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Review

Fluorogenic reactions for biomedical chromatography

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Abstract

A number of fluorogenic reactions, which have been used for HPLC detection systems by means of pre- and/or postcolumn derivatization, are surveyed with respect to both sensitivity and selectivity for the determination of biomedically important substances. For the derivatization of the substances, two types of fluorogenic reactions, fluorescence-generating and fluorescence-tagging, have been studied. The former are usable in most instances for both pre- and postcolumn derivatization methods, and the latter only for precolumn derivatization methods. HPLC methods utilizing the fluorogenic reactions allow analytes to be detected at picomole–subfemtomole levels. In the fluorescence-generating reactions, several fluorogenic reagents possessing two or more reactive sites in the molecule, which show molecular recognition for a variety of analytes, permit facile and reproducible detection in HPLC because there are fewer interferences from biological matrices.

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List of abbreviations

ADAM	9-anthryldiazomethane
AE-OTf	2-(2,3-anthracenedicarboximido)ethyl trifluoromethanesulphonate
BA	benzamidine
Boc-L-Cys	N-*tert*.-butyloxycarbonyl-L-cysteine
Br-DMC	4-bromomethyl-6,7-dimethoxycoumarin
Br-DMEQ	3-bromomethyl-6,7-dimethoxy-1-methylquinoxalin-2(1*H*)-one
Br-MAC	4-bromomethyl-7-acetoxycoumarin
Br-MDC	4-bromomethyl-6,7-methylenedioxycoumarin
Br-MMC	4-bromomethyl-7-methoxycoumarin
Dansyl-Cl	1-dimethylaminonaphthalene-5-sulphonyl chloride
Dansyl-BAP	N-bromoacetyl-N′-(dansyl)-piperazine
DBD-PZ	4-(N,N-dimethylaminosulphonyl)-7-N-piperazino-2,1,3-benzoxazole
DMB	1,2-diamino-4,5-methylenedioxybenzene
DMEQ-hydrazide	6,7-dimethoxy-1-methyl-2(1*H*)-quinoxaline-3-propionylcarboxylic acid hydrazide
DPE	1,2-diphenylethylenediamine
ED	ethylenediamine
Em	fluorescence emission wavelength
Ex	fluorescence excitation wavelength
FMOC-Cl	9-fluorenylmethyl chloroformate
HPLC	high-performance liquid chromatography
NBD-Cl	4-chloro-7-nitrobenzo-2-oxa-1,3-diazole
NBD-F	4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole
NDA	2,3-naphthalenedialdehyde
NE-OTf	2-(2,3-naphthalimido)ethyl trifluoromethanesulphonate
OMB-COCl	2-(5-chlorocarbonyl-2-oxazolyl)-5,6-methylenedioxybenzofuran
OPA	*o*-phthalaldehyde
p-MBA	4-methoxybenzamidine
p-MOED	*meso*-1,2-bis(4-methoxyphenyl)ethylenediamine
PGO	phenylglyoxal
RP	reversed-phase
THI	trihydroxyindole

1. Introduction

High-performance liquid chromatography (HPLC), one of the most effective separation methods, has been widely used in biomedical and many other fields. Among various detection methods in HPLC, fluorescence detection is sensitive and selective, and thus useful especially for the determination of trace levels of bioactive compounds in complicated matrices such as mammalian blood, urine and tissue. Its increasing use is due not only to improvements in the fluorescence detection systems but also to the development of various fluorogenic reactions [1–5].

Fluorogenic reactions can be classified into two groups by reaction type: "fluorescence generation" and "fluorescence tagging or labelling". In the former, the fluorogenic reagents are generally non-fluorescent and react with target compounds to form conjugated-ring molecules,

resulting in production of fluorescence. In the latter, the reagents are composed of a highly fluorescent aromatic moiety and a reactive moiety, and the reactive moiety attaches to an analyte to form a fluorescence-tagging derivative.

Fluorogenic reactions can also be categorized according to the timing of the reaction in HPLC: precolumn derivatization (before separation) and postcolumn derivatization (after separation). In the former, the reaction should produce individually the different derivatives from the analytes. However, the optimum reaction conditions are not limited by the HPLC separation conditions. In the latter, the reaction should proceed rapidly because a prolonged reaction time causes peak broadening in chromatography. Fluorogenic reagents for postcolumn derivatization need to be non-fluorescent or markedly different from the derivatives in their fluorescence excitation and/or emission spectra in the mobile phase; they are allowed to give multiple fluorescent derivatives, provided that their reactions are reproducible. In general, the reagents for fluorescence generation have the possibility of being used for both pre- and postcolumn derivatization methods, although fluorescence-tagging reagents are used only for precolumn derivatization.

In this review, analytical features of fluorogenic reactions of biologically important compounds and their applications to HPLC with fluorescence detection are surveyed.

2. Amines and amino acids

Amines and amino acids are frequently measured throughout all the life science fields, and a variety of fluorogenic reactions and modified methods have been proposed for pre- and postcolumn derivatizations. The amino moieties of the compounds are so reactive that the fluorogenic reactions generally proceed under mild conditions.

2.1. General considerations on amino compounds

o-Phthalaldehyde (OPA) reacts with primary amines and amino acids in the presence of 2-mercaptoethanol to form highly fluorescent isoindole derivatives (Fig. 1A) [6]. The reaction proceeds rapidly (within 2 min) in borate buffer (pH 6–8 for amines, pH 9.5–10 for amino acids). The reaction can be applied to postcolumn derivatization [7] and precolumn derivatization [8] of amino acids in HPLC. As OPA derivatives are not sufficiently stable to afford reproducible results, the postcolumn OPA reaction coupled with cation-exchange chromatography has most commonly been utilized for amino acid analysis. An automated system for the precolumn derivatization makes the method highly sensitive and reproducible [9]. 2-Ethanethiol [10], 3-mercaptopropionic acid [11] and N-acetyl-L-cysteine [12,13] afford more stable fluorescent derivatives than 2-mercaptoethanol. The detection limits [signal-to-noise ratio (S/N) = 3] of amino acids in precolumn derivatization HPLC are *ca.* 1 pmol on-column.

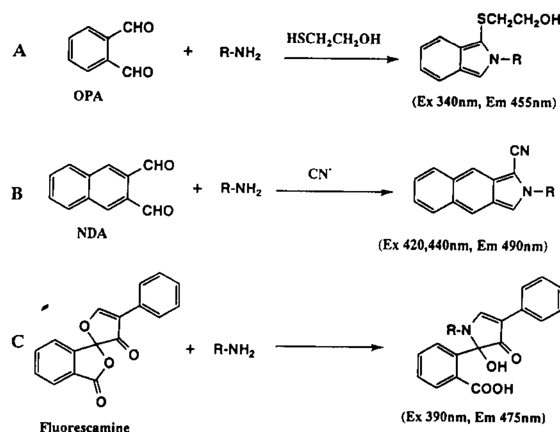

Fig. 1. Fluorogenic reactions of primary amino compounds with (A) OPA, (B) NDA and (C) fluorescamine.

The OPA reaction is applicable to the separation of the enantiomers of amino compounds by using a homochiral thiol compound such as N-acetyl-L-cysteine [12,13], N-*tert.*-butyloxycarbonyl-L-cysteine (Boc-L-Cys) [12], 1-thiol-D-glucose [14] and D-3-mercapto-2-methylpropionic acid [15]. In these methods, enantiomeric amino compounds are converted into diastereomeric isoindole compounds that can be separated by conventional RP-HPLC (Fig. 2). The OPA and Boc-L-Cys reaction was successfully applied to the determination of the enantiomeric amino acids in rat brain and serum samples [16].

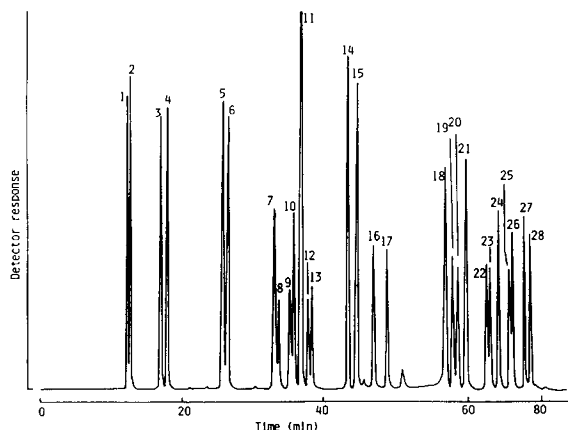

Fig. 2. Chromatogram of OPA–S-acetyl-L-cysteine derivatives of standard amino acid enantiomers. Column: Develosil ODS (5 μm; 200 × 6 mm I.D.). Mobile phase: methanol (0–60%)–50 mM sodium acetate. Peaks: 1 = D-Asp; 2 = L-Asp; 3 = L-Glu; 4 = D-Glu; 5 = D-Ser; 6 = L-Ser; 7 = D-Thr; 8 = L-His; 9 = D-His; 10 = L-Thr; 11 = Gly; 12 = L-Arg; 13 = D-Arg; 14 = D-Ala; 15 = L-Ala; 16 = L-Tyr; 17 = D-Tyr; 18 = L-Val; 19 = D-Met; 20 = L-Met; 21 = D-Val; 22 = D-Phe; 23 = L-Phe; 24 = L-Ile; 25 = D-Lys; 26 = D-Ile; 27 = D-Leu; 28 = L-Leu. Concentrations: 5 ng each. (Reproduced with permission from ref. 13.)

OPA-analogous reagents, 2,3-naphthalenedialdehyde (NDA) (Fig. 1B) [17] and 3-(2-furoyl)quinoline-2-carbaldehyde [18], also react with primary amino compounds in a similar manner except that cyanide ion is used instead of the thiol compound. Although these reactions require longer reaction times (15–20 and 45 min, respectively) than OPA reaction, the resulting derivatives are so stable that both reactions are suitable for precolumn derivatization. The detection limits [at a signal-to-noise ratio (S/N) of 3] of the NDA method [17] for amino acids are 15–30 fmol on-column, and a laser-induced detection system using an argon ion laser can increase the sensitivity *ca.* 100-fold [19].

Fluorescamine reacts with primary amines and amino acids in borate buffer (pH 9.5–10) at room temperature (Fig. 1C), and this reaction is complete in a few minutes [20,21]. Since the reagent is non-fluorescent, the reaction is used for precolumn [22] and postcolumn [23,24] derivatizations of amino acids. The fluorescent derivatives are not very stable, and the sensitivity is 2–5 times lower than in the OPA method. However, this method is useful for the determination of labile substances. An analogous reagent, 2-methoxy-2,4-diphenyl-3[2*H*]-furanone, reacts with primary amino compounds in a similar manner [25]. The completion of the reaction requires 30 min at room temperature, although the derivatives are very stable.

1-Dimethylaminonaphthalene-5-sulphonyl chloride (Dansyl-Cl) reacts with both secondary and primary amino compounds under weakly alkaline conditions (Fig. 3); the optimized reaction times (30–120 min) vary depending on the type of amino compound [26,27]. Dansyl derivatives are fairly stable and have long Stokes shifts. The reagent is hydrolysed in the derivatization procedure to produce highly fluorescent 1-dimethylaminonaphthalene-5-sulphonic acid. Hence this reaction is used mainly for precolumn derivatization. The derivatives of amino acids can be separated on an RP column [27]. The sensitivity of this method is comparable to that of the OPA method. As Dansyl-Cl also acts on a phenolic hydroxyl moiety, amino compounds containing this moiety such as catecholamines, tyramine and tyrosine, give multiple fluorescent derivatives [28]. Analogous sulphonyl chlorides, 1-di-*n*-butylaminonaphthalene-5-sulphonyl chloride [29], 2-methylanilinonaphthalene-6-sulphonyl chloride [30] and 4-(N-phthalimidyl)benzenesulphonyl chloride [31], also act on primary and secondary amino compounds in a similar manner.

4-Chloro- and 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazoles (NBD-Cl and NBD-F) [32,33] work on primary and secondary amino compounds (Fig. 4) at 50–60°C under alkaline conditions (pH 8–9). The reaction with NBD-F is more than ten times faster than that with NBD-Cl, and

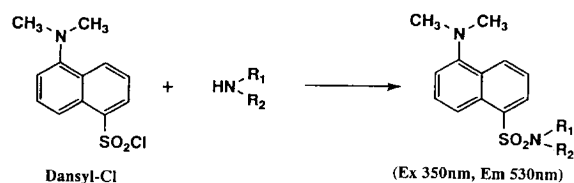

Fig. 3. Fluorogenic reaction of primary and secondary amino componds with Dansyl-Cl.

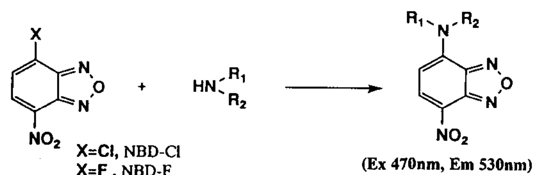

Fig. 4. Fluorogenic reaction of amino compounds with NBD-Cl or NBD-F.

is complete within 1 min. As the hydrolysed product of the reagents (NBD-OH) also fluoresces intensely, both reagents are used only for precolumn derivatization. The derivatives of amino acids other than tryptophan can be separated by RP-HPLC and their detection limits are in the subpicomole range. 9-Fluorenylmethyl chloroformate (FMOC-Cl) is used as a precolumn derivatization reagent for primary and secondary amino compounds (Fig. 5) [34]. The reaction proceeds in borate buffer (pH 8) within 2 min, and the derivatives are stable. However, FMOC-Cl and its hydrolysed product (FMOC-OH) are highly fluorescent, hence they have to be removed by extraction with an organic solvent, pentane. As with Dansyl-Cl, FMOC-Cl is also reactive towards a phenolic hydroxyl moiety. Further, the reagent combines with the imidazole ring of amino compounds. In this reaction, tyrosine and histidine give the corresponding mono- and disubstituted derivatives. Despite these disadvantages, this reaction has become popular through simplification [35,36] and/or automation [35,37] of the derivatization procedure. The sensitivity is comparable to that of the OPA method.

Analogous reagents, 2-naphthyl chloroformate [38], 2-(1-pyrenyl)ethyl chloroformate [39], 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride [40], 6-methoxy-2-methylsulphonylquinoline-4-carbonyl

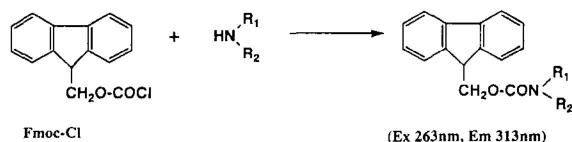

Fig. 5. Fluorogenic reaction of amino compounds with FMOC-Cl.

chloride [41] and 7-dimethylaminocoumarin-3-carbonyl fluoride [42], have been developed. These reactions proceed in an aprotic solvent, benzene or acetonitrile, and are complete within 5 min at room temperature, except for 2-naphthyl chloroformate (100°C, 1 h). The reactions may be useful for the precolumn derivatization of hydrophobic amines.

Edman's reagents are used not only in the precolumn determination of amino acids but also in the microanalysis of peptide sequences. The following reagents have been developed: fluorescein isothiocyanate [43], 4-(N,N-dimethylamino)-1-naphthyl isothiocyanate [44], 4-(1-dimethylaminonaphthalene-5-sulphonylamino)phenyl isothiocyanate [45], 3- and 4-(2-phenanthraoxazolyl)phenyl isothiocyanates [46] and 7-N,N-dimethylaminosulphonyl- and 7-aminosulphonyl-4-(2,1,3-benzoxadiazolyl) isothiocyanates [47]. These reagents do not show great differences in sensitivity and reactivity.

2.2. Particular amines and amino acids

The measurement of catecholamines in biological samples requires high selectivity and sensitivity, because many precursors and metabolites co-exist in the samples. The reagents for general amino compounds do not have sufficient selectivity for this purpose. Trihydroxyindole (THI) [48,49], ethylenediamine (ED) [50,51] and 1,2-diphenylethylenediamine (DPE) [52] methods have developed as highly selective reactions for catecholamines (Fig. 6). Of these methods, the THI method is the most selective, but it cannot afford fluorescence with dopamine. The DPE method is the most sensitive for all the catecholamines and is the best reaction in practical use. DPE derivatives of catecholamines can be separated on an RP column. By use of this reaction for precolumn derivatization, catecholamines in human plasma (0.5 ml) can be measured (Fig. 7) [52]. The method requires only simple clean-up of plasma by solid-phase extraction using a cation-exchange cartridge. Clean-up by liquid–liquid extraction is also effective [53,54]. An on-line automated precolumn

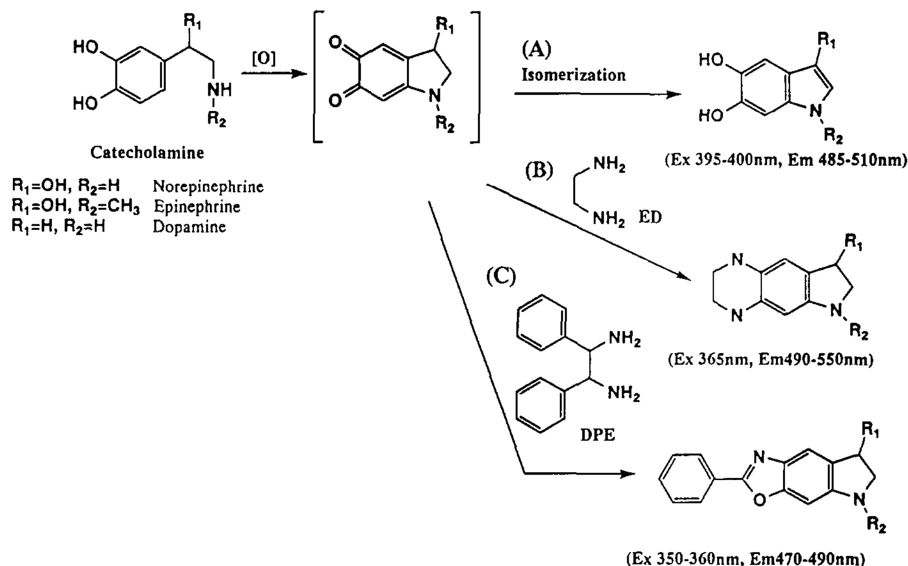

Fig. 6. Fluorogenic reactions of catecholamines by methods (A) with formation of THI and with (B) ethylenediamine and (C) DPE reagents.

derivatization has been devised for reproducible results [55].

Histamine can be selectively converted into a fluorescent derivative by reaction in an alkaline medium with OPA in the absence of a thiol compound [56,57] (Fig. 8). This reaction is applicable to precolumn [58] and postcolumn [59] derivatization HPLC for the determination of histamine in human serum and rat tissues.

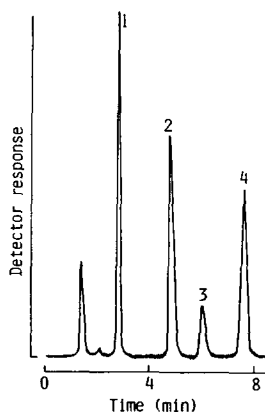

Fig. 7. Chromatogram of DPE derivatives of catecholamines in human plasma. Column: TSK gel ODS-120T (5 μm; 150 × 4.6 mm I.D.) Mobile phase: acetonitrile–methanol–50 m*M* Tris–hydrochloric acid buffer (pH 7.0) (5:1:4, v/v/v). Peaks with concentrations (pmol/ml plasma) in parentheses: 1 = norepinephrine (1.72); 2 = epinephrine (0.56); 3 = dopamine (0.21); 4 = isoproterenol (internal standard, 0.5). (Reproduced with permission from ref. 52.)

Benzylamine reacts with 5-hydroxyindolamines under fairly mild conditions (pH 9.0, 37°C, 20 min) [60] (Fig. 9), and this reaction can be applied to postcolumn derivatization HPLC [61]. DPE reacts with the indoleamine under similar conditions to those for the benzylamine reaction. By applying the reaction to postcolumn derivatization, 5-hydroxytryptamine and its metabolites (5-hydroxyindoles) in rat brain tissues are measured together with catecholamines [62].

Guanidino compounds and arginine produce fluorescence selectively by reaction with 9,10-phenanthraquinone [63] or benzoin [64,65] (Fig. 10). They are applicable to postcolumn derivatization HPLC, but only benzoin is useful for the precolumn derivatization HPLC of biogenic guanidino compounds in human serum and urine [66].

Tryptophan and indolamines can be converted into fluorescent derivatives by reactions with formaldehyde [67], chloroacetaldehyde [68] and

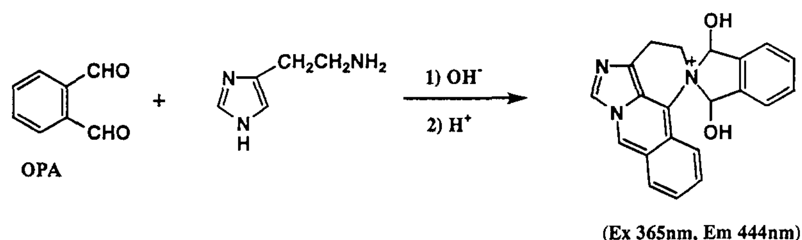

Fig. 8. Fluorogenic reaction of histamine with OPA.

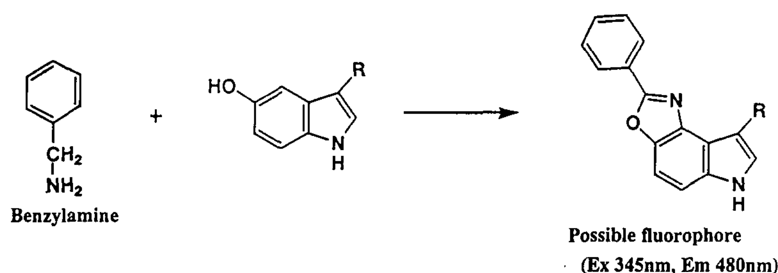

Fig. 9. Fluorogenic reaction of 5-hydroxyindoles with benzylamine.

methoxyacetaldehyde [69]. The reaction conditions are fairly drastic (acidic medium, 80–100°C, 15–60 min) in the presence of an oxidizing agent, and they are used for precolumn derivatization. Phenylglyoxal reacts selectively with tryptophan [70] (Fig. 11) and this reaction was applied to the determination of free and total tryptophan in human serum [71].

3. Organic acids

As the carboxyl moiety shows low reactivity, high activity is required of fluorogenic reagents (Fig. 12) for organic acids (carboxylic acid): bromomethyl, diazomethyl and amino moieties are used as reactive sites. Table 1 shows the reagents used and features of the fluorogenic

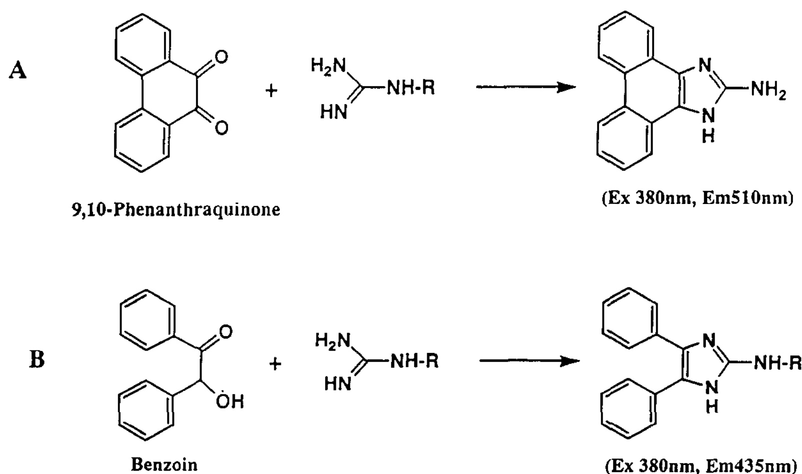

Fig. 10. Fluorogenic reactions of guanidino compounds with (A) 9,10-phenanthraquinone and (B) benzoin.

Table 1
Features of fluorogenic reactions for carboxylic acids

Reagent[a]	Reaction conditions			Catalyst or coupling reagent[c]	Ex (nm)	Em (nm)	DL[d] (fmol)	Ref.
	Solvent	Temperature (°C)[b]	Time (min)					
Aryl bromomethanes ($-CH_2Br$)								
Br-MMC	Acetonitrile	60	15	K_2CO_3, 18-crown-6	360	415	10 000	72,73
Br-DMC	Acetonitrile	70	30	K_2CO_3, 18-crown-6	340	425	500	74
Br-MAC	Acetone	50	30	K_2CO_3, 18-crown-6	365	460	10	75
Br-DMEQ	Acetonitrile	80	20	K_2CO_3, 18-crown-6	370	460	1	76
9-Bromomethylacridine	Phosphate buffer (pH 7)–chloroform	60	5	Micellar phase-transfer catalyst	362	418	<1000	77
9-Bromomethylanthracene	Acetonitrile	80	20	K_2CO_3, 18-crown-6	365	412	100	78
Br-MDC	Acetonitrile	40	60	K_2CO_3, 18-crown-6	355	435	15	79
Acyl bromomethanes ($-COCH_2Br$)								
Panacyl bromide	Acetonitrile	25–37	60–180	Triethylamine	375	470	250	80
Bromoacetylpyrene	Acetonitrile	40	30	K_2CO_3, 18-crown-6	365	412	100	81
(N,9-Acridinyl)bromo-acetamide	Phosphate bufer (pH 7.2)–chloroform	90	30	Micellar phase-transfer catalyst	358	482	10	82
Dansyl-BAP	Acetonitrile	RT	20	$KHCO_3$, 18-crown-6	350	530	1000	83
Aryl diazomethanes ($-CHN_2$)								
ADAM	Acetonitrile	RT	60	None	365	412	100	84
4-Diazomethyl-7-methoxycoumarin	Acetonitrile	80	60	None	325	385		85
Fluorescent amines ($-NH_2$)								
Dansyl semipiperazine	Chloroform	RT	30	DCC	350	530	1000	86
Dansyl cadaverine	Dimethylformamide	RT	15	DEPC	340	518	100	87
DBD-PZ	Acetonitrile	RT	20	Mukaiyama A	440	569	3–5	88
Miscellaneous								
DMEQ-hydrazide	Water	RT	15	EDC–pyridine	365	447	3–5	89
NE-OTf	Acetonitrile	RT	10	KF, 18-crown-6	259	394	4	90
AE-OTf	Acetonitrile	RT	10	TEAC	298	456	0.5–3	91

[a] Br-MMC = 4-bromomethyl-7-methoxycoumarin; Br-DMC = 4-bromomethyl-6,7-dimethoxycoumarin; Br-MAC = 4-bromomethyl-7-acetoxycoumarin; Br-DMEQ = 3-bromomethyl-6,7-dimethoxy-1-methylquinoxalin-2(1*H*)-one; Br-MDC = 4-bromomethyl-6,7-methylenedioxycoumarin; Dansy-BAP = N-bromoacetyl-N'-(dansyl)piperazine, ADAM = 9-anthryldiazomethane; DBD-PZ = 4-(N,N-dimethylaminosulphonyl)-7-N-piperazino-2,1,3-benzoxazole; DMEQ-hydrazide = 6,7-dimethoxy-1-methyl-2(1*H*)-quinoxaline-3-propionylcarboxylic acid hydrazide; NE-OTf = 2-(2,3-naphthalimido)ethyl trifluoromethanesulphonate; AE-OTf = 2-(2,3-anthracenedicarboximido)ethyl trifluoromethanesulonate.

[b] RT = room temperature.

[c] DCC = dicyclohexylcarbodiimide; DEPC = diethyl phosphorocyanidate; EDC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; Mukaiyama A = pyridine–2,2'-dipyridyl disulphide–triphenylphosphine; TEAC = tetraethylammonium carbonate.

[d] Detection limit (S/N = 3) on-column.

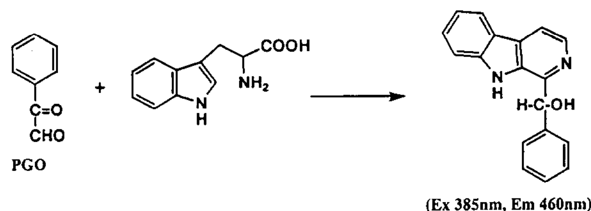

Fig. 11. Fluorogenic reaction of tryptophan with phenylglyoxal (PGO).

reactions, with literature references. All reagents are of the fluorescence tagging type and highly fluorescent. Further, they are easily decomposed on exposure to moisture or light to form fluorescent by-products. Therefore, these reactions are used for precolumn derivatization and the derivatives are generally separated on an RP column.

Bromomethyl reagents [72–83] react with carboxylic acids in acetonitrile under fairly drastic conditions and the reactions are catalysed by a crown ether and potassium ion, except that 9-bromomethylacridine [77] and (N-9-acridinyl)-bromoacetamide [82] react in an aqueous matrix. ADAM [84] works under mild conditions without catalysis. Recently, "fluorescent amine"-type reagents [86–88] have been developed. Various coupling reagents make the reaction proceed under mild conditions (room temperature, 10–30 min).

Serum fatty acids can be measured by using the following reagents: Br-MMC [73], Br-MAC [75], Br-DMEQ [92], ADAM [93], Dansyl semipiperazine [86] and DMEQ-hydrazide [94]. Only the DMEQ-hydrazide method does not require organic solvent extraction of fatty acids from a serum sample. Measurement of prostaglandins in biological samples requires higher sensitivity than that of fatty acids. Several methods have been developed using the following reagents: Br-MAC [95], Br-DMEQ [96], ADAM [97], Dansyl semipiperazine [98], Dansyl cadaverine [87] and DBD-PZ [99]. As prostaglandins are generally labile, derivatizations under mild conditions (like the fluorescent amine type) are preferable. The reagents, Br-MAC and Br-DMEQ are usable for the determination of

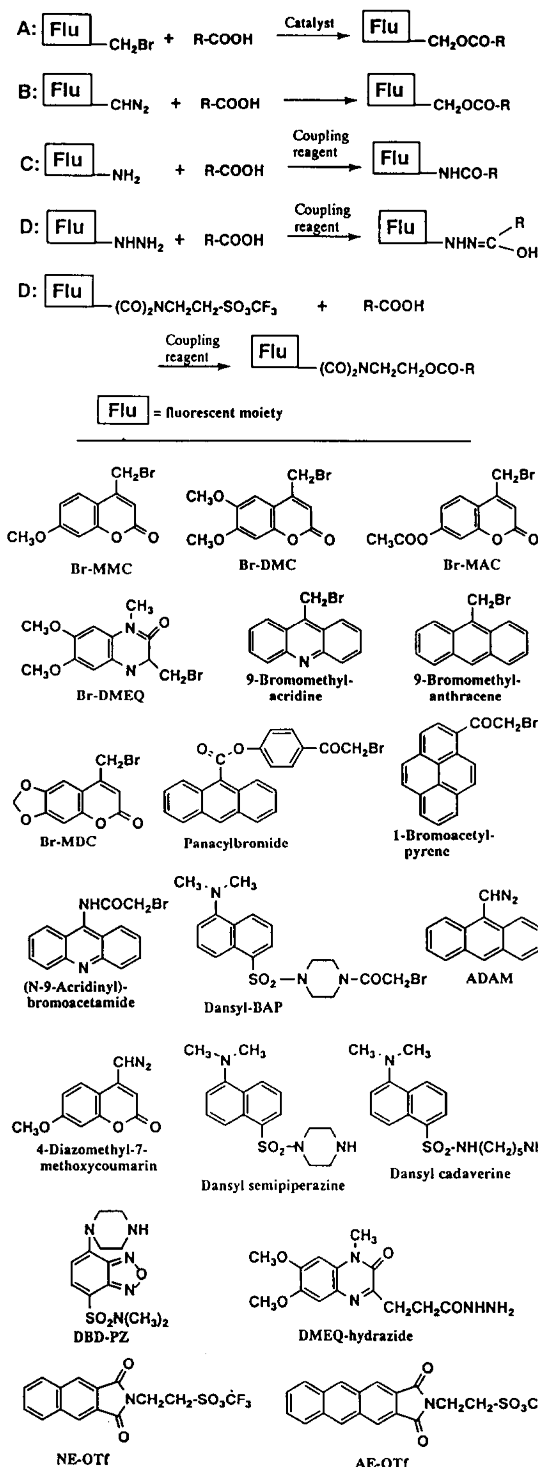

Fig. 12. Five types of fluorogenic reactions and their reagents for carboxylic acids. For abbreviations, see Table 1.

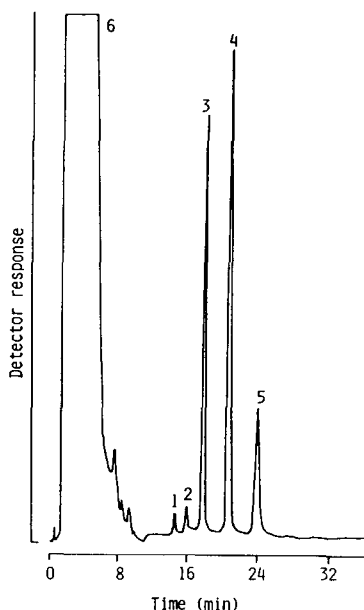

Fig. 13. Chromatogram of DMEQ derivatives of prostaglandins (PGs) in human seminal fluid. Column: YMC Pack C_8 (10 μm; 150 × 6 mm I.D.). Mobile phase: acetonitrile–methanol–water (35:10:55, v/v/v). Peaks with concentrations (nmol/ml) in parentheses: 1 = PGF_2 (5.5); 2 = PGF_1 (8.2); 3 = PGE_2 (84.1); 4 = PGE_1 (94.1); 5 = 16-methyl-PGF_1 (internal standard); 6 = Br-DMEQ and endogenous carboxylic acids. (Reproduced with permission from ref. 96.)

endogenous prostaglandins in human seminal fluids [95,96]. Fig. 13 shows a chromatogram of the DMEQ derivatives of synthetic and biogenic prostaglandins. The detection limits in the method are at the femtomole level; even higher sensitivity is required for the measurement of serum prostaglandins.

4. Steroids

As biological steroids have hydroxyl, carbonyl and/or carboxyl groups, fluorogenic reactions selective for these groups are used in their determinations. Most of these reagents are of the "fluorescence-tagging" type. The fluorescence derivatives can be separated on an RP column.

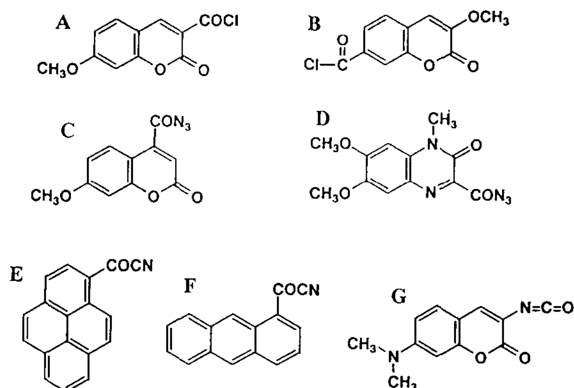

Fig. 14. Fluorogenic reagents for alcohols.

4.1. Reactions for hydroxyl group

Fig. 14 shows fluorogenic reagents for alcohols: 7-methoxycoumarin-3-carbonyl chloride (A) [100], 3-methylcoumarin-7-carbonyl chloride (B) [101], 7-methoxycoumarin-4-carbonyl azide (C) [102], 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl azide (D) [103], pyrene-1-carbonylnitrile (E) [104], 1-anthroylnitrile (F) [105] and 7-dimethylaminocoumarin 3-isocyanate (G) [106]. They react with primary and secondary alcohols in aprotic solvents such as benzene and chloroform. The conditions of fluorogenic reactions are drastic (80–100°C, 30–60 min), except that the pyrene-1-carbonyl chloride reaction is complete within 30 min at 25°C in the presence of triethylamine. The reagents all act on hydroxysteroids such as cholesterol, 17-oxosteroids and bile acids. The detection limits for the hydroxysteroids are at the femtomole level.

Dansyl-Cl reacts with the phenolic hydroxyl moiety and also amino compounds. The reagent reacts with estrogens in alkaline media (pH 10.5) at 100°C within 15 min [107]. The detection limits for estrogens are *ca.* 1 pmol.

4.2. Reactions for carbonyl group

Dansylhydrazine forms fluorescent hydrazone (Ex 365 nm, Em 505 nm) with 17-oxosteroids in

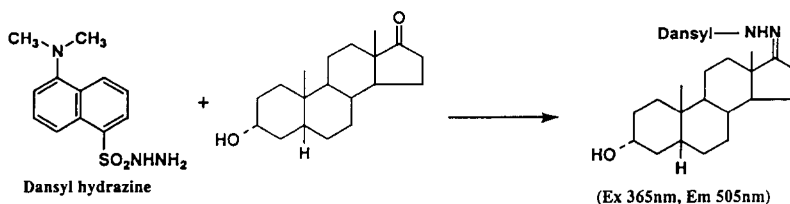

Fig. 15. Fluorogenic reaction of etiocholanolone with Dansyl hydrazine.

benzene in the presence of trichloroacetic acid [108] (Fig. 15). The reaction terminates within 20 min at 60°C. Bile acids are also derivatized by this reaction following enzymatic conversion of the acids into 3-oxo-bile acids [109]. The detection limits are *ca.* 1 pmol on-column.

3-Oxo-bile acids react with O-(1-, 2- or 9-anthrylmethyl) hydroxylamine in a similar manner to form the corresponding fluorescent oximes [110] (Fig. 16). The sensitivities are 20–50 fmol on-column.

Corticosteroids can be derivatized to fluorescent compounds by reaction with 1,2-diamino-4,5-methylenedioxybenzene (MDB) [111,112], after oxidative conversion of the steroids into the corresponding glyoxal compounds (Fig. 17). The derivatives are separated on an RP column, and their detection limits (at a signal-to-noise ratio of 3) are *ca.* 1 pmol on-column.

4.3. Reactions for carboxyl and diene groups

1-Bromoacetylpyrene reacts with potassium salts of bile acids to form fluorescent esters [113] (Fig. 18). The reaction is carried out in acetonitrile in the presence of dicyclohexyl-18-crown-6 at 40°C for 30 min. The derivatives are separated by RP-HPLC.

Cookson-type reagents such as 4-(1-pyrenyl)-1,2,4-triazoline-3,5-dione [114] and 4-(6-methoxy-2-phenylbenzoxazolyl)-1,2,4-triazoline-3,5-dione [115] act on conjugated dienes (7-dehydrocholesterol and provitamin D_3 to

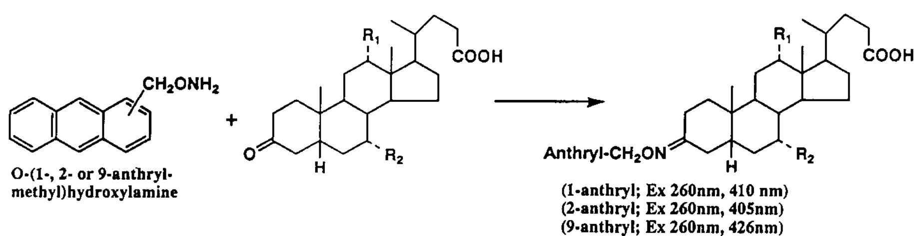

Fig. 16. Fluorogenic reaction of 3-oxo-5β-cholanoic acid with O-(1-, 2- or 9-anthrylmethyl)hydroxylamine.

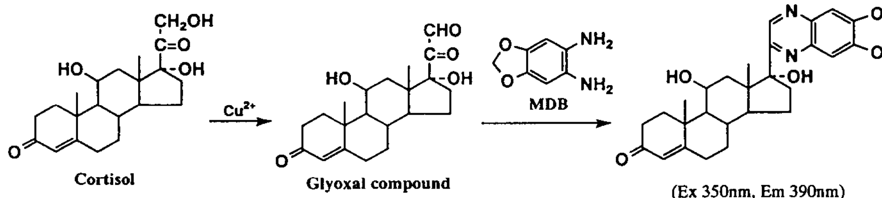

Fig. 17. Fluorogenic reaction of cortisol with MDB following oxidation.

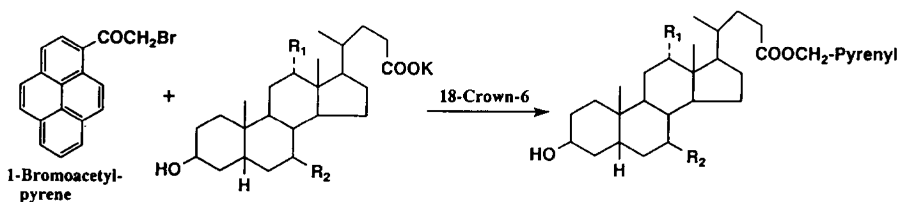

Fig. 18. Fluorogenic reaction of bile acid with 1-bromoacetylpyrene.

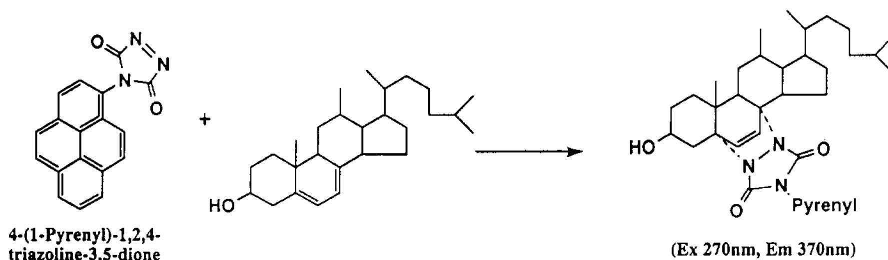

Fig. 19. Fluorogenic reaction of 7-dehydrocholesterol with 4-(1-pyrenyl)-1,2,4-triazoline-3,5-dione.

form fairly stable adducts (Fig. 19), which can be separated by RP-HPLC.

5. Carbohydrates

HPLC analyses of carbohydrates can be greatly improved in sensitivity and selectivity by utilizing post- or precolumn fluorescence derivatization for the detection systems.

5.1. *Postcolumn derivatizations*

Many fluorogenic reagents have been proposed for reducing carbohydrates. The reagents are classified into two groups with respect to their reaction conditions. One is reagents such as ethylenediamine [116], 2-ethanolamine [117,118], 2-cyanoacetamide [119,120] and arginine [121], which allow the fluorimetric detection of reducing carbohydrates in a weakly alkaline or neutral medium with a reaction period longer than 30 min. The reagents of the other group are arylamidines [122] and *meso*-1,2-diarylethylenediamines [123], which require strongly alkaline conditions with a reaction period of less than 15 min. Of these reagents, 2-cyanoacetamide, benzamidine (BA), 4-methoxybenzamidine (*p*-MBA) and *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine (*p*-MOED) (Fig. 20) appear to be most favourable in terms of sensitivity and reactivity towards various reducing carbohydrates, including amino sugars, uronic acids and sialic acids.

2-Cyanoacetamide requires the participation of the hydroxy group at the 2-position of the carbohydrate in the reaction [124] (Fig. 21) Although the derivatization cannot afford fluorescence with 2-deoxy sugars, the postcolumn derivatization HPLC method has been widely used for the determination of various carbohy-

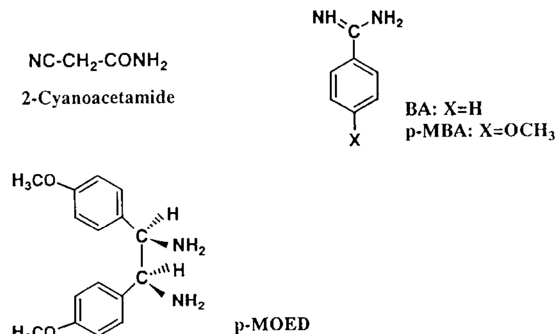

Fig. 20. Fluorogenic reagents for reducing carbohydrates.

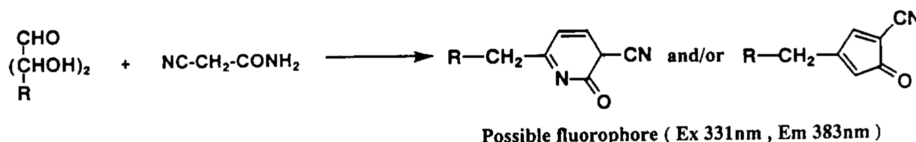

Fig. 21. Fluorogenic reaction with 2-cyanoacetamide.

drates in biological samples. In the method, the detection limits (S/N = 2) are 0.1–4.2 nmol on-column [119,120].

BA and p-MBA offer the advantage of rapidity in reaction over the other fluorogenic reagents: the reaction is completed within 3 min at 100°C [122]. The reagents provide fluorescence for 2-deoxy sugars, although their intensities are 10–15% of that given by glucose. Reducing carbohydrates in biological matrices such as human serum, mustard plants and wines are determined by the post-column derivatization HPLC methods using BA [125,126] or p-MBA [127]. The detection limits (S/N = 3) for the compounds are 2–63 pmol on-column.

On the other hand, p-MOED reacts with all kinds of reducing carbohydrates, especially 2-deoxy sugars such as 2-deoxyribose and 2-deoxyglucose [123]. The postcolumn derivatization HPLC method is useful for the determination of reducing sugars in human urine (Fig. 22) and serum [128] and of 2-deoxyglucose in rat serum for pharmacokinetic studies [129]. Both p-MOED and p-MBA can be used similarly to the postcolumn reactor system in the HPLC, by means of which glycated albumin in human serum can be determined [130].

5.2. Precolumn derivatizations

Reagents reported for precolumn fluorescence derivatization in HPLC for reducing carbohydrates include 2-aminopyridine [131,132], Dansyl hydrazine [133] and 9-fluorenylmethyl chloroformate (Fmoc)–hydrazine [134]. The reactions are all based on the derivatization of the hydrazine moiety of the reagents with the aldehyde or keto group of the carbohydrates (Fig. 23). Precolumn HPLC methods using these reagents have not been widely used for the determination of biogenic sugars in complex matrices.

6. Peptides

Mammalian tissues and fluids contain many physiologically important peptides at low concentrations. For the chromatographic analyses of the bioactive peptides, several fluorogenic reactions have been proposed in order to obtain high sensitivity and selectivity in detection [135].

Sensitive detection in HPLC for the determination of general peptides can be performed by use of fluorogenic reagents such as OPA (Fig. 1A), NDA (Fig. 1B) and fluorescamine (Fig. 1C). These reagents react with primary amines,

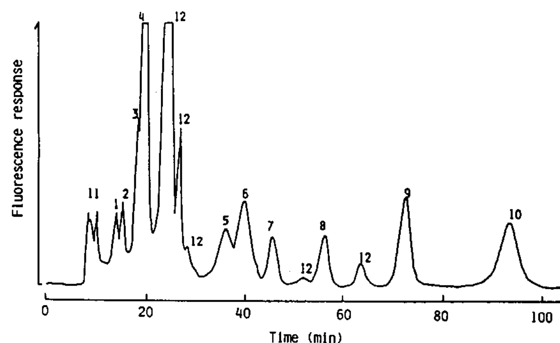

Fig. 22. Chromatogram obtained by postcolumn fluorescence derivatization detection for reducing carbohydrates in human urine. Column: TSK gel Sugar AXG (150 × 4.6 mm I.D.). Mobile phase (flow-rate 0.4 ml/min): 0.5 M sodium borate buffer (pH 8.7). Peaks with concentrations (nmol/ml urine) in parentheses: 1 = N-acetyl-D-glucosamine (170); 2 = cellobiose (130); 3 = maltose (180); 4 = lactose and/or L-rhamnose; 5 = D-fructose (200); 6 = D-arabinose and/or L-fucose; 7 = D-galactose (280); 8 = D-xylose (230); 9 = D-glucose (370); 10 = α-melibiose (internal standard); 11 = endogenous fluorescent substances; 12 = unidentified, probably reducing carbohydrates. (Reproduced with permission from ref. 128.)

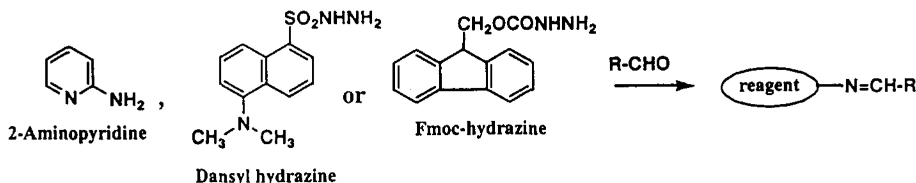

Fig. 23. Fluorogenic reactions with hydrazine derivatives.

so that peptides containing free α- and ε-amino moieties can be detected by pre- or postcolumn derivatization methods [136–139].

Using chromatographic methods, the peptides are detectable at the 0.05–100-pmol level on-column. However, these reagents are not selective with respect to peptides because nearly all peptides possess a primary amino group. Therefore, the separation conditions necessitate a high resolution for peptides when endogenous peptides in biological matrices are determined by HPLC. Fig. 24 shows chromatograms obtained

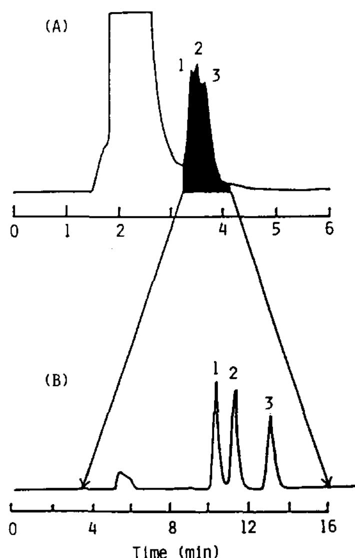

Fig. 24. Column-switching chromatography of NDA derivatives of enkephalins. The derivatives were first separated on (A) a Spherisorb Phenyl column (5 μm; 150 × 4.6 mm I.D.) and subsequently on (B) a ODS Hypersil column (5 μm; 150 × 4.6 mm I.D.). Both columns were eluted with 45% acetonitrile in 26.5 m*M* trifluoroacetic acid (pH 3.5) at a flow-rate of 1.0 ml/min. Peaks: 1 = methionine-enkephalin; 2 = [D-^{2}Ala]methionine-enkephalin; 3 = leucine-enkephalin. (Reproduced with permission from ref. 138.)

by precolumn fluorescence derivatization HPLC for enkephalin peptides using NDA reagent [138]. In the method, the column-switching technique was necessary for the separation of the biogenic enkephalins.

6.1. *Arginine-containing peptides*

Arginine-containing peptides can be rapidly converted into their respective fluorescent derivatives by condensation of the guanidino moiety of an arginyl residue with benzoin (Fig. 10B) in an alkaline solution (100°C, 90 s) [140].

The postcolumn derivatization HPLC method has been studied for the determination of arginine-containing peptides [141,142]. Fig. 25 shows chromatograms of seven arginine-containing peptides and various other substances obtained with UV detection and subsequent postcolumn fluorescence derivatization with benzoin. The fluorescence detection is specific for the arginine-containing peptides, and this method can detect them down to *ca.* 10 pmol on-column (S/N = 2). This postcolumn HPLC method can be applied not only to tryptic mapping of a large peptide by means of enzymatic digestion with trypsin [141], but also to the determination of endogenous substance P, a neuropeptide, in hypothalamus tissue of rat brain [142].

The precolumn derivatization HPLC method using benzoin is more sensitive for arginine-containing peptides and permits possible detection at levels as low as 100 fmol on-column [140]. The method can be applied to enzyme assays in a renin angiotensin system, *e.g.* renin activity (liberation of angiotensin I from angiotensinogen) in human plasma [143] and angiotensin-converting enzyme activity (cleavage of the carboxy-terminal His–Leu of angiotensin I, generat-

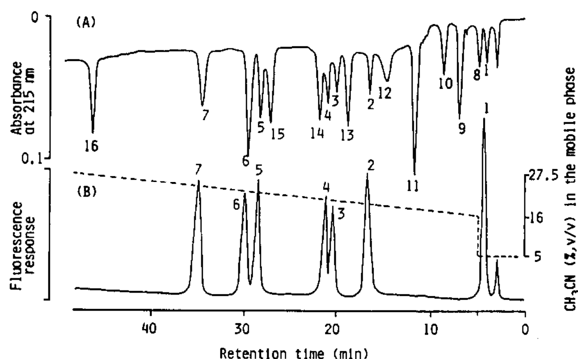

Fig. 25. Chromatograms obtained with (A) UV detection and (B) postcolumn fluorescence derivatization detection for arginine-containing peptides and several other biological substances. Column: TSK gel ODS-120T (5 μm; 150 × 4.6 mm I.D.). Mobile phase (flow-rate 1.0 ml/min): acetonitrile (5–27.5%)–0.2 *M* phosphate buffer (pH 2.3) (95–72.5%). Peaks with concentrations (nmol per injection volume of 50 μl) in parentheses: 1 = kyotorphin (1.0); 2 = kallidin (0.5); 3 = angiotensin II (0.25); 4 = angiotensin III (0.25); 5 = angiotensin I (0.5); 6 = β-melanocyte stimulating hormone (1.0); 7 = substance P (0.5); 8 = tyrosine (1.0); 9 = propionic acid; 10 = phenylalanine (2.0); 11 = tryptophan (0.5); 12 = phenylpyruvic acid (10); 13 = methionine-enkephalin (1.0); 14 = sorbic acid (2.5); 15 = estriol (1.5); 16 = estrone 3-sulphate (3.0). (Reproduced with permission from ref. 141.)

ing angiotensin II) in human serum [144]. With slightly modified reaction conditions, the benzoin precolumn derivatization method is also usable for the determination of peptide-like inhibitors of proteinases, leupeptin [145] and antipain [146] in mouse serum and muscle.

6.2. Tyrosine-containing peptides

A fluorescence derivatization of tyrosine-containing peptides, which is based on the formylation of the phenolic moiety in the tyrosyl residue by means of the Reimer–Tiemann reaction and subsequent conversion of the aldehyde thus formed into a fluorescent derivative with 1,2-diamino-4,5-dimethoxybenzene (Fig. 26), is applicable to the precolumn derivatization HPLC method for the determination of synthetic bioactive peptides such as angiotensins I, II and III and methionine- and leucine-enkephalins [147,148]. This method permits the determination of the endogenous leucine-enkephalin at concentrations as low as 5.6 pmol/g present in rat brain tissues [149].

Opioid peptides such as methionine- and leucine-enkephalins play a role in the control of pain sensation. Most of the opioid peptides have a tyrosyl residue at the N-terminus in their amino acid sequence. Such peptides can be derivatized into the corresponding fluorescent compounds by reaction with hydroxylamine, cobalt(II) ion and borate at 100°C for 1–5 min in a weakly alkaline solution (pH 8–9) [150] (Fig. 27). The molecular structures of the fluorescent

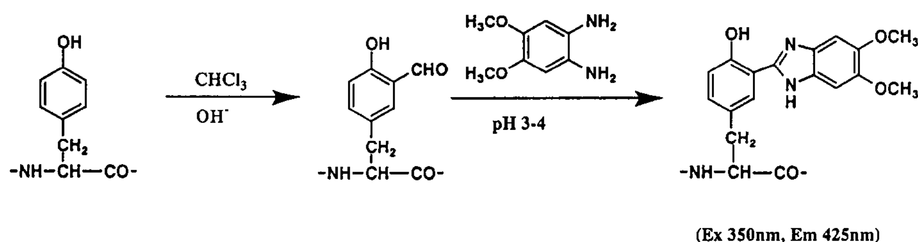

Fig. 26. Fluorogenic reaction for tyrosine-containing peptides.

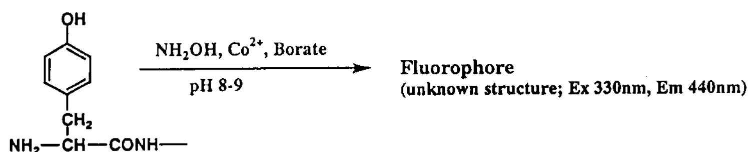

Fig. 27. Fluorogenic reaction for N-terminal tyrosine-containing peptides.

compounds remain unknown. The reaction can be applied to both pre- and postcolumn derivatization systems in HPLC.

The precolumn HPLC method using an RP column is useful for the determination of endogenous methionine- and leucine-enkephalins [151,152] and also methionine-enkephalin–Arg–Phe and methionine-enkephalin–Arg–Gly–Leu [153] in rat brain tissues. In the method, the detection limits (S/N = 3) for the peptides are 0.33–1.2 pmol on-column.

The postcolumn derivatization HPLC method is more useful with respect to facility and reproducibility for the determination of biogenic opioid peptides [154,155]. In the method the detection limits (S/N = 3) for the peptides are 0.5–1.5 pmol on-column. Seven endogenous opioid peptides in rat brain tissues can be simultaneously determined by the method [155] (Fig. 28). This method is also applicable to the assay of enkephalin-related enzymes, enkephalin-degrading peptidases (enkephalinases A and B) [156] and enkephalin-generating enzymes [157]. For determining the distribution of three different opioid peptide precursors, namely proopiomeranocortine and proenkephalines A and B, in various tissues of rat brain, the fragment peptides released from these opioid peptide precursors by tryptic enzyme reaction are determined by the method [158].

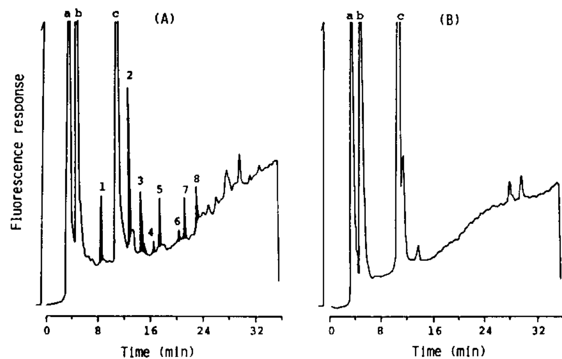

Fig. 28. Chromatograms obtained with (A) postcolumn fluorescence derivatization detection and (B) non-derivatization detection for opioid peptides in striatum tissue (30 mg) of rat brain. Column: Asahipak ODP-50 (5 μm; 150 × 6 mm I.D.). Mobile phase (flow-rate 1.0 ml/ml): acetonitrile (12–36%)–50 m*M* sodium borate buffer (pH 10) (20%)–water. Peaks with concentrations (pmol/g of tissue) in parentheses: 1 = Trp-Phe (internal standard); 2 = methionine-enkephalin (400); 3 = leucine-enkephalin (187); 4 = α-endorphin 83); 5 = leucine-enkephalin–Arg (108); 6 = γ-endorphin (94); 7 = methionine-enkephalin–Arg–Gly–Leu (136); 8 = methionine-enkephalin–Arg–Phe (135); a–c = endogenous fluorescent substances. (Reproduced with permission from ref. 155.)

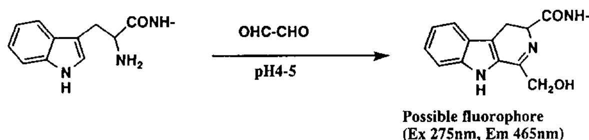

Fig. 29. Fluorogenic reaction for N-terminal tryptophan-containing peptides.

6.3. *N-Terminal tryptophan-containing peptides*

Glyoxal is a fluorogenic reagent for N-terminal tryptophan-containing peptides [159]. The fluorogenic reaction forms single fluorescent derivatives of the respective N-terminal tryptophan-containing peptides and free tryptophan when the compounds are heated with glyoxal at 100°C for 30 min in a weakly acidic buffer (pH 4–5) [160] (Fig. 29). The precolumn derivatization HPLC method using an RP column allows the sensitive detection of the peptides at the 55–382-fmol level on-column, and also the facile identification of an N-terminal tryptophyl fragment in the tryptic digest of dynorphin A [160].

7. Nucleic acid-related compounds

Nucleic acids (DNA and RNA) are macromolecular compounds possessing genetic information. The monomeric constituents of the nucleic acids are nucleotides, which consist of nucleosides (nucleobases and ribose or deoxyribose moieties) and phosphate esters. The nucleobases, nucleosides and nucleotides in physiological fluids have been determined by HPLC for biomedical studies. Fluorogenic reactions adequate for selective and sensitive detection in the HPLC of nucleic acid-related compounds are generally based on the derivatization of the sugar moiety or nucleobases.

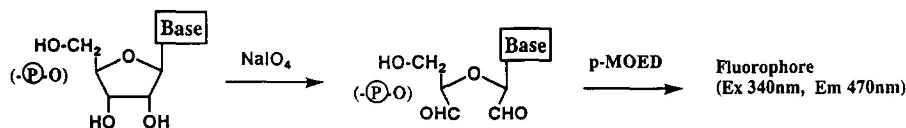

Fig. 30. Fluorogenic reaction of ribonucleosides and ribonucleotides with *p*-MOED following periodate oxidation.

7.1. General considerations on nucleosides and nucleotides

The sugar moiety in nucleosides and nucleotides can be derivatized with a few fluorogenic reagents. *p*-MOED (Fig. 20), which is a fluorogenic reagent for reducing carbohydrates, can derivatize selectively the ribose moiety in various ribonucleosides and nucleotides [161]. The fluorescence derivatization is performed by heating with *p*-MOED at 140°C for 15 min in 10 m*M* hydrochloric acid containing 0.85 m*M* sodium periodate (Fig. 30). This reaction is applied to the postcolumn derivatization system of the HPLC of ribonucleosides and nucleotides [162,163]. The HPLC method permits the determination of pseudouridine (a modified nucleoside as a tumour marker) in human urine and serum [162], and of several ribonucleotides in human erythrocytes [163]. In the method, the detection limits (S/N = 3) for the nucleosides and nucleotides are 4–67 pmol on-column.

2 - (5 - Chlorocarbonyl - 2 - oxazolyl) - 5,6 - methylenedioxybenzofuran (OMB-COCl) as a fluorescence-tagging reagent for alcoholic hydroxy groups [164] can react selectively with the 5′-hydroxy group of the sugar moiety in nucleosides and nucleotides to produce the corresponding fluorescent ester derivatives (Fig. 31). The derivatives of nucleosides and nucleotides are formed by heating with OMB-COCl at 100°C for 30 min in non-aqueous pyridine–benzene (1:9, v/v). The fluorescent derivatives of ten ribo- and deoxyribonucleosides and -nucleotides are separated by isocratic RP-HPLC [165]. This precolumn derivatization HPLC is useful for the determination of 2′,3′-dideoxyinosine (an active drug against immunodeficiency virus) in rat plasma for pharmacokinetic studies [166]. Tri- and tetraoligonucleotides that have a 5′-hydroxy group in the respective molecules can also be derivatized with OMB-COCl in the presence of sodium azide, and the resulting derivatives are separable by RP-HPLC [167,168].

7.2. Adenine nucleosides and nucleotides

Chloroacetaldehyde [169] or bromoacetaldehyde [170] reacts with adenine moiety in the nucleosides and nucleotides to provide the corresponding fluorescent 1,N^6-etheno derivatives (Fig. 32). The derivatives of the adenine nucleosides and nucleotides that are formed by heating with the reagent at 80–100°C for 10–30 min in an acidic buffer (pH 4.5) are separated by RP-HPLC [170,171]. Precolumn HPLC methods have been applied to the determination of adenine and its nucleosides and nucleotides in complex biological samples [172,173]. The detec-

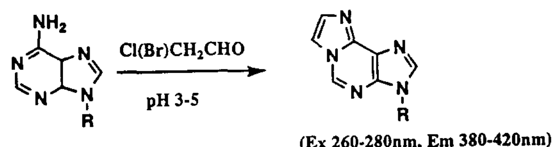

Fig. 32. Fluorogenic reaction of adenine nucleosides and nucleotides with chloro(or bromo)acetaldehyde.

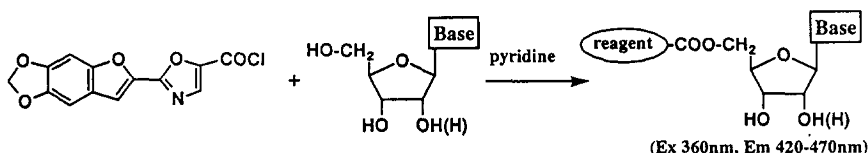

Fig. 31. Fluorogenic reaction of nucleosides with OMB-Cl.

tion limits (S/N = 3) are 0.5–10 pmol on-column. These reagents are also usable for post-column fluorescence derivatization HPLC [174].

7.3. Guanine nucleosides and nucleotides

Phenylglyoxal affords fluorescent derivatives selectively for guanine and its nucleosides and nucleotides by reacting with the guanine moiety of the compounds at 60°C for 30 min in an acidic buffer (pH 4.0) [175] (Fig. 33), which can be applied to the postcolumn HPLC method for the determination of guanine and its nucleosides and nucleotides in human erythrocytes [176]. Fig. 34 shows the chromatograms obtained by UV detection and the postcolumn derivatization fluorescence detection in the HPLC of various nucleobases, nucleosides, nucleotides and related compounds. The postcolumn HPLC method gives detection limits (S/N = 3) of 3.2–10 pmol on-column.

The phenylglyoxal reaction is also applicable to precolumn derivatization HPLC when the reaction is modified to milder conditions, in which guanine nucleosides and nucleotides are warmed with the reagent at 37°C for 15 min in 12.5 m*M* phosphate buffer (pH 6.0) [177]. The precolumn HPLC method permits the determination of biogenic guanine nucleotides in human erythrocytes and rat brain tissues, and the detection limits (S/N = 3) are 0.14–0.72 pmol on-column [178].

Arylglyoxals which have a phenyl moiety substituted with electron-donating methoxy or methylenedioxy groups can give more highly fluorescent derivatives for guanine and its nucleosides and nucleotides [179]. Of the tested

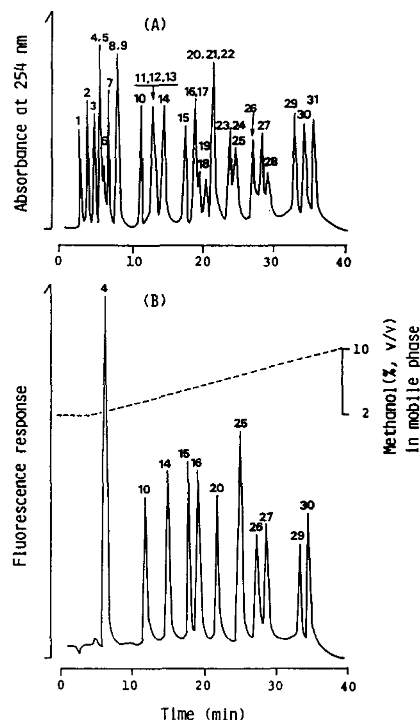

Fig. 34. Chromatograms obtained by (A) UV detection and (B) postcolumn fluorescence derivatization detection for various nucleobases, nucleosides, nucleotides and related compounds (1.0 nmol per injection volume). Column: TSK gel ODS-120T (5 μm; 150 × 4.6 mm I.D.). Mobile phase (flow-rate 1.0 ml/min): methanol (2–10%)–10 m*M* tetra-*n*-propylammonium phosphate (pH 6.0) (17%)–50 m*M* sodium phosphate buffer (pH 6.0) (20%)–water. Peaks: 1 = cytosine; 2 = uracil; 3 = cytidine; 4 = guanine; 5 = hypoxanthine; 6 = CMP; 7 = uridine; 8 = thymine; 9 = UMP; 10 = GMP; 11 = adenine; 12 = XMP; 13 = inosine; 14 = guanosine; 15 = deoxyguanosine; 16 = GDP; 17 = thymidine; 18 = cAMP; 19 = AMP; 20 = dGMP; 21 = NAD; 22 = CTP; 24 = UTP; 25 = 9-ethylguanine; 26 = GTP; 27 = dGDP; 28 = adenosine; 29 = cGMP; 30 = dGTP; 31 = ATP. (Reproduced with permission from ref. 176.)

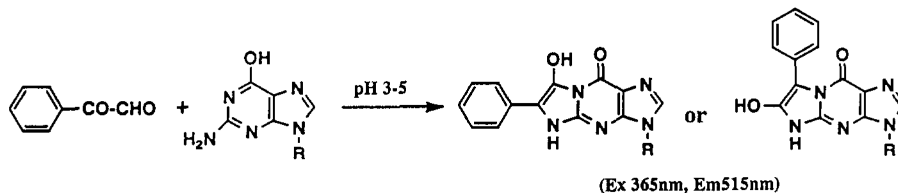

Fig. 33. Fluorogenic reaction of guanine nucleosides and nucleotides with phenylglyoxal.

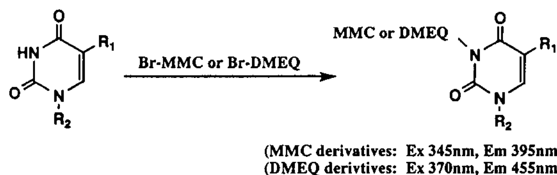

Fig. 35. Fluorogenic reaction of pyrimidine nucleosides and nucleotides with Br-MMC or Br-DMEQ.

reagents, 3,4-dimethoxyphenylglyoxal is the most sensitive, and its reaction conditions (37°C, 5–7 min, pH 7.0 phosphate buffer) are much milder. In precolumn derivatization HPLC using this reagent, the detection limits (S/N = 3) are 0.04–0.4 pmol on-column.

7.4. Pyrimidine nucleosides and nucleotides

Nucleobases of uracil and thymine have an active imino hydrogen in their molecules. The imino groups in the compounds are reactive to Br-MMC or Br-DMEQ (Fig. 35) that are fluorescence-tagging reagents for carboxylic acids (Fig. 12). The derivatives of the pyrimidine nucleosides with Br-MMC [180,181] or Br-DMEQ [182] can be determined by RP-HPLC. In the method with Br-MMC, the detection limits (S/N = 2) are 0.5–1 pmol on-column. An antitumour agent, 5-fluorouracil, and its prodrug, 5-fluoro-2′-deoxyuridine, in human serum can be derivatized to the corresponding fluorescent compounds by reaction with Br-DMEQ [183]. The derivatives are determined by RP-HPLC for the pharmacokinetic study. The detection limits (S/N = 5) are 375–575 fmol on-column.

8. Conclusions

Many substances that exhibit bioactivity at extremely low concentrations occur in biological fluids and tissues. Therefore, a number of fluorogenic reactions, surveyed in this review, have been studied to gain both selectivity and sensitivity for HPLC detection.

For obtaining a high selectivity for the analytes, molecular recognition techniques in the fluorogenic reactions have been devised on the basis of the principle that vicinal or geminal, homo or hetero bifunctional (diamino, amino-imino, aminohydroxyl) compounds react with 1,2-dioxo compounds (1,2-ketols, glyoxals, 1,2-diketones or 1,2-quinones) to yield fluorescent cyclic products. These derivatization methods can minimize the chromatographic interferences from various constituents of the biological samples such as mammalian body fluids and tissues.

Regarding the sensitivity for the biogenic analytes, much more sensitive fluorogenic reactions have been required to be introduced into HPLC detection. However, detection at femtomole or subfemtomole levels seems to be the lower limit of sensitivity that can be obtained by HPLC with a conventional fluorescence detector.

Therefore, techniques based on instrumental principles, such as the use of a laser as the excitation source for fluorescence detectors and time-resolved fluorimetry, have been introduced into the HPLC system. Laser excitation offers not only enhanced fluorescence but also suppressed background fluorescence, because of the high intensities and well defined monochromaticity of laser beams. Time-resolved fluorimetry can be used to detect selectively compounds having longer fluorescence lifetimes, and shorter lifetime fluorescences from co-existing compounds; light scattering does not cause interference. A combination of the two techniques, laser-induced time-resolved fluorimetry, offers a good possibility of improving the detection limit in the attomole range for the HPLC analysis of biosubstances. This requires the development of a more practical laser and a time-resolved fluorimeter. In addition, laser-induced time-resolved fluorimetry may require reagents that can afford long-lifetime fluorescent derivatives with excitation maxima at wavelengths longer than *ca.* 450 nm. In the near future, such fluorescence detection systems as described above will enable HPLC to be applied to ultrahigh sensitivity analyses of bioactive compounds.

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