization, in which the derivatization reaction is performed continuously after chromatographic separation of the analytes, can mostly be carried out when the resultant fluorescence derivative is unstable, but the rate of derivatization is rapid. A representative reagent often adopted in post-column derivatization is a dialdehyde-type reagent, *o*-phthaldialdehyde (OPA), which is also used as pre-column derivatizing reagent. OPA reacts with amino compounds in the presence of thiol compound as the co-reagent to form a fluorescence derivative [34–36]. The post-column derivatization method has been employed in an automated column-switching HPLC system, which affords to determine a compound in biosample such as plasma without any manual pre-treatments [34].

In this section, the recent fluorescence derivatization of endogenous compounds or some drugs having an amino, thiol, hydroxyl or carboxyl group, is described, and the derivatization methods are summarized in Table 1.

2.1. Amino acids and amino compounds

The sensitive determination of amino acids in body fluids is important for the diagnosis of diseases, such as inborn errors of amino acid metabolism, phenylketonuria, tyrosinemia or maple syrup urine disease. Most amino acids have originally no intrinsic chromophores and fluorophores in their structures while, because of their hydrophilicity, affording a low affinity to a reversed-phase column. Therefore, most studies to determine amino acids in body fluids have employed a pre-column derivatization with fluorescence reagents followed by simultaneous determinations of derivatized amino acids by the reversed-phase HPLC. The pre-column derivatization also offers the advantage of increasing the hydrophobicity of the analytes sufficiently to retain on the reversed-phase stationary phase.

The following fluorescence reagents have already been applied to the sensitive determination of amino acids: 9-fluorenylmethyl chloroformate (FMOC) [37,38], 1-(9-fluorenyl)ethyl chloroformate (FLEC) [39], 1-dimethylamino-naphthalene-5-sulfonyl chloride (Dns-Cl) [40], 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) [41,42], 2-(9-anthryl)ethyl chloroformate (AEOC) [43,44], 4-(*N*-phthalimidyl)benzenesulphonyl chloride (Phisyl-Cl) [45], 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl chloride (DPS-Cl) [46], 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (DMS-Cl) [47], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (6-AQC) [48,49], 1-methoxycarbonylindolizine-3,5-dicarbaldehyde (IDA) [50,51], *N*-hydroxysuccinimidyl-α-(9-acridine)-acetate (HSAA) [52], acridone *N*-acetyl chloride (ARC-Cl), carbazole-9-acetyl chloride (CRA-Cl) and carbazole-9-propionyl chloride (CRP-Cl) [53]. Among them, ARC-Cl, CRA-Cl and CRP-Cl (Scheme 1), developed by Fan et al. [53], have a prominent feature on the stability of the resultant derivatives. Less than 4% decomposition was observed after heating of the derivatives with these reagents at 40 °C for 24 h. These derivatives are therefore considered to be well suited for the pre-column derivatizing reagent. The detection wavelengths for these reagents were at 430, 360 and 365 nm with the excitation wavelengths of 404, 335 and 340 nm, respectively. The detection limits for amino acids attained by these reagents were 14–76 fmol for CRA and CRP, and 2.3–13 fmol for ARC, respectively [53].

Recently, in addition to L-amino acids, D-amino acids were also found in mammals at very low concentrations as compared to L-amino acids [54–56]. Among the D-amino acids found in mammals, the physiological roles of D-Asp [57] and D-Ser [58,59] have been gradually clarified. Since the concentration of D-amino acid occurring in mammals is very low, the fluorescence derivatization has been effectively employed, together with the use of enantiomeric separation methods. OPA with *N*-isobutyryl- or *N*-*tert*-butyloxycarbonyl-L-cysteine have been utilized for the determination of D-amino acids in the biosamples [60,61]. FLEC [39] was used for the detection of D-amino acids in the tissues of crustaceans [62,63]. Toyo'oka et al. developed benzofurazan reagents with a chiral center, *R*(−)- and *S*(+)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazoles (DBD-PyNCS, Scheme 1), and used them for the determination of D-amino acids in fermented drink and foods [64,65]. How-

Table 1
Fluorescence derivatizations used in HPLC

Analyte	Derivatizing reagent	Reaction condition, catalyst	Reaction time	Detection, ex., em. (nm)	Detection limit	Validation data			Matrices	Refs.
						Precision (%)		Accuracy (%)		
						Intra-day	Inter-day			
Amino acids	NBD-F	pH 8.0, 60 °C	1 min	470, 530	10–100 fmol	0.57–5.6			Rabbit pyruvate kinase-M1, rabbit aldolase A, papain	[41]
Amino acids	NBD-F	pH 8.0, 60 °C	1 min	470, 530		0.8–9.5			Human serum, dried blood disc	[42]
Imino acids	NBD-F	pH 8.0, 60 °C	1 min	470, 530		8.4			Human serum, dried blood disc	
Amino acids	CRA-Cl	pH 8.5	90–120 s	335, 360	14–76 fmol	1.0–4.0			–	[53]
	CRP-Cl	pH 8.5	90–120 s	340, 365	14–76 fmol	1.0–4.5				
	ARC-Cl	pH 8.5	30–60 s	404, 430	2.3–13 fmol	1.0–3.0				
Fluoxetine	NBD-COCl	60 °C	2 h	478, 537	10 nM	1.47–7.61	1.53–10.60	97.6–105.3	Rat plasma	[71]
(*S*)-Methamphetamine	DIB-Cl	pH 9.0, r.t.	10 min	330, 440	8.0 fmol	6.1	4.3	99–109	Human urine	[72]
(*R*)-Methamphetamine	DIB-Cl	pH 9.0, r.t.	10 min	330, 440	8.8 fmol	6.7	4.9		Human urine	
(*S*)-Amphetamine	DIB-Cl	pH 9.0, r.t.	10 min	330, 440	3.2 fmol	4.4	6		Human urine	
(*R*)-Amphetamine	DIB-Cl	pH 9.0, r.t.	10 min	330, 440	3.1 fmol	4.5	5.9		Human urine	
(*S*)-Hydroxymethamphetamine	DIB-Cl	pH 9.0, r.t.	10 min	330, 440	2.8 fmol	6.1	7.2		Human urine	
(*R*)-Hydroxymethamphetamine	DIB-Cl	pH 9.0, r.t.	10 min	330, 440		7.4	6		Human urine	
D-Fenfluramine	DIB-Cl	pH 9.0, 60 °C	10 min	325, 430	27 fmol	9.0–10.9	6.8–6.3	108	Human plasma	[73]
L-Fenfluraimine	DIB-Cl	pH 9.0, 60 °C	10 min	325, 430	47 fmol	5.6–7.5	6.1–7.9	92–105	Human plasma	
Phentermine	DIB-Cl	pH 9.0, 60 °C	10 min	325, 430	19 fmol	3.6–4.3	3.8–7.1	98–102	Human plasma	
D-Fenfluramine	DIB-Cl	pH 9.0, 60 °C	10 min	325, 430	19 fmol	4.0–5.6	5.7–10.1	104–106	Rat plasma	
L-Fenfluraimine	DIB-Cl	pH 9.0, 60 °C	10 min	325, 430	57 fmol	3.8–4.1	6.2–9.4	102–104	Rat plasma	
Phentermine	DIB-Cl	pH 9.0, 60 °C	10 min	325, 430	23 fmol	3.8–5.4	4.9–8.4	93–108	Rat plasma	
Homocysteine	mBBr	pH 7.4, 50 °C	10 min	270, 474	2.4 pmol	0.7–3.9	0.7–4.0	95.6–102.2	Human plasma	[75]
Homocysteine	ABD-F	pH 8.0, 50 °C	10 min	386, 516	3.3 pmol	2.8–4.6	3.6–4.9		Human plasma	[76]
GSH	ThioGlo^{TM}3	pH 7.0, r.t.	5 min	365, 445	50 fmol	1.08–2.94	4.31–8.61			[77]
GSSG	ThioGlo^{TM}3	pH 7.0, r.t.	5 min	365, 445	100 fmol	0.85–7.29	3.02–7.64			
Cysteine	ThioGlo^{TM}3	pH 7.0, r.t.	5 min	365, 445	50 fmol	0.58–4.13	2.07–7.55			

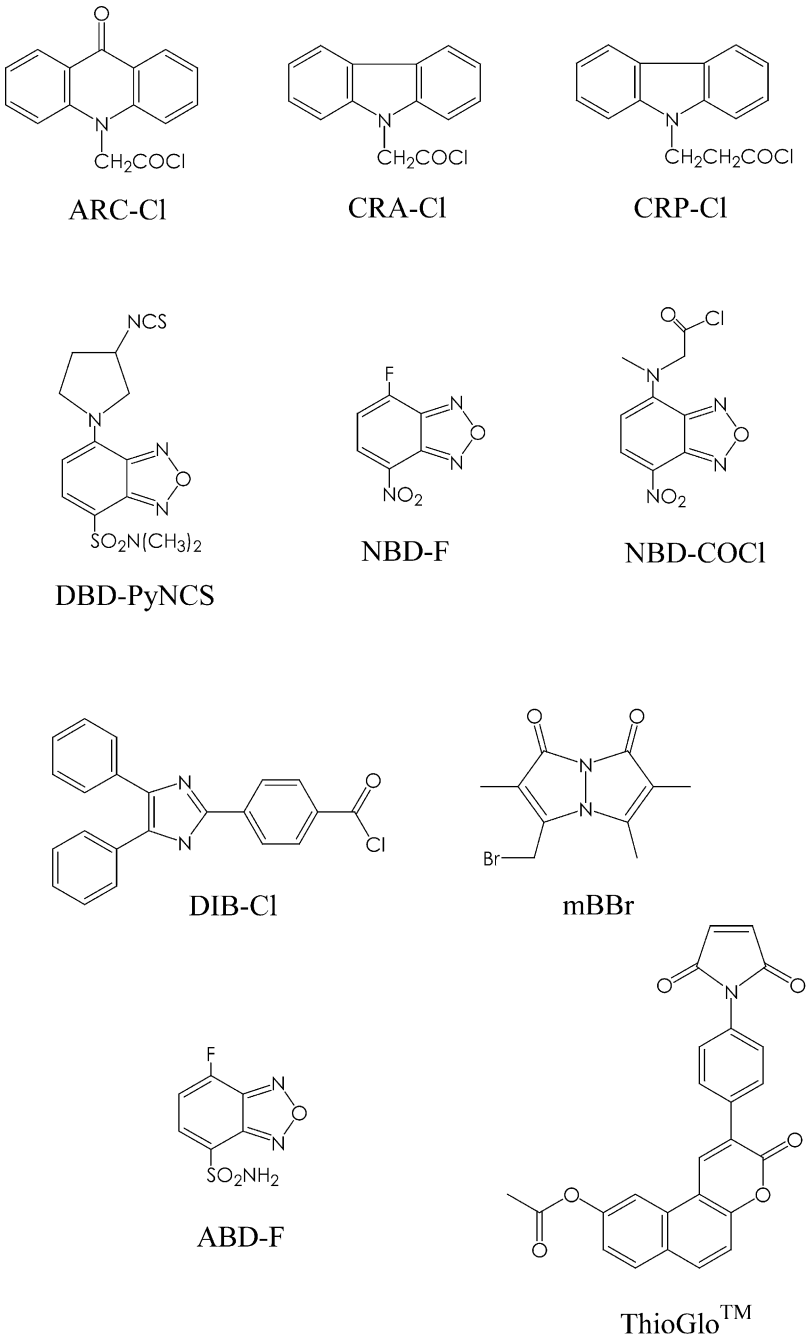
Table 1 (*Continued*)

Analyte	Derivatizing reagent	Reaction condition, catalyst	Reaction time	Detection, ex., em. (nm)	Detection limit	Validation data			Matrices	Refs.
						Precision (%)		Accuracy (%)		
						Intra-day	Inter-day			
Captopril	ThioGlo^{TM}3	r.t.	25 min	365, 445	200 fmol	1.48	0.7	99.3±2.5	Rat liver	[78]
						4.12	1.02	99.4±6.3	Rat lung	
						1.67	2.8	94.0±3.3	Rat kidney	
						1.99	1	107.5±11.6	Rat plasma	
Free fatty acid	DBD-ProCZ	r.t.	90 min	450, 550	19–176 fmol	0.5–5.6			Rat serum	[79]
Fatty acids	9-BMA	r.t.	>40 min	320, 450					Dietary fat	[83]
Caproic acid	NOEBPES	95 °C	1.5 h	305, 354	0.1 pmol	0.79–1.64	1.19–1.94		–	[85]
Fatty acids	NOEPES	95 °C	30 min	235, 350	8 nM	6	6.4		Milk	[84]
LA	APMB	60 °C	5 min	343, 423	0.5 ng/ml				Human plasma, human urine	[86]
DHLA	APMB	60 °C	5 min	343, 423	0.1 ng/ml				Human plasma, human urine	
Anandamide	DBD-COCl	60 °C	2 h	450, 560	10 fmol	6.47	3.57		Rat brain homogenate	[88]
Erythromycin	FMOC-Cl	pH 7.5, 45 °C	1 h	260, 305	50 mg/kg				Meat, fish	[89]
					50 mg/kg				Kidney, liver	
					50 mg/kg				Egg	
					25 mg/kg				Milk	
Oleandomycin	FMOC-Cl	pH 7.5, 45 °C	1 h	260, 305	50 mg/kg				Meat, fish	
					100 mg/kg				Kidney, liver	
					50 mg/kg				Egg	
					50 mg/kg				Milk	
Phenol	DIB-Cl	60 °C, triethylamine	30 min	340, 450	0.2–1.6 pmol	2.6–6.9	2.5–6.7	94.3–105.9	Human urine	[90]
Cresol	DIB-Cl	60 °C, triethylamine	30 min	340, 450					Human urine	
Xylenol	DIB-Cl	60 °C, triethylamine	30 min	340, 450						
Bisphenol A	DIB-Cl	r.t., triethylamine	20 min	350, 475	0.3 ppb	7.0–10.9	5.5–6.9		Rat brain microdialysis sample	[91]
					4.6 ppb	1.0–2.2	5.6–6.3		Rat plasma	
Bisphenol A	CDB	40 °C, IDC[a]/4-piperidinopyridine	60 min	336, 440	0.1 pg/ml	7.4	7.8		Rat serum	[92]
Insulin chain BI	SBD-F	pH 9.0, 40 °C	3 h	380, 505	3.4 fmol				Rodent islet of Langerhans	[95]
Insulin chain BII	SBD-F	pH 9.0, 40 °C	3 h	380, 505	3.7 fmol				Rodent islet of Langerhans	

Table 1 (*Continued*)

Analyte	Derivatizing reagent	Reaction condition, catalyst	Reaction time	Detection, ex., em. (nm)	Detection limit	Validation data			Matrices	Refs.
						Precision (%)		Accuracy (%)		
						Intra-day	Inter-day			
Bradykinin	*m*-BS-ABD-F	pH 7.0, 70 °C	100 min	426, 564	35 fmol	5.4–5.5	2.5–5.2	95–111	Rat urine	[97]
Progesterone	BODIPY FL hydrazide	r.t., CF_3COOH	15 h	495, 516	550 fmol	5.9–6.4	6.7–7.6		Human serum	[108]
17-Hydroxyprogesterone	BODIPY FL hydrazide	r.t., CF_3COOH	15 h	495, 516	550 fmol	5.9–6.4	6.7–7.6		Human serum	
Ascorbate	Benzamidine	pH 10.5, 100 °C[b]	1.5 min	325, 400	0.2 μM	2.5			*Erigeron canadensis*	[115]
Dehydroascorbate	Benzamidine	pH 10.5, 100 °C[b]	1.5 min	325, 400	0.1 μM	2.8				
2,3-Diketogluonate	Benzamidine	pH 10.5, 100 °C[b]	1.5 min	325, 400	0.2 μM	2.2				
Carbamyl ascorbate	Benzamidine	pH 10.5, 100 °C[b]	1.5 min	325, 400	0.4 μM	2.5				
CDA	Benzamidine	pH 10.5, 100 °C[b]	1.5 min	325, 400	0.2 μM	2.7				
25-Hydroxyvitamin D_3	DMEQ-TAD	r.t.	30 min	370, 440	1 fmol	4.2	5		Human plasma	[112]
24*R*,25-Hydroxyvitamin D_3	DMEQ-TAD	r.t.	30 min	370, 440	1 fmol					
LLU-α	DBD-PZ, CH_3COCl	r.t., TPP[c]/DPDS[d]	120 min	450, 560	100 fmol	3.7–8.0	6.3–13.6	93.1–116	Rat plasma	[115]

[a] IDC: 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide perchlorate.
[b] Post-column reaction.
[c] TPP: triphenylphosphine.
[d] DPDS: 2,2′-dipyridyl disulfide.



Scheme 1. Chemical structures of derivatizing reagents described in Sections 2.1 and 2.2.

ever, in the case of using these chiral reagents, care should be taken in ensuring the purity of the reagent: even a few % of optical impurity in the reagents may lead to an incorrect quantification of D-amino acid. This is because most D-amino acid exists at a low percentage of the L-amino acid.

Hence, the use of achiral fluorescence reagent with a chiral stationary phase seems to be a facile way for the determination of D-amino acids. Among the commercial chiral columns, a Pirkle-type chiral column (Sumichiral OA-series) was shown to separate each enantiomer of the amino acids derivatized with an achiral reagent, NBD-F (Scheme 1) [66]. The combination of HPLC using Sumichiral OA-series with the derivatization of amino acids with NBD-F was applied to the search of D-amino acids in mammals [67–69].

The fluorescence reagents used for amino acids are also applicable to the determination of drugs having an amino group. The fluorescence derivatization of drugs with a combined use of enantiomeric separation technique is useful for the study on the stereo-selective metabolism of racemic drugs in animals. In most cases, the fluorophore bearing a rich π-electron and hydrophobic property, bound covalently to chiral analytes may interact easily with chiral moieties having aromatic rings in the chiral stationary phases to accomplish the sufficient enantiomeric separations [70].

Fluoxetine, a potent anti-depressant drug, is currently marketed as racemate and the determinations of each enantiomer in body after its oral administration become necessary. 4-(*N*-Chloroformylmethyl-*N*-methyl)amino-7-nitro-2,1,3-benzoxadiazole (NBD-COCl, Scheme 1) reacted with secondary amino group of fluoxetine extracted from rat plasma at 60 °C for 2 h, and the enantiomeric separation and determination of fluoxetine derivatives can be conducted on an amylose-type chiral column, CHIRALPAK AD-RH to trace the plasma concentration of each enantiomer [71].

Al-Dirbashi et al. achieved the enantiomeric determination of methamphetamine and its major metabolite, amphetamine, and *p*-hydroxymethamphetamine derivatized with 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl, Scheme 1) on a cellulose-type chiral column (CHIRALCEL OD-R) [72]. Kaddoumi et al. reported that enantiomeric separation of sympathomimetic amines, D,L-fenfluramine and D,L-norfenfluramine was achieved on the cellulose-type chiral column following derivatization with DIB-Cl [73]. The fluorescence detection was made at 440 nm with the excitation wavelength of 330 nm, and the detection limits were at 2.8–8.8 fmol range on column.

The other drugs prescribed as racemates of β-blockers, propranolol, metoprolol, and atenolol were derivatized with 4-(*N*-chloroformylmethyl-*N*-methyl)amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-COCl), followed by chromatographic separations on the C_{18} and cellulose-type chiral column, CHIRALCEL OD-R and OJ-R [74].

2.2. Thiol compounds

Because its elevated plasma concentration is a risk factor for myocardial infarction, stroke, and peripheral vascular disease, homocysteine, an intermediary amino acid formed during methionine metabolism, is of recent clinical interest. Homocysteine has a thiol group, which can be specifically derivatized with the fluorescence reagent. Since 70% of homocysteine is bound to protein in plasma, a pre-column fluorescence derivatization can be performed after the reduction of the disulfide bond. Chou et al. used methanolic monobromobimane (mBBr, Scheme 1) following the reduction of dithiothreitol treatment, and derivatized thiols at 50 °C for 10 min [75]. The optimum wavelengths for the homocysteine derivative were 270 and 474 nm for the excitation and emission, respectively. The detection limit was 2.4 pmol per 20 μl on column (signal-to-noise ratio was 3). Using the proposed HPLC system, patients with occluded coronary artery disease (13.62 ± 5.43 μmol/l) or hemodialysis (21.28 ± 4.32 μmol/l) were found to have significantly higher ($P < 0.05$) homocysteine levels than controls (11.02 ± 2.85 μmol/l) [75].

Homocyteine in human plasma has also been determined by HPLC with pre-column derivatization with 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F, Scheme 1) [76]. The detection wavelength was 516 nm with the excitation wavelength of 386 nm, and the concentration detection limit was 3.3 μM.

9-Acetoxy-2-[4-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)phenyl]-3-oxo-3*H*-naphtho[2,1-*b*]pyran (ThioGlo^{TM} 3, Scheme 1) was developed for the

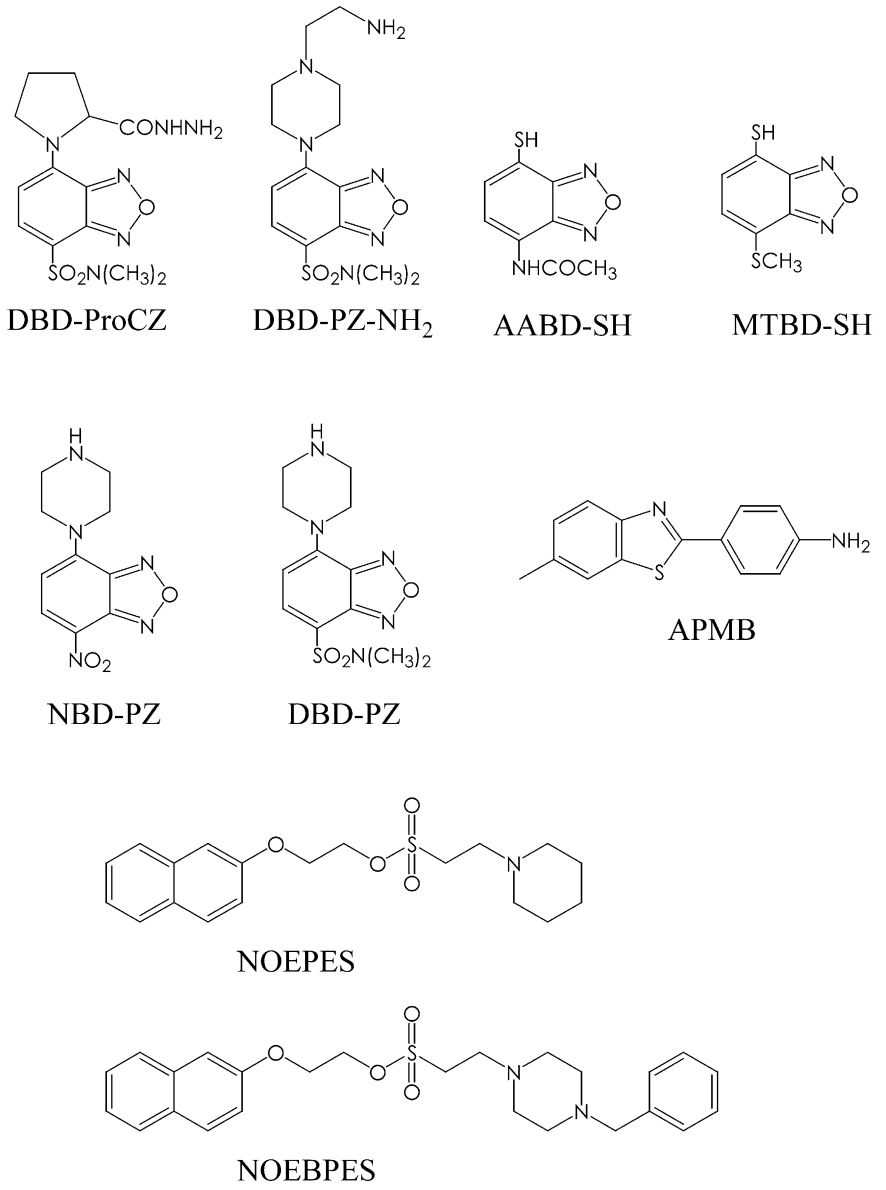
determination of thiols. ThioGlo™ 3 has a maleimide group, which reacts rapidly with thiol to yield fluorescent adducts detected at 445 nm with 365 nm of excitation wavelength [77]. The resultant derivative with GSH was shown to have an approximately 50 fmol of detection limit, and remained stable for 1 month at 4 °C. ThioGlo™ 3 was also applied to the determination of captopril, an inhibitor of angiotensin converting enzyme, in the biological samples such as rat brain, lung, kidney and plasma [78]. The detection limit for the derivative was 200 fmol on column.

2.3. Acidic compounds having a carboxyl group

A number of fluorescence reagents for carboxyl group have also been developed [5,6]. For discussion purposes, as a representative of compounds having a carboxyl group, fatty acid was selected—as it is frequently employed as a test compound for evaluating the newly developed fluorescence reagent to label with carboxyl group. Most reagents for the carboxyl group have a primary or secondary amino group as nucleophiles to form an amide bond with the carboxyl group. Of these reagents, there have been several benzofurazan-type reagents developed. 4-(2-Carbazoylpyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulphonyl)-2,1,3-benzoxadiazole (DBD-ProCZ, Scheme 2) was used for the fluorescence labeling of free fatty acids in rat serum and human plasma, under mild conditions at room temperature for 90 min in DMF containing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, 0.2 M)/2% pyridine [79]. The detection wavelength was 550 nm with excitation of 450 nm, and the on-column detection limit (signal-to-noise ratio 3) was in the range of 19 (palmitic acid)–176 fmol (palmitoleic acid). The other benzofurazan reagents include 4-nitro-7-*N*-piperazino-2,1,3-benzoxadiazole (NBD-PZ), 4-*N*,*N*-dimethylaminosulfonyl-7-*N*-piperazino-2,1,3-benzoxadiazole (DBD-PZ), 4-aminosulfonyl-7-*N*-piperazino-2,1,3-benzoxadiazole (ABD-PZ), 4-nitro-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (NBD-Apy), 4-*N*,*N*-dimethylaminosulfonyl-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (DBD-Apy), 4-aminosulfonyl-7-(3-aminopyrrolidin-1-yl)-2,1,3-benzoxadiazole (ABD-Apy), 4-(2-aminoethylamino)-7-nitro-2,1,3-benzoxadiazole (NBD-ED), 4-*N*,*N*-dimethylaminosulfonyl-7-*N*-(2-aminoethyl)amino-2,1,3-benzoxadiazole (DBD-ED), *N*-(4-nitro-2,1,3-benzoxadiazoyl-7-yl)-*N*-methyl-2-aminoacetohydrazide (NBD-COHz), 4-(*N*-hydrazinoformylmethyl-*N*-methyl)amino-7-*N*,*N*-dimethylaminosulfonyl-2,1,3-benzoxadiazole (DBD-COHz), 7-acetylamino-4-mercapto-2,1,3-benzoxadiazole (AABD-SH), 4-mercapto-7-methylthio-2,1,3-benzoxadiazole (MTBD-SH) and 7-(*N*,*N*-dimethylaminosulfonyl)-4-*N*-(4-*N*-aminoethyl)piperazino-2,1,3-benzoxadiazole (DBD-PZ-NH_2). Among them, AABD-SH [80] and MTBD-SH [81] (Scheme 2) were theoretically designed and synthesized on the basis of the fluorescence characteristics and Hammett substituent constants or PM3 calculation of the S_1–T_2 energy gaps. Both have a thiol group at 4-position as a nucleophile to react rapidly with carboxyl group within 1–5 min. These reagents themselves showed no fluorescence, but the resultant derivatives afforded a strong fluorescence at 524 and 519 nm with the excitation wavelengths at 368 and 391 nm, respectively. The detection limits were 10–20 and 2.4–5.0 fmol for the derivatives with AABD- and MTBD-SH, respectively.

DBD-PZ-NH_2 (Scheme 2) has a feature of water-solubility due to having a tertiary amino group in the structure [82]. In acidic medium, the amino group can be protonated to increase the water-solubility. The detection wavelengths for the resultant derivatives of fatty acids (C_4–C_{20}) were 544–563 nm with the excitation wavelengths at 415–422 nm. The detection limits for the derivatives were in the range of 1.0–9.1 fmol on column. Owing to its water-solubility, DBD-PZ-NH_2 is expected to be used for CE as described in Section 4.

The derivatization techniques of fatty acids for the sensitive determination are also applied to evaluate the activity of lipase, which hydrolyzes triglyceride to release fatty acids. Tsuzuki et al. reported the use of 9-bromomethylacridine (9-BMA) for the labeling of the carboxyl group of fatty acids released from glyceride treated with porcin pancreatic lipase [83]. The derivatization with 9-BMA was achieved in the presence of tetramethylammonium carbonate. The resultant



Scheme 2. Chemical structures of derivatizing reagents described in Section 2.3.

9-acridinylmethyl derivative of the released fatty acids was separated and determined by HPLC. The method can be applicable to investigate kinetic properties of the other hydrolases.

2-(2-Naphthoxy)ethyl 2-[1-(4-benzyl)piperazyl]ethanesulfonate (NOEBPES, Scheme 2) [84] and 2-(2-naphthoxy)ethyl 2-(piperidino)ethanesulfonate (NOEPES, Scheme 2) [85] were developed for the highly sensitive determination of compounds having a carboxyl group. The complete derivatization was attained in the presence of 18-crown-6 and potassium carbonate at 95 °C for 1.5 h. The detection limit for the derivative was about

0.1 pmol. Using NOEPES as the fluorescence reagent, Lu et al. determined the free fatty acids in milk [85].

α-Lipoic acid (LA) and dihydrolipoic acid (DHLA) function as a redox couple in living organisms, and also act as a coenzyme in the glycine cleavage system and dehydrogenase complex. Haj-Yehia et al. reported that simultaneous determination of LA and DHLA in human plasma and urine was accomplished using 2-(4-aminophenyl)-6-methylbenzothiazole (APMB, Scheme 2) as the fluorescence reagent following a protection procedure of thiol groups with the treatment of ethylchloroformate [86]. The detection wavelength was set at 343 and 423 nm for the excitation and emission, respectively. The concentration detection limits were 0.1 and 0.5 ng/ml for LA and DHLA, respectively.

D-Lactate is an end-product in the methylglyoxal pathway, and has been suggested to be an indicator for diseases such as diabetes. D- and L-Lactic acid in rat plasma were derivatized with a fluorescence reagent, NBD-PZ (Scheme 2), separated enantiomerically on a phenylcarbamoylated β-cyclodextrin-type chiral column and determined. Only 10 μl of rat plasma was needed for the simultaneous determination of D- and L-lactate, and a significant increase of D-lactate was observed in streptozotocin-induced diabetic rats [87].

2.4. Compounds having a hydroxyl group

Although many fluorescence derivatization reagents for compounds with a hydroxyl group have been reported, none of them reacted specifically with hydroxyl groups. This was because the reagents used could also react with any compounds with amino groups to give fluorescence derivatives. Therefore, an efficient removal of the derivatized amino compounds before the HPLC separation is most desirable for the sensitive determination of a compound with a hydroxyl group.

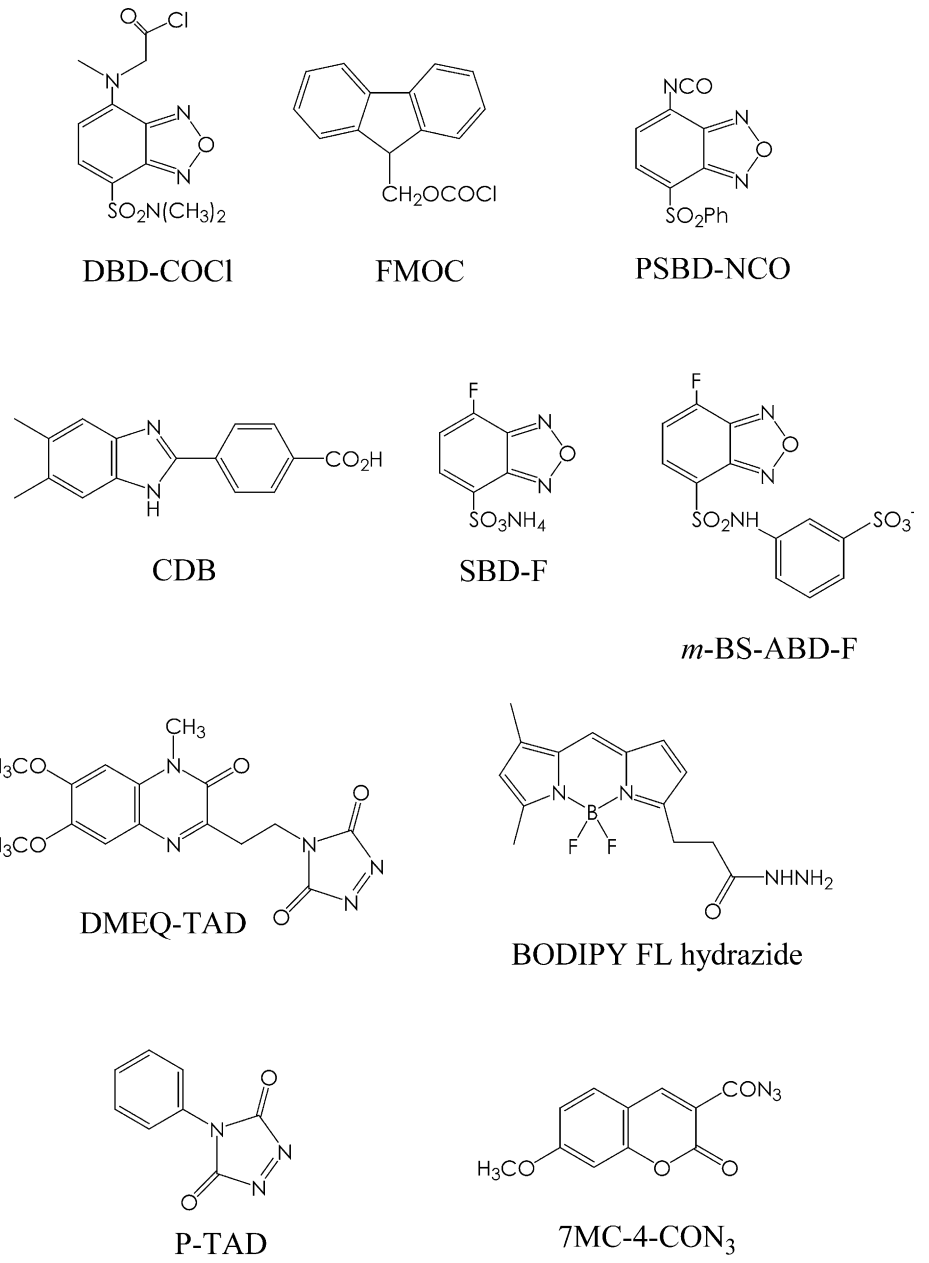
An acid halide type reagent has been frequently used for hydroxyl group owing to its high reactivity. The determination of anandamide, an endogenous ligand for cannabinoid receptor in rat brain homogenate was performed by the use of DBD-COCl (Scheme 3) as the fluorescence reagent followed by the separation on the column-switching HPLC system, which was effective for removing the interfering peaks [88].

An HPLC with pre-column derivatization with FMOC (Scheme 3) afforded the determination of erythromycin and oleandomycin, antibiotics against a wide range of gram-negative bacteria, in food of animal origin, such as meat, liver, kidney, raw milk and egg [89]. FMOC reacted with the hydroxyl groups in the antibiotics, indicating that FMOC was useful as fluorescence labeling reagent for not only amino group, but also hydroxyl group. The antibiotics were pretreated with a cation exchange cartridge followed by liquid–liquid extractions with CH_2Cl_2, and the derivatization reaction was performed at 45 °C for 1 h. The detection wavelength was at 305 nm with the excitation at 260 nm.

4-(4,5-Diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) was also used for the fluorescence derivatization of phenolic hydroxyl group [90], and applied to determine bisphenol A (BPA), an endocrine disruptor, in the brain microdialysis samples of rats. BPA was derivatized with DIB-Cl at room temperature for 10 min, and determined using a column-switching HPLC consisting of dual C_{18} semi-microcolumns [91]. As shown in Fig. 1, it was shown by the proposed method that BPA was permeable through the blood-brain barrier of rat.

BPA was also derivatized with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB, Scheme 3) at 40 °C for 10 min, and determined by an HPLC with fluorescence detection [92].

7-Phenylsulfonyl-4-(2,1,3-benzoxadiazolyl) isocyanate (PSBD-NCO, Scheme 3), developed theoretically as mentioned above [93], is non-fluorescence itself, and reacts with 1-octanol, 1-nonanol, 1-decanol and 1-undecanol at 60 °C for 4 h to produce their corresponding fluorescence derivatives, which showed an intense fluorescence at 475 nm with excitation wavelength at 366 nm. The detection limits for these derivatives were 5.6–10.7 fmol on column.



Scheme 3. Chemical structures of derivatizing reagents described in Sections 2.4, 2.5 and 2.6.

2.5. Peptides

Bioactive peptides acting as hormones, neurotransmitters, or immunomodulators exist at very low concentrations in tissues or biological fluids. Therefore, a sensitive determination method should be necessary for studying these peptides in tissues. Although an immunoassay, RIA or ELISA, has mostly been employed, it still has the problems of cross-reactivity. The chromatographic techniques for the determination of peptides are preferred owing to their high selectivity. However,

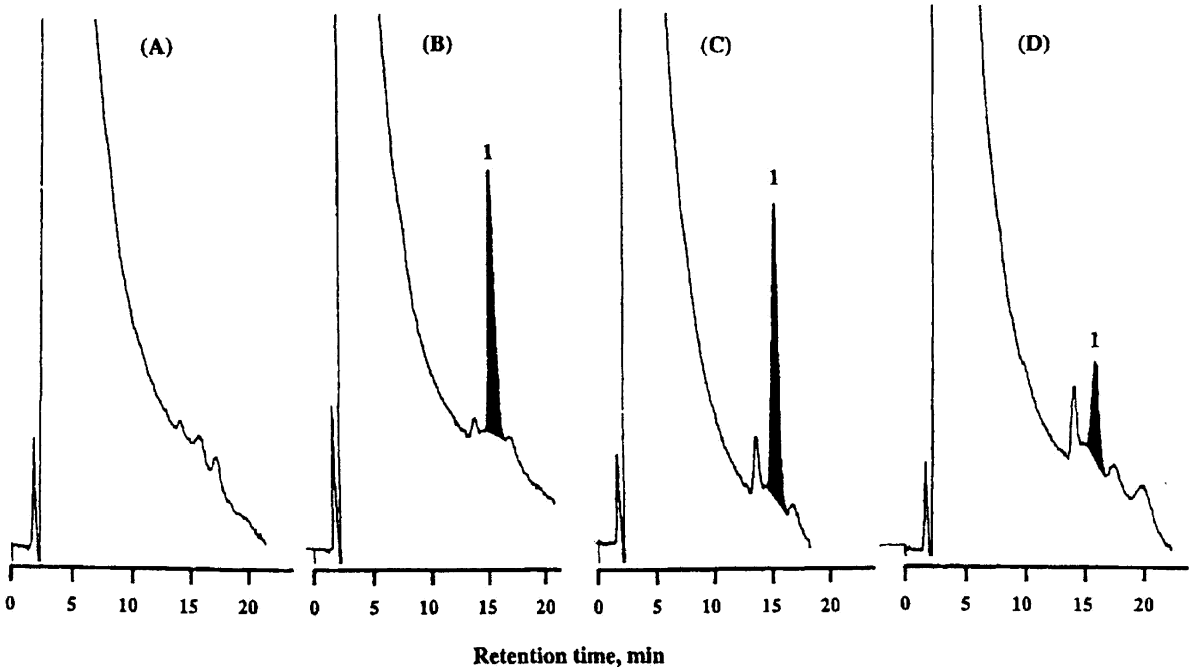


Fig. 1. Typical chromatograms of rat brain microdialysate samples obtained from a single i.v. administration of BPA (10 mg/kg). (A) Sample collected before administration; (B) sample spiked with 5.0 ppb BPA; (C) sample containing BPA (5.6 ppb) collected 20 min after BPA administration; (D) sample containing BPA (1.1 ppb) collected 180 min after BPA administration. 1, BPA. Reproduced from Ref. [91] with permission.

it is known that some peptides adsorb onto the inner surface of the tube during the process of sample handling. When the peptides are derivatized with a fluorescence reagent, an undesired adsorption would result from a further increase of the hydrophobicity of the analyte. Therefore, the development of a fluorescence reagent having a hydrophilic property, such as a water-soluble reagent, is desirable.

A water-soluble fluorescence reagent, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F, Scheme 3), was developed in 1984, and has largely been used for proteins and small compounds having a thiol group [94]. The rodents have two types of insulins, types I and II, which consist of chain A and isomeric chain BI (5804 Da) and BII (5796 Da), respectively. The insulins in a single islet of Langerhans of Wistar and GK rats and ICR mice were successfully derivatized with SBD-F, separated on an ODS column with less organic solvent and determined fluorometrically at 520 nm with the excitation at 390 nm [95]. Each single islet of Langerhans tissue was dissolved in 0.1 M NaOH, treated with tris(2-carboxyethyl)phosphine (TCEP) to form reduced thiol groups, and then reacted with SBD-F at pH 9.0 and 40 °C for 3 h. Representative chromatograms are shown in Fig. 2. The detection limits for chain A, BI and BII were in the range of 2.2–3.7 fmol. The method is feasible for investigation of pathophysiological roles of the insulin isomers in diabetic rats.

In order to derivatize the N-terminal amino group of peptide, the benzofurazan type reagents were developed with a sulfonyl group, 2-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)ethanesulfonic acid (ES-ABD-F) and 3-(7-fluoro-2,1,3-benzoxadiazole-4-sulfonamido)benzenesulfonic acid (*m*-BS-ABD-F, Scheme 3) [96]. These water-soluble fluorescence reagents reacted selectively with amino group in the peptide. Among them, *m*-BS-ABD-F was successfully applied to determine bradykinin, a nanopeptide generated by kinin–kallikrein system, in rat urine [97]. After solid-

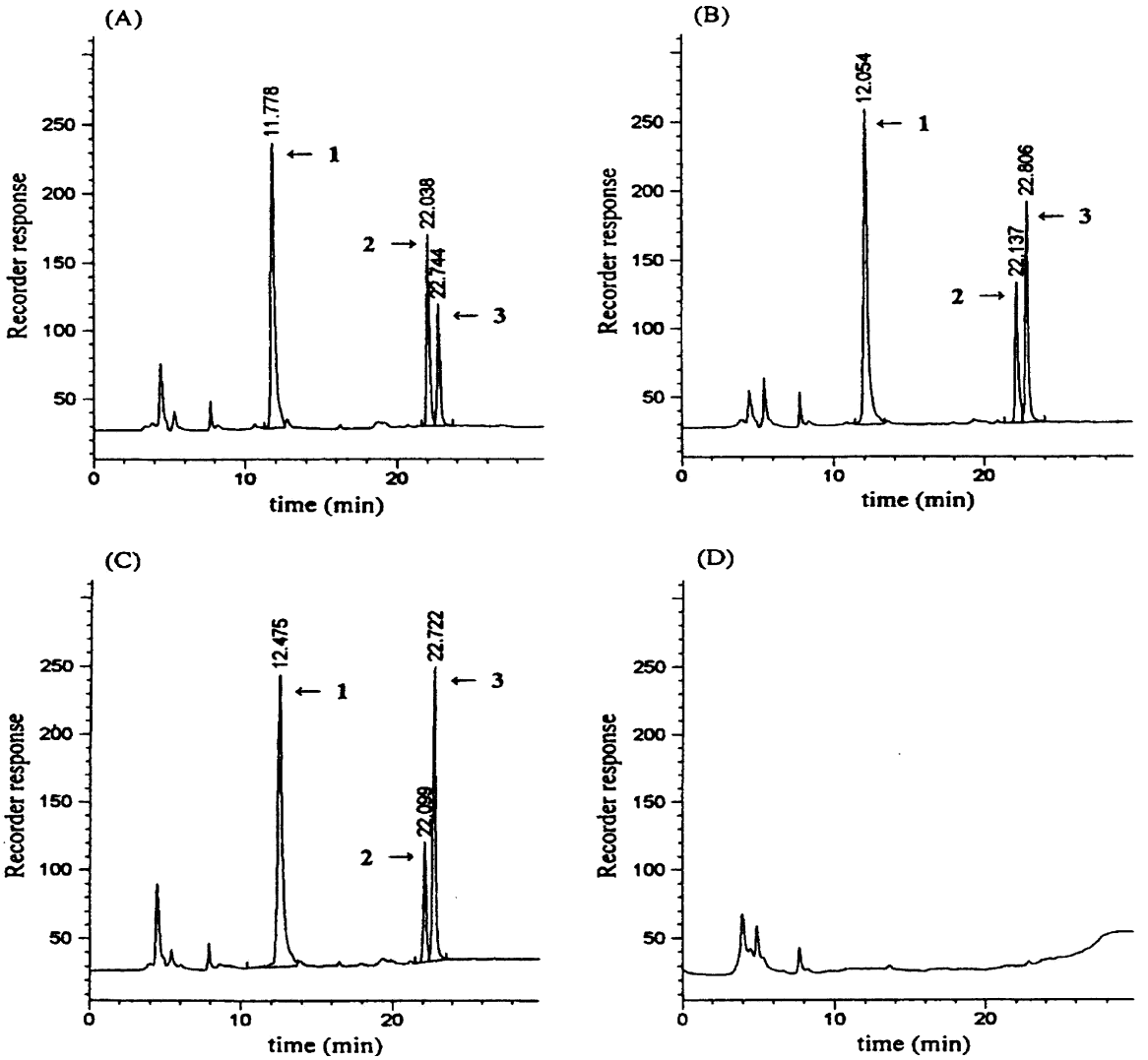


Fig. 2. Chromatograms of the SBD-insulin derivatives obtained from a single islet of Langerhans and a nonislet tissue. Islet from (A) ICR mouse, (B) islet from Wistar rat, (C) islet from GK rat, and (D) nonislet tissue (10 μg) from Wistar rat (as a negative control): 1. chain A derivative; 2. chain BII derivative; 3. chain BI derivative. Islets and nonislet tissue were derivatized with 1.7 mM SBD-F at pH 9.0 and 40 °C for 3 h in the presence of 0.7 mM TCEP, 2 mM EDTA, and 1 mM DM. Reproduced from Ref. [95] with permission.

phase extraction with a C_{18} cartridge, bradykinin in rat urine was derivatized with *m*-BS-ABD-F at 70 °C for 100 min, and determined by a column-switching HPLC system, consisting of cation exchange and ODS columns. The detection limit was 35 fmol on column.

As demonstrated above, an HPLC method using pre-column derivatization with water-soluble reagent could be widely applied to the analysis of peptides in biosamples.

As regard with peptide sequence analysis, a fluorescence reagent having an isothiocyanate moiety capable of performing Edman degradation has been developed [98,99]. In the 1990s, it had been shown that biologically active peptides including D-amino acid residue were present in

invertebrates and amphibians, and that certain mammalian peptides, α-crystalin and β-amyloid, contained a racemized amino acid, especially D-Asp related to aging and some diseases. Considering these backgrounds, Edman degradation methods have been developed to determine the configuration of amino acids in the process of sequential analysis [100–104]. For these HPLC methods, see the previous review [105].

2.6. Steroids and vitamins

Steroids and vitamins are biologically important and should be targets for determination. As their physiological concentrations are extremely low, a highly sensitive determination method should be necessary. Until recently, however, most steroids and vitamins were difficult to determine without any derivatization procedure, for the lack of specific chromophores or fluorophores.

Progesterone and 17-hydroxyprogesterone in serum of woman could be an indicator for pregnancy or hormone disease. Katayama et al. reported that progesterone and 17-hydroxyprogesterone in serum of woman were determined in HPLC after pre-column derivatization with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionohydrazine (BODIPY FL hydrazide, Scheme 3) in the presence of 0.08% trifluoroacetic acid at room temperature [106]. Hydrazine moiety of BODIPY FL hydrazide reacted with carbonyl group of the steroids to form a hydrazone. The detection wavelength was at 495 and 516 nm for the excitation and emission, respectively. The detection limits for progesterone and 17-hydroxyprogesterone were 550 fmol per 10 μl injection. They reported that the BODIPY FL hydrazide showed the larger peak area of these steroids as compared to the other fluorescence reagents having dansyl, FMOC and NBD skeletons. It should be noted that the derivatization of carbonyl group at 3-position of steroid with the hydrazine-type reagent has been shown to produce isomers of *syn*- and *anti*-hydrazone to give two peaks on the chromatogram [107–109].

Saisho et al. reported an HPLC method for the determination of 7α-hydroxycholesterol in dog plasma after pre-column derivatization with 7-methoxycoumarin-3-carbonyl azide (7MC-4-CON_3, Scheme 3) [110]. The resultant fluorescence derivative was shown to contain two coumarin moieties introduced to the carbonyl groups at 3- and 7-positions of 7α-hydroxycholesterol. The detection limit obtained was 4 pg (signal to noise ratio 5), four times lower than that of derivative with 1-anthroylcyanide. The method was successfully applied to the investigation of the plasma concentration of 7α-hydroxycholesterol after oral administration of cholestyramine to dogs.

Determination of vitamin D metabolites in body are extensively carried out by HPLC with derivatization with Cookson-type reagent. The derivatives with fluorescence Cookson-type reagents, DMEQ-TAD (Scheme 3) [111,112] were sensitively detected. The detection limits for the derivative were 1 fmol on column. Recently, the derivatizing reagent for electrospray mass-spectrometry, P-TAD (Scheme 3), has also been used for the determination of vitamin D metabolites [113,114].

Vitamin C (ascorbate), dehydroascorbate, carbamyl ascorbate and carbamylated dehydroacsorbate (CBA) were specifically reacted with benzamidine in alkaline solution (pH 10.5) at 100 °C to produce their fluorescence products. Using the specific reaction, an HPLC method with the post-column reaction with benzamidine for the determination of the ascorbate related compounds was developed [115]. The detection wavelength was set at 400 nm with the excitation at 325 nm. The concentration detection limit was less than 0.5 μM. The method was applied to determine these compounds in the leaves of *Erigeron canadensis*.

Vitamin E family consists of α-, β-, γ- and δ-tocopherol (Toc) and -tocotrienol (T3). Among the isoforms of vitamin E family, γ-Toc and -T3 have recently been receiving more attention, because their metabolite, 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxy chroman (γ-CEHC, LLU-α) exhibits unique biological activities such as natriuresis and inhibition of cyclooxygenase II. The carboxyl group of LLU-α was derivatized with DBD-PZ (Scheme 2), in the presence of condensing agents, triphenylphosphine and 2,2′-dipyridinyl disulfide followed by *O*-acetylation with acetyl chloride [116–118]. The sensitive

determination of LLU-α in the biological fluids, such as plasma, urine and bile was achieved utilizing a column-switching HPLC system. Subsequently, the enantiomeric separation of LLU-α derivative was accomplished on a cellulose-type chiral column (CHIRALCEL OD-RH). Using the HPLC system, only *S* form of LLU-α was found to be produced from the administration of vitamin E, γ-Toc and -T3 (Fig. 3) [117], which were excreted into not only urine, but also into bile in rats [116].

3. HPLC with chemiluminescence detection

Chemiluminescence (CL) is a light emitted from a compound such as a fluorophore excited by chemical reactions. The absence of light source for excitation observed in a usual fluorescence detection allows the decrease of the background noise, and thus attains a highly sensitive detection due to the increase of signal to noise ratio. Owing to the highly sensitive detectability in CL, the CL detection has been applied to the immunoassay and the detection system in HPLC in the last two decades [119]. In this section, three representative CL reagents, peroxyoxalate ester, tris(2,2′-bipyridine)ruthenium (II) [$Ru(bpy)_3^{2+}$] and luminol, are reviewed, and the methods are listed in Table 2.

3.1. Peroxyoxalate ester

As one of the CL detections used in HPLC, the reaction of a peroxyoxalate ester (PO, Scheme 4) with hydrogen peroxide in the presence of a base such as imidazole as the catalyst had been employed in the post-column HPLC [120–122]. The PO-CL reaction gives a potential intermediate (1,2-dioxetane dione) of energy, by which a co-existing fluorophore is excited to emit CL. Using the PO-CL reaction, an automated HPLC method with post-column derivatization was developed for catecholamines (CAs), derivatized with ethylenediamine to form fluorophores and subsequently, detected by the PO-CL using bis[2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO, Scheme 4) with hydrogen peroxide [123,124]. Furthermore, both CAs and their *O*-methyl metabolites in plasma were able to be simultaneously determined by the improvement of the HPLC-PO-CL system, which included pre-treatment of plasma sample with a weak anion-exchange column, a post-column oxidative conversion by a coulometric cell, fluorescence derivatization with ethylenediamine and the post-column PO-CL

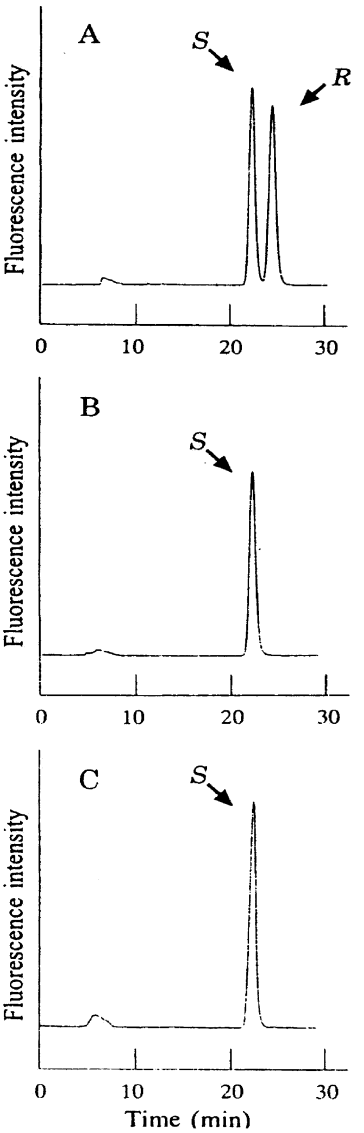


Fig. 3. Representative chromatograms of enantiomeric separation of LLU-α (γ-CEHC) derivatives. Racemic LLU-α standard (A), LLU-α stemmed from γ-T3 (B) and from γ-Toc (C). The arrows indicate S- and R-LLU-α derivative, respectively. Reproduced from Ref. [117] with permission.

Table 2
CL derivatizations used in HPLC

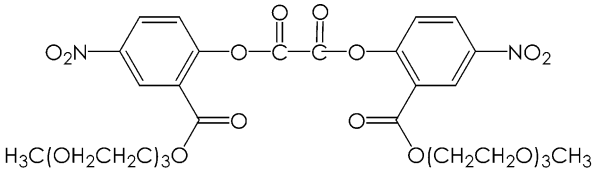
Analyte	Derivatizing reagent	Reaction condition, catalyst	Reaction time (min)	CL	Detection limit	Validation			Matrices	Ref.
						Precision (%)		Accuracy (%)		
						Intra-day	Inter-day			
CAs, 3-*O*-methyl metabolites	Ethylenediamine	90 °C[a]		TDPO, H_2O_2	3–10 fmol	2.6–7.6	7.7–9.1	86–97	Rat plasma	[124]
	Ethylenediamine	80 °C[a]		TDPO, H_2O_2	0.3–2.0 fmol	4.9–5.5	5.8–11.1	88–105	Rat plasma	[125]
Myristic and palmitic acids	NAPP	r.t., BEPT[b]/MDPP[c]	30	$[Ru(bpy)_6]^{2+}_3$	70 fmol				Human plasma	[133]
Ibuprofen	NAPP	r.t., BEPT[b]/MDPP[c]	30	$[Ru(bpy)6]^{2+}_3$	45 fmol				Human plasma	
Histamine	DEAP	r.t., DCC[d]/HOOBt[e]	60	$[Ru(bpy)_6]^{2+}_3$	70 fmol					[133]
Myristic acid	PROB	r.t., EDC[f]/pyridine	40	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	11.9 fmol	2.7–5.5			Human plasma	[135]
Linolenic acid	PROB	r.t., EDC[f]/pyridine	40	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	13.2 fmol	2.7–5.5			Human plasma	
Palmitoleic acid	PROB	r.t., EDC[f]/pyridine	40	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	14.8 fmol	2.7–5.5			Human plasma	
Linoleic acid	PROB	r.t., EDC[f]/pyridine	40	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	19.9 fmol	2.7–5.5			Human plasma	
Palmitic acid	PROB	r.t., EDC[f]/pyridine	40	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	23.9 fmol	2.7–5.5			Human plasma	
Oleic acid	PROB	r.t., EDC[f]/pyridine	40	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	47.0 fmol	2.7–5.5			Human plasma	
Stearic acid	PROB	r.t., EDC[f]/pyridine	40	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	64.4 fmol	2.7–5.5			Human plasma	
Methyl-*n*-octylamine	TPB-Suc	80 °C, triethylamine	20	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	560 amol	2.9–4.8				[136]
n-Nonylamine	TPB-Suc	80 °C, triethylamine	20	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	210 amol	2.9–4.8				
n-Decylamine	TPB-Suc	80 °C, triethylamine	20	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	410 amol	2.9–4.8				
5-Hydroxytryptophan	6-AMP	pH 9.0, r.t., $K_3Fe(CN)_6$	2	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	1.96 fmol	6.0–7.5				[137]
Serotonin	6-AMP	pH 9.0, r.t., $K_3Fe(CN)_6$	2	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	2.50 fmol	6.0–7.5				

Table 2 (*Continued*)

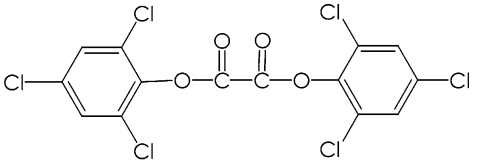
Analyte	Derivatizing reagent	Reaction condition, catalyst	Reaction time (min)	CL	Detection limit	Validation			Matrices	Ref.
						Precision (%)		Accuracy (%)		
						Intra-day	Inter-day			
5-HIAA	6-AMP	pH 9.0, r.t., $K_3Fe(CN)_6$	2	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	0.18 fmol	2.6		93.5–95.5	Human urine	[138]
α-Methylserotonine	6-AMP	pH 9.0, r.t., $K_3Fe(CN)_6$	2	H_2O_2, OH^-, $K_3[Fe(CN)_6]$	0.20 fmol					

[a] Post-column reaction.
[b] BEPT: 2-bromo-1-ethylpyridinium tetrafluoroborate.
[c] MDPP: 9-methyl-3,4-dihydro-2*H*-pyrido[1,2-*a*]-pyrimidin-2-one.
[d] DCC: *N*,*N*-dicyclohexylcarbodiimide.
[e] HOOBt: 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazole.
[f] EDC: 1-(ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride.

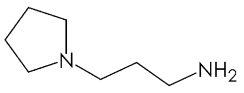
detection [125]. The detection limits for CAs and their metabolites were 0.3–2.0 fmol on column [126]. The HPLC-PO-CL system developed is useful for the study of CA metabolism in experimental animals [125–127] as well as human erythrocyte [128].



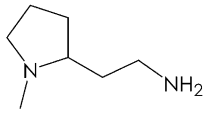
TDPO



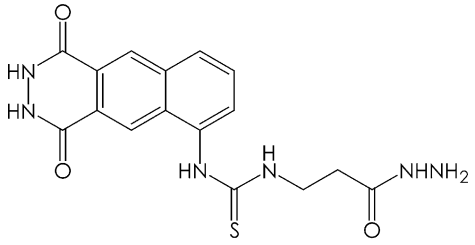
TCPO



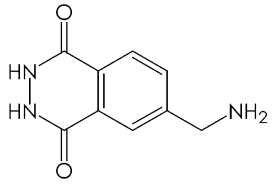
NAPP



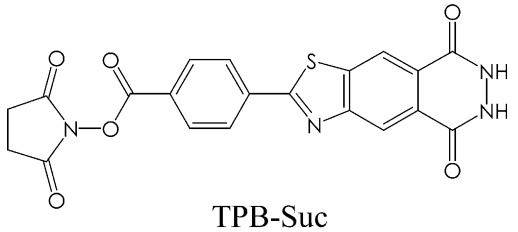
AEMP



PROB



6-AMP



TPB-Suc

Scheme 4. Chemical structures of the reagents described in Section 3.

Hayakawa et al. developed an HPLC-PO-CL method for the determination of nitropolycyclic aromatic hydrocarbons (NPAHs) in airborne particulates [129]. They used a bis(2,4,6-trichlorophenyl)oxalate (TCPO, Scheme 4) as the peroxyoxalate ester. The concentration detection limits for NPAHs were $1–40 \times 10^{-11}$ M.

3.2. Tris(2,2′-bipyridine)ruthenium (II)

As the other CL detection in HPLC, an electrogenerated chemiluminescence (ECL) using $Ru(bpy)_3^{2+}$ has frequently been utilized [130–132]. The reaction mechanism for emitting ECL is as follows [132]:

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-$$

$$Ru(bpy)_3^{3+} + \text{reductant} \rightarrow \text{product} + [Ru(bpy)_3^{2+}]^*$$

$$[Ru(bpy)_3^{2+}]^* \rightarrow Ru(bpy)_3^{2+} + h\nu$$

$Ru(bpy)_3^{2+}$ is electrochemically oxidized to $Ru(bpy)_3^{3+}$ on the platinum electrode surface. Since the CL intensity ($h\nu$) is theoretically proportional to the amount of reductant, the determination of reductant can be performed by measuring the CL intensity.

Morita et al. developed an HPLC method for the determination of carboxylic acids by an ECL detection using post-column reaction with $Ru(bpy)_3^{2+}$ [133]. In ECL using $Ru(bpy)_3^{2+}$, a tertiary amino group is active for emitting CL [131], and thus, they developed the pre-column CL derivatizing reagents, 2-(2-aminoethyl)-1-methylpyrrolidine (AEMP) and *N*-(3-aminopropyl)pyrrolidine (NAPP) (Scheme 4), both of which have a tertiary amino group in their structures. As a representative of carboxylic acids, myristic acid and ibuprofen extracted with chloroform/*n*-heptane (1/1) from 5 μl of human plasma was derivatized with NAPP in the presence of the condensing reagents, 2-bromo-1-ethylpyridinium tetrafluoroborate (BETP) and 9-methyl-3,4-dihydro-2*H*-pyrido[1,2-*a*]-pyrimidin-2-one (MDPP), and subjected to the HPLC with post-column ECL system. The detection limits for myristic acid and ibuprofen were 70 and 45 fmol, respectively. For the determination of amino compounds, 3-(diethylamino)propionic acid (DEAP) was used for the derivatization of amino compounds. The detection limit for histamine derivative with DEAP was 70 fmol on column [133].

3.3. Luminol

Luminol is one of the most widely used reagents which emits CL in the presence of H_2O_2 and metal ion such as Co(II), Cu(II) and Fe(III). There are several luminol-type CL reagents developed for the sensitive determination of endogenous substances [134].

6-[*N*-(3-Propionohydrazino)thioureido]benzo[*g*]-phthalazine-1,4(2*H*,3*H*)-dione (PROB, Scheme 4) was developed for the CL reagent for carboxyl group [135]. PROB reacted with fatty acids in the presence of EDC and pyridine at room temperature for 40 min to form the corresponding acid hydrazide derivatives. The resultant derivative also produced a strong CL by the reaction with H_2O_2 in the presence of potassium hexacyanoferrate (III) in an alkaline medium. The fatty acids in 20 μl of human serum were derivatized with PROB after deproteinization with ethanol, and determined by CL detection system. The values obtained were in good agreement with those obtained by the HPLC method previously reported. The detection limits for the fatty acids were in the range of 11.9–64.4 fmol for the injection volume of 20 μl.

Another luminol-type reagent for amino group, 4-(6,7-dihydro-5,8-dioxothiazolo[4,5-*g*]phthalazin-2-yl)benzoic acid *N*-hydroxysuccinimide ester (TPB-Suc, Scheme 4) was also developed by Yoshida et al. [136]. The derivatization proceeded at 80 °C for 20 min in the presence of 50 mM triethylamine. The detection limits were 560, 210 and 410 amol for methyl-*n*-octylamine, *n*-nonylamine and *n*-decylamine, respectively. The method was applied to investigate the concentration of amantadine hydrochloride, a drug for Parkinson's disease, after oral administration to human [136].

6-Aminomethylphthalhydrazide (6-AMP, Scheme 4) was developed for the determination

of 5-hydroxyindoles [137]. The reagent has a benzylamine moiety to react with 5-hydroxyindole skeleton at room temperature for 2 min. The resultant benzoxazole derivatives produced a strong CL by the reaction with H_2O_2 in the presence of 100 mM potassium hexacyanoferrate (III). The detection limits for 5-HT and the metabolite, 5-HIAA were 2.50 [137] and 3.67 fmol [138] on column, respectively.

4. CE with laser-induced fluorescence

Since theoretical plate numbers of peaks obtained in CE are nearly 1.0×10^6 (/m), the mass detection limits in CE are lower than those in HPLC. However, the concentration detection limits in CE become significantly higher than those in HPLC, because the injection volume are in the range of a few nl. Thus, a derivatization with a suitable fluorescence reagent is frequently carried out to enhance the sensitivity. In CE, a LIF detector with Ar ion or He–Cd laser source is usually adopted, taking advantage of its high energy and collimation to focus the beam into the inner diameter of capillary.

As for the derivatizing reagents employed for CE, a hydrophilic reagent is desired, as a hydrophilic solute in aqueous running buffer generally gives a well-sharped peak due to the avoidance of adsorption into the inner surface of capillary. Therefore, a fluorescence reagent bearing a polar functional group such as an amino, carboxyl, or sulfonyl group has been somewhat preferred, particularly because it can possess a positive or negative charge in the running buffer during the separation.

In this section, the derivatization methods for CE–LIF detection are reviewed, and summarized in Table 3.

4.1. Amino acids and amino compounds

Using CE–LIF system, amino acids or amino compounds in the biological samples such as microdialysis and single cells have been determined with fluorescence derivatization [139].

Naphthalene-2,3-dicarboxaldehyde (NDA, Scheme 5) can react rapidly with amino compounds in the presence of cyanide ion (CN^-) as a co-reagent, and can produce a fluorescence derivative, *N*-substituted 1-cyanobenzo[*f*]isoindoles (CBI), which emits at 490 nm by irradiation with He–Cd laser at 442 nm. Gilman et al. reported that the on-column derivatization with NDA and CN^- at the inlet of the capillary was successfully applied to study the change of concentration of CA and amino acids in a single rat pheochromocytoma (PC12) cell [140]. The front end of the capillary was used as a reaction chamber for the derivatization, and gave a merit to limit the dilution of the fluorescence reagents. The addition of the cell lysing reagents (50 μM digitonin with 5% ethanol and 5% acetonitrile) in the running buffer was necessary to assist the lysing of a single PC12 cell. The peaks of taurine, Glu and Asp were clearly observed within 30 min on the electropherogram.

The isoindole derivative (CBI) formed from NDA and CN^- shows the potential use of not only LIF, but also electrochemical detection (ED) [141,142]. The detection limits for amino acids were comparable to those obtained by LIF. On-column derivatization of amino acids with NDA and CN^- followed by CE–ED system was successfully applied to the analysis of a single cell such as human erythrocyte [141] and mouse peritoneal macrophages [142].

CE–LIF system is also applicable to connect to the microdialysis probe via a flow-gated interface [143–145]. Microdialysis is a useful technique, in which the change of the concentration of a compound of interest in tissues of living animals can be monitored. However, since the dialysate of microdialysis is pumped at a slow flow rate (usually 1–2 μl/min), it takes much time, mostly every 5–15 min, for collecting the mass amount necessitated to detect the compounds. These temporal resolutions are too slow to monitor the rapid changes of the compounds of interest in tissues. Therefore, it is desirable to establish a method that is sensitive enough to detect a compound of interest in a fraction collected for just a few minutes. Robert et al. reported the continuous flow derivatization of neurotransmit-

Table 3
LIF derivatizations used in CE

Analyte	Derivatizing reagent	Reaction condition, catalyst	Reaction time	LIF	Detection limit	Validation		Matrices	Refs.
						Precision (%)			
						Intra-day	Inter-day		
Amino acids	NDA/CN^- [a]	pH 9.5		He–Cd laser, 442 nm				PC12 cell	[140]
Dopamine	NDA/CN^- [a]	pH 9.5		He–Cd laser, 442 nm					
Noradrenaline	NDA/CN^-	pH 8.7		He–Cd laser, 442 nm	0.8 amol	10.6	14.9	Microdialysis sample	[143]
Glu	NDA/CN^-	pH 8.7		He–Cd laser, 442 nm	92 amol	6.3	7.9		
Glu	OPA/β-mercaptoethanol[a]	pH 10.5	1.5 min	Ar ion laser, 351 nm	45 nM			Microdialysis sample of rat brain	[144]
Asp	OPA/β-mercaptoethanol[a]	pH 10.5	1.5 min	Ar ion laser, 351 nm	49 nM				
GABA	OPA/β-mercaptoethanol[a]	pH 10.5	1.5 min	Ar ion laser, 351 nm	17 nM				
Gly	OPA/β-mercaptoethanol[a]	pH 10.5	1.5 min	Ar ion laser, 351 nm	13 nM				
Dopamine	OPA/β-mercaptoethanol[a]	pH 10.5	1.5 min	Ar ion laser, 351 nm	36 nM				
GABA	FITC	40 °C	6 h	Ar ion laser, 448 nm	1 nM	7.8		Microdialysis sample of cat brain	[145]
Glu	FITC	40 °C	6 h	Ar ion laser, 448 nm	12 nM	4.7			
Asp	FITC	40 °C	6 h	Ar ion laser, 448 nm	19 nM	5.4			
γ-Carboxyglutamic acid	FITC	pH 9.0	24 h	Ar ion laser, 448 nm	5.0×10^{-11} M			Hydrolysate of human prothrombin, blood coagulation factor X, and bovine osteocalcin human urine and plasma	[146]
GABA	CFSE	pH 9.28, r.t.	Overnight	Ar ion laser, 448 nm	0.3 nM				[147]
Putrescine	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	2.6 nM	1	2.3		[148]
Histamine	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	2.8 nM	0.9	2.5		
Tyramine	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	2.2 nM	0.2	3.3		

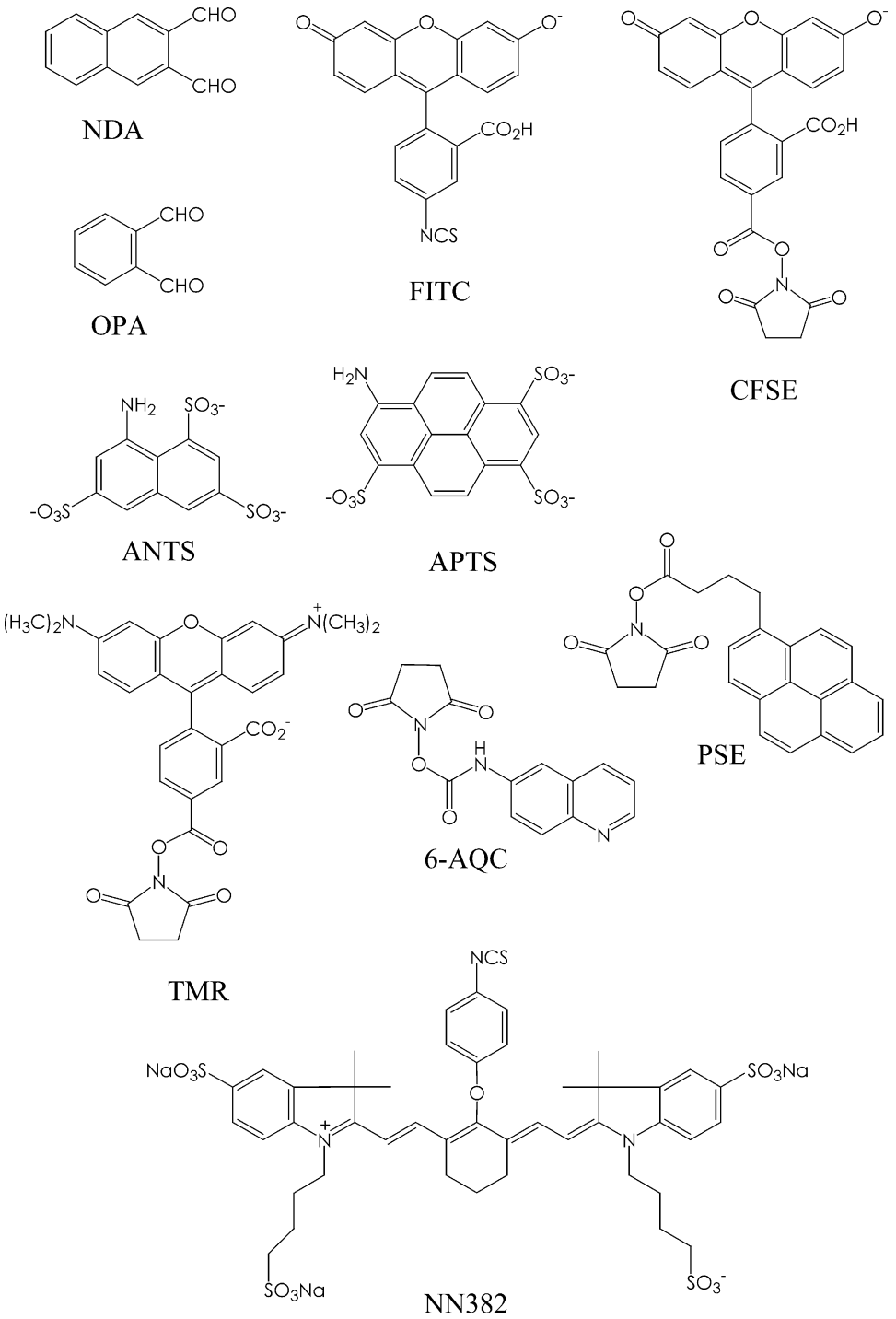
Table 3 (*Continued*)

Analyte	Derivatizing reagent	Reaction condition, catalyst	Reaction time	LIF	Detection limit	Validation		Matrices	Refs.
						Precision (%)			
						Intra-day	Inter-day		
Cadaverine	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	0.83 nM	1.8	2.6		
2-Phenylethylamine	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	0.72 nM	0.7	6		
Tryptamine	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	0.63 nM	7.3	16		
1,6-Diaminohexane	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	4.3 nM	1.7	9.2		
Spermidine	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	8.0 nM	1.5	5.4		
Spermine	FITC	pH 10, r.t.	5–12 h	Ar ion laser, 448 nm	350 nM	8	13.6		
Putrescine	PSE	95 °C, K_2CO_3	40 min	He–Cd laser, 325 nm	6 nM	0.2	7.9		[149]
Cadaverine	PSE	95 °C, K_2CO_3	40 min	He–Cd laser, 325 nm	5 nM	0.8	4.1		
Spermine	PSE	95 °C, K_2CO_3	40 min	He–Cd laser, 325 nm	13 nM	0.8	6		
Spermidine	PSE	95 °C, K_2CO_3	40 min	He–Cd laser, 325 nm	15 nM	1.1	3.1		
Oligosaccharide	ANTS	40 °C, aq. AcOH/$NaBH_3CN$	15 h	He–Cd laser, 325 nm	500 amol				[153]
Oligosaccharide	APTS	75 °C, aq. AcOH/$NaBH_3CN$	1 h	Ar ion laser, 488 nm	0.8 amol				[154]
Oligosaccharide	TMR	pH 8.5	4 h	He–Ne laser, 543.5 nm	80 ymol			Lysed yeast spheroplast	[155,156]
	TMR	pH 8.5	4 h	He–Ne laser, 543.5 nm	10^{-20} mol			Human epidermoid cell line, A431	[155,157]
Lysozyme	6-AQC	55 °C	10 min	He–Cd laser, 325 nm	29.6±0.6 ng/ml	0.28			[161]
AHF	6-AQC	55 °C	10 min	He–Cd laser, 325 nm	150±7 ng/ml	3.6			
HRF	6-AQC	55 °C	10 min	He–Cd laser, 325 nm	11.8±1.0 ng/ml	4.4			

Table 3 (*Continued*)

Analyte	Derivatizing reagent	Reaction condition, catalyst	Reaction time	LIF	Detection limit	Validation		Matrices	Refs.
						Precision (%)			
						Intra-day	Inter-day		
Insulin	6-AQC	55 °C	10 min	He–Cd laser, 325 nm	53.7±3.7 ng/ml	2.2			
Substance P	NDA/CN^-	pH 10	2 min	He–Cd laser, 442 nm	2.5–26.5 nM			Rat microdialysis sample	[164]
	NDA/CN^-	pH 10	2 min	He–Cd laser, 442 nm	3.2–28.6 nM			Bovine brain microvessel endothelial cells	[165]
Amino acids	NN382	pH 9.4, r.t.	12–16 h	GaA1As diode laser	16.5–65.3 zmol				[168]
Histidine	NN382	pH 9.4, r.t.	12–16 h	GaA1As diode laser	401 zmol				
Angiotensin I variants	NN382	pH 8.5, 45 °C, NaCl	1 h	GaA1As diode laser, 787 nm	100–300 zmol				[169]
Amino sugars	NBD-F	pH 6.0, 40 °C	20 min	Ar ion laser, 488 nm	0.5 fmol			Bovine submaxillary mucin	[180]
GlcN						4.9			
GalN						3.4			
GalNol						4.7			
GlcNol						4.9			

[a] On-line reaction.



Scheme 5. Chemical structures of derivatizing reagents described in Section 4.

ters, CAs and amino acids with NDA and CN^- in microdialysis sample from frontal cortex of rat [143]. The fractions collected every 2 min were subjected to CE–LIF system, and the peaks of these neurotransmitters were clearly detected in the electropherograms. While the method by Robert et al. was carried out by off-line mode, Bowser et al. achieved the on-line CE–LIF system coupled to microdialysis using OPA (Scheme 5) and β-mercaptoethanol as fluorescence reagents [144]. The CE–LIF system allowed to determine simultaneously Glu, GABA, Gly, Asp, Ser, taurine, Gln and dopamine in microdialysis sample collected in vivo from rat striatum every 20 s.

Fluorescein isothiocyanate (FITC, Scheme 5) has been frequently used for the fluorescence derivatization of amino group in amino acids or biogenic amines followed by CE–LIF system. The excitation wavelength of FITC is compatible with a commercial Ar ion laser at 488 nm to emit fluorescence at 520 nm. Li et al. used FITC for the derivatization of neurotransmitters, GABA, Glu and Asp in the microdialysis sample from cat brain [145]. However, it took over 6 h at 40 °C to complete the derivatization.

Britz-Mckibbin et al. reported the determination of γ-carboxyglutamic acid, a biochemical marker for osteoporosis, deep-vein thrombosis and liver diseases etc. in protein, human urine and plasma by CE with LIF system after pre-capillary derivatization with FITC [146]. The detection limit was about 5.0×10^{-11} M. It took 24 h at room temperature in the dark to derivatize γ-carboxyglutamic acid with FITC. Accordingly, a drawback of using FITC was that it required a long reaction time. Furthermore, FITC can also react with amino group in the hydrolysis product of isothiocyanate moiety of FITC itself to form a dimer through the thiourea bond [147]. Therefore, large peaks of the dimers, FITC itself, and the hydrolysis product of FITC appeared in the electropherogram. In complementing the demerit of FITC to form dimerization, 5-carboxyfluorescein succinimidyl ester (CFSE, Scheme 5), which has an activated ester for the reaction site instead of isothiocyanate, was developed by Lau et al. [147]. They chose GABA as the test compound. As a result, the peak of derivative of GABA with CFSE and the hydrolysis product of CFSE were observed in the chromatogram. The concentration detection limit with Ar ion laser for GABA derivative was 0.3 nM. However, the problem of requiring a long time for the derivatization with FITC still remains to be solved as it took overnight to complete the derivatization [147].

Biogenic amines, putrescine, histamine, tyramine, cadaverine, etc. produced by microorganism can be used as an index of microbial contamination in foods. Furthermore, these amines themselves cause various actions such as headache, nausea, hypo- or hypertension and cardiac palpitation following absorption to human body. Because of the toxicities of biogenic amines, many papers describing the determinations of the biogenic amines have been published, and most of them employed fluorescence derivatization techniques.

Rodriguez et al. reported that biogenic amines, putrescine, histamine, tyramine, cadaverine, and so on in soy sauce sample was derivatized with FITC and determined by CE with the running buffer containing 30 mM sodium dodecyl sulfate (SDS) at pH 9.4 [148]. The derivatization reaction was performed at pH 10.0 for 5–12 h in the dark at room temperature. The concentration detection limits for these amines were about 10^{-10} M.

Putrescine, cadaverine, spermine and spermidine were determined by micellar electrokinetic chromatography (MEKC) with LIF detection after derivatization with 1-pyrenebutanoic acid succinimidyl ester (PSE, Scheme 5) [149]. The main advantage of using PSE for the determination of polyamines is to form an intramolecular excimer complex, which fluoresces at longer wavelengths (450–520 nm) than mono-labeled analytes (360–420 nm). This was the result of an increase of selectivity of poly-amino compounds, allowing it to be discriminated from mono-amino compounds. The separation of the four polyamines was achieved within 10 min using the buffer consisting of 10 mM phosphate (pH 7.2), 30 mM cholate and 30% acetonitrile as the running buffer. The concentration detection limits for putrescine, cadaverine, spermine and spermidine were 6, 5, 15 and 13 nM, respectively. The unique property of PSE skeletons to form the excimer (to fluoresce at

a longer wavelength) is also used for the determination of polyamine by using HPLC with fluorescence detection [150,151].

4.2. Oligosaccharides

Oligosaccharides play important roles in a variety of biological recognition processes, such as cell–cell interactions, cell development and differentiation, etc. The analysis of an oligosaccharide is considered to be essential for the investigation on its actions in biological events. However, it is challenging to determine the oligosaccharides, because of their complicated structural diversity, which are composed of 8–14 monosaccharide units through *O*-linkage and they contain no specific chromophore or fluorophore. As described above, CE–LIF system with pre-capillary derivatization is one of the most promising methodologies for the determination of the oligosaccharide due to the excellent resolving power and high sensitivity [152]. Fluorescence derivatization of oligosaccharide has mostly been performed through the reductive amination, of which aldehyde group of the saccharide reacted with amino group of the fluorescence reagents. As to the fluorescence reagent for oligosaccharide, aromatic sulfonic acids such as 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and 8-amino-2-naphthalenesulfonic acid (8,2-ANS), have been used [152]. These reagents are highly negatively charged in even low pH buffer and thus are conveniently used in CE separation. Based on their naphthalene skeleton, LIF detection can be adopted using He–Cd laser at 325 nm. Oligomannose-type mixtures were derivatized with ANTS (Scheme 5) as the derivatization reagent, and separated less than 8 min using running buffer at pH 2.5 [153]. The concentration and mass detection limits were 5×10^{-8} M and 500 amol, respectively [153]. Oligosaccharide derivatized with 9-aminopyrene-1,4,6-trisulfonate (APTS, Scheme 5), which has a pyrene skeleton instead of naphthalene, fluoresce at 512 nm with excitation of 488 nm of Ar ion laser [154]. Excitation at 488 nm gave a merit for high sensitivity over that at 325 nm of He–Cd laser, because of less production of interferences originated from the endogenous compounds. The detection limit was 0.4 nM, being estimated to be 0.8 amol.

In contrast, the use of tetramethylrhodamine (TMR, Scheme 5) has given more sufficient results [155–157]. A He–Ne laser was used for the excitation of TMR adducts at 543.5 nm. As shown in Fig. 4, 350 molecules (top) of each TMR adduct were sensitively detected. The proposed CE method allowed to give the detection limit of ca. 80 yoctomol, or 50 molecules of the TMR-labeled saccharides [156]. The TMR adducts were well separated on the CE with a running buffer containing borate and phenylboronic acid, as a result of forming a complex with hydroxyl groups

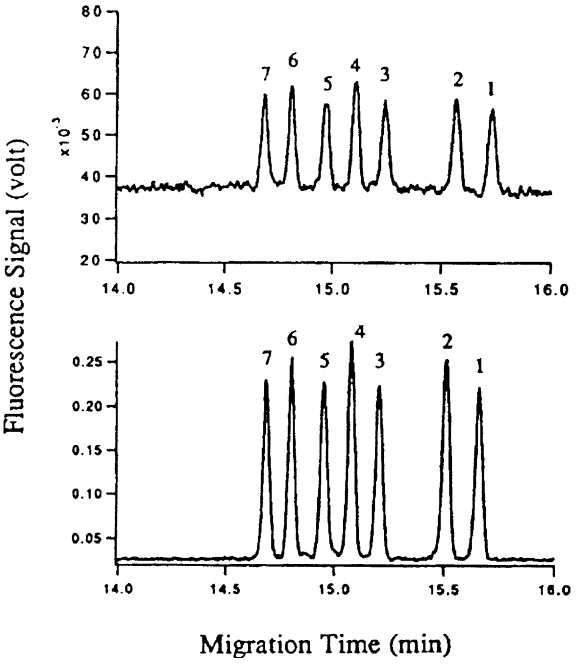


Fig. 4. CE–LIF of six fluorescently labeled saccharides (peaks 2–7) and the linker arm (peak 1). Capillary, 42 cm × 10 μm I.D. × 144 μm O.D.; running buffer, 10 mM phosphate-borate-phenylboronic acid-SDS; running voltage, 16.8 kV (400 V/cm); injection time, 5 s; injection voltage, 500 V; injection volume, 5.5 pl; concentration of each component, 10^{-10} M (top) and 10^{-9} M (bottom). Peaks: 1 = $HO(CH_2)_8CONHCH_2CH_2NH$-CO-TMR (linker arm: -O-TMR); 2 = *N*-acetyl-β-D-glucosaminide-O-TMR (GlcNAc-O-TMR); 3 = βGal(1→3)βGlcNAc-O-TMR (Lewis C); 4 = βGal(1→4)βGlcNac-O-TMR (LacNAc); 5 = αFuc(1→2) βGal(1→4)βGlcNAc-O-TMR (H-Type II); 6 = βGal(1→4)[αFuc(1→3)]βGlcNAc-O-TMR (Lewis X); 7 = αFuc(1→2)βGal(1→4)[αFuc(1→3)]βGlcNAc-O-TMR (Lewis Y). Reproduced from Ref. [156] with permission.

of sugar to enhance the separatability of the TMR adducts [157].

4.3. Peptides and proteins

Analyses of peptides by CE were extensively reviewed by Knull et al. [158], Koller et al. [159] and Kašička [160]. It appears that CE separation of peptides is one recent interesting topic in bioscience.

A bioactive peptide/protein has mostly been derivatized with a suitable fluorescence or electrochemical reagent to increase the detection sensitivity. However, when a peptide/protein is derivatized with the reagent, multiple peaks would appear on the electropherogram.

The appearance of multiple peaks is caused by heterogeneous derivatization of the functional groups in a single peptide/protein. Three dimensional structure of the peptide/protein makes it difficult for several functional groups in a single peptide/protein to be equally accessible to the fluorescence reagent. As a result, the derivatization results in a mixture of mono-, di-, tri-,...labeled products—the very cause of the problem that an identification or a sensitive detection of the peptide/protein is difficult. One of the many ways to overcome the multiple labeling is to explore the uniform derivatization at all functional groups.

For the fluorescence derivatization of peptide/protein in CE, 6-aminoquinoyl-*N*-hydroxysuccinimidyl carbamate (6-AQC, Scheme 5) has been used by Liu et al., who reported that an addition of SDS would help the derivatization and CE separation of such protein and peptide [161]. They reported that the addition of 50 mM SDS in the reaction medium prior to the addition of 6-AQC was effective for the fluorescence derivatization of proteins, such as human insulin, lysozyme and adrenocorticotropic hormone fragment [161]. Furthermore, in the case of larger proteins such as α-chymotrypsinogen A, ovalbumin and bovine serum albumin, the reduction with dithiothreitol and alkylation with sodium iodoacetate were effective [162]. According to our experience, *n*-dodecyl β-D-maltopyranoside (DM) is more effective than SDS for the completion of derivatization [95]. 6-AQC derivative has also been applicable to the ED [163].

Substance P, a dodecapeptide in tachykinin family, was pre-column derivatized with NDA/CN^-, followed by CE separation (MEKC) with LIF detection, which was accomplished using a 32 mW He/Cd laser at 442 nm for the excitation [164,165]. The concentration detection limit ranged from 2.5 to 26.5 nM. The proposed method was applied to the study of metabolism of substance P in rat striatum using microdialysis technique [164], and to that on permeability into blood–brain barrier using bovine brain microvessel endothelial cells [165].

Lunte et al. utilized the biuret reaction to form a complex of copper ion (Cu^{2+}) and a peptide for the determination of peptide in CE followed by ED [166,167]. The reaction of the complexation took place inside the capillary, and the separation was performed using borate–tartrate buffer (pH 9.8) including 1.0 mM $CuSO_4$ as a running buffer. The detection limit was 200 nM for the Cu^{2+}–angiotensin II complex. The separation efficiency of the peptide was also improved by forming the complexation with Cu^{2+}. The method was successfully applied to monitor the in vitro metabolism of angiotensin I in human plasma [167].

Legendre et al. derivatized amino acids with a near-infrared (NIR) fluorescence dye, NN382 (Scheme 5) followed by CE separation with methanol–water (50:50) as the running buffer (40 mM borate, pH 9.4) including 0.55 mM cetyltrimethylammonium bromide [168]. The detection limits for amino acids were in the range of 16.5–401 zmol.

Baars et al. reported the pre-capillary derivatization of angiotensin I and closely related peptides with NIR fluorescence dye (NN382) followed by CE separation using a running buffer (pH 7.2) containing 8 mM Triton X-100 and 1 M urea. The use of the dye gave a successful separation with highly sensitive detection (Fig. 5) [169]. NN382 is a hydrophilic reagent and has a fluorescence feature of absorption and emission maxima in water around 780 and 800 nm, respectively. The detection limits for these angiotensins were in the range of 100–300 zmol. It is expected the proposed CE method can be applied to many biological studies.

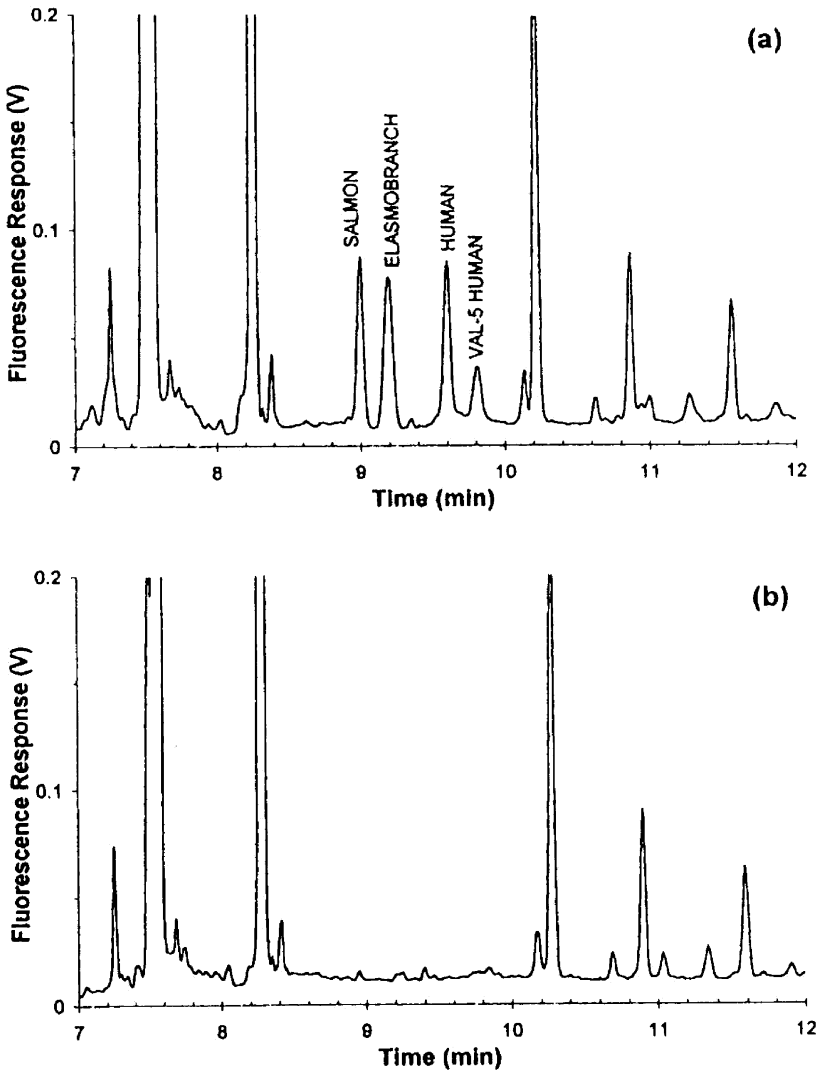


Fig. 5. (a) MEKC separation of 13 amol of salmon, elasmobranch, human, and Val-5 Ang-I peptide injected on column. A standard peptide mixture was prepared in water containing each peptide at 5×10^{-7} M. A 50 μl sample of the mixture was derivatized and diluted 310-fold, to a derivative concentration of 5.4×10^{-10} M, prior to injection. About 23.5 nl of the sample was injected on column during a 5-s hydrodynamic injection at 0.5 psi. Separation was conducted at 16 kV and 28 °C on a bare fused-silica capillary (50 cm to detector, 75-μm i.d.) with a 100 mM phosphate run buffer (pH 7.2) containing 8 mM Triton X-100 and 1 M urea. (b) MEKC separation of reagent blank solution. Sample was prepared as in (a) but 50 μl of water was substituted for the peptide mixture. Reproduced from Ref. [169] with permission.

In the last few years, new trends of chromatographic separation techniques such as capillary electrochromatography (CEC) [170–174] or microchip electrophoresis (ME) [175–179], have emerged and been applied to determine some compounds. Suzuki et al. developed a highly sensitive and rapid determination of aminosugars, D-glucosamine, D-galactosamine, and their reduced forms, derivatized with NBD-F on a microfabricated quartz chip with Ar ion LIF detection [180]. The separation of the aminosugars was achieved within 60 s, and 0.5 fmol of aminosugar was detected. The proposed method was successfully applied to determine aminosugars

in the acid hydrolysate of bovine submaxillary mucin.

5. Future trends

In this article, recent derivatization methods for the fluorescence and CL detection in HPLC, and for LIF detection in CE, were the subjects of interest. In HPLC, a fluorescence derivatization has been extensively employed for increasing the detection sensitivity. The hydrophilic fluorescence reagents were effective for the avoidance of undesired adsorption, especially in the case of determination of peptides, and had been applied to the determination of insulin chain A, BI and BII [95] and bradykinin [97] in biosamples. Currently, developments of water-soluble fluorescence reagents are eagerly awaited. It is possible that these reagents may emit a strong fluorescence even in the medium of high percentage of H_2O.

As concerned with the detectability and separatability in CE, extremely low detection limit ranging from atto to yocto mol level and the analysis time of less than a few minutes have been achieved. In particular, CE–LIF and –NIR detections were shown to be appropriate for the separation and detection of a trace amount of oligosaccharides and peptides. Although the determinations in single cell or microdialysis sample have been reported, there have been a few papers determining these analytes in biosamples such as plasma or urine. This is perhaps due to the complicated matrices of biosamples such as plasma and urine, which could also react with the reagent to give many interfering peaks, overlapping a small peak corresponding to a trace amount of the analyte. Considering this, a development of the methodology for the removal of a number of the interfering substances should also be critical in CE.

Recently, new trends of chromatographic separation techniques, CEC and ME can be expected to improve the separatability and the required analysis time. The derivatization methods in the HPLC, CE, CEC, and ME will further enhance the advancement of the research in pharmaceutical, environmental, clinical, and biochemical fields.

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