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Journal of Chromatography A, 950 (2002) 1–19

JOURNAL OF CHROMATOGRAPHY A

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Review

Luminol-type chemiluminescence derivatization reagents for liquid chromatography and capillary electrophoresis

Masatoshi Yamaguchi*, Hideyuki Yoshida, Hitoshi Nohta

Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Johnan, Fukuoka 814-0180, Japan

Received 24 September 2001; received in revised form 19 December 2001; accepted 20 December 2001

Abstract

The present paper provides the principles for chemiluminescence of luminol-type compounds and their wide and powerful application to the detection system in liquid chromatography and capillary electrophoresis as derivatization reagents. The reagents can be classified into two types, chemiluminescence labeling and chemiluminogenic reagents. The former reagents are highly chemiluminescent themselves and used for tagging their intense chemiluminophores to analytes, whereas the latter are weakly chemiluminescent but generate intense chemiluminescence by reaction with analytes. The liquid chromatographic methods utilizing chemiluminescence derivatizing reactions with luminol-type reagents allow the analytes to be detected at pmol–sub-fmol levels. Furthermore, the chemiluminogenic reactions show high selectivity owing to their selective reaction against the analytes permitting facile and reproducible detection.

Keywords: Reviews; Derivatization, LC; Derivatization, CE; Detection, LC; Detection, CE; Chemiluminescence detection; Luminol; Carbonyl compounds; Hydroxyindoles; Catecholamines; Amines; Carboxylic acids; Amino acids; Peptides

Contents

*Corresponding author. Tel.: +81-92-871-6631; fax: +81-92-863-0389.
E-mail address: masayama@fukuoka-u.ac.jp (M. Yamaguchi).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00004-3

1. Introduction

A great variety of biological substances that exhibit bioactivities even at extremely low concentrations, occur in biological fluids and tissues, and their concentrations in bio-matrices are generally controlled by their related enzymes. Therefore, it is very important to measure these substances in biological materials over wide fields in life science. Since such bioactive substances occur at only small quantities in highly complex matrices, analysis methods should be both selective and sensitive. Selectivity and sensitivity have been achieved only by a combination of effective separation technique [liquid chromatography (LC), gas chromatography (GC), capillary electrophoresis (CE), immune reaction or hybridization reaction] and a highly sensitive detection method (fluorescence, chemiluminescence, electrochemical, radioisotopic or mass-spectrometric methods).

Of the detection methods, chemiluminescence is highly sensitive and selective, and becomes essential in liquid chromatography, immunoassay and hybridization assay. Chemiluminescence can be defined as emission of light (ultraviolet, visible or infrared) from a molecule or atom in an electronically excited state, which occurs by a chemical reaction. The chemiluminescence from synthetic compounds, including lophine derivatives, luminol-type compounds, acridinium esters, adamantyldioxetane compounds, peroxyoxalate esters and ruthenium complexes, have been known for a long time and many analytical applications of them have been reviewed [1–13]. Among these chemiluminescent compounds, luminol-type derivatives have been well examined concerning their reaction mechanism, apparatus and so on, and thus have been used for various applications.

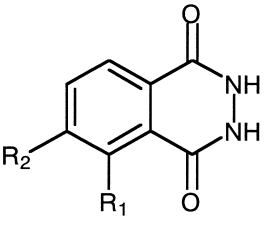
In the present review, we describe the chemiluminescence derivatization methods of mainly bioactive substances using luminol-type reagents in flow systems, LC and CE.

2. Chemiluminescence of luminol-related compounds

2.1. Chemiluminescence of luminol and related compounds

The chemiluminescence of luminol [5-aminophthalazine-1,4(2*H*,3*H*)-dione] was first reported by Albrecht in 1928 [14]. Since that time, luminol, isoluminol [6-aminophthalazine-1,4(2*H*,3*H*)-dione] (Fig. 1) and their derivatives have been examined extensively. All chemiluminescence reactions of (iso)luminol derivatives are oxidation reactions, which are carried out in either aprotic solvents [dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide] or protic solvents (water, lower alcohols) [15,16]. Although the chemiluminescence reaction in aprotic media needs only oxygen and a strong base, the reaction in protic media usually requires a base, an oxidizing agent, and either a peroxide or oxygen.

The chemiluminescent quantum yield (Φ_{CL}) of luminol is about 5% in DMSO and about 1–2% in aqueous systems [15–17]. Isoluminol shows 10–100 times weaker chemiluminescence than that of



	R_1	R_2
5-Aminophthalazine-1,4(2*H*,3*H*)-dione (Luminol)	NH_2	H
6-Aminophthalazine-1,4(2*H*,3*H*)-dione (Isoluminol)	H	NH_2

Fig. 1. Luminol and isoluminol.

luminol [18,19]. The Φ_{CL} of luminol-type compounds has been known to depend on the structure and the oxidation conditions. Several derivatives of (iso)luminol have been studied on the relationships between their structures and chemiluminescence properties. Modifications of the cyclic phthalhydrazide moiety of (iso)luminol led to complete loss of the chemiluminescence property [16,17]. Therefore, some enzymatic substrates, of which cyclic phthalhydrazide is modified by certain leaving group and which recover chemiluminescence by triggered enzyme reactions, have been reported (Fig. 2) [20–22].

Luminol analogues of which the aromatic ring is modified are generally found to be chemiluminescent [16,17,23]. Alkylations of the amino group in isoluminol increase the chemiluminescence intensity, whereas the substitutions in luminol cause decreases due to stereoscopic hindrance. Since the *N*-alkyl derivatives of isoluminol were found to be highly chemiluminescent, many isoluminol-type compounds *N*-alkylated with methyl, ethyl, butyl or hexyl have been used as the chemiluminescence derivatization reagents [16,17,24].

Many cyclic acylhydrazides originated in aromatic *o*-dicarboxylic acids show chemiluminescence, and substitution of the aromatic amino group has a great influence on the Φ_{CL} of (iso)luminol derivatives. For example, 7-[*N*-(4-aminobutyl)-*N*-ethyl]naphthalene-1,2-dicarboxylic acid hydrazide (Fig. 3A) [15,17], benzo[*ghi*]perylene-1,2-dicarboxylic acid hydrazide (Fig. 3B) [16,17], 4-(9-acridonyl-10-methylene)-

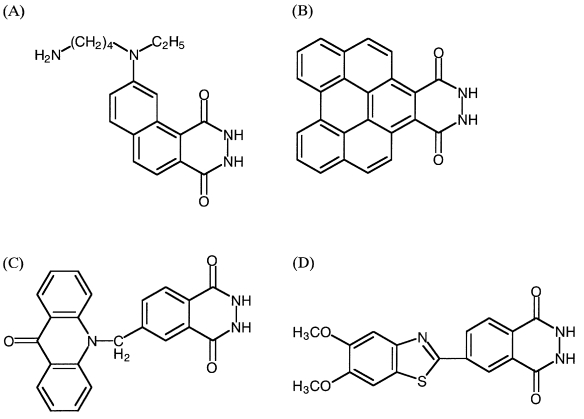


Fig. 3. Chemiluminogenic acylhydrazides. (A) 7-[*N*-(4-aminobutyl)-*N*-ethyl]naphthalene-1,2-dicarboxylic acid hydrazide; (B) benzo[*ghi*]perylene-1,2-dicarboxylic acid hydrazide; (C) 4-(9-acridonyl-10-methylene)phthalhydrazide; (D) 4-(5′,6′-dimethoxybenzothiazolyl)phthalhydrazide.

phthalhydrazide (Fig. 3C) [16], and 4-(5′,6′-dimethoxybenzothiazolyl)phthalhydrazide (Fig. 3D) [25] have been known as intense chemiluminogenic compounds based on their highly fluorescent structures, naphthalene, benzo[*ghi*]perylene, *N*-methylacridone, and 2-phenylbenzothiazole, respectively.

2.2. Chemiluminescence reaction mechanism

The chemiluminescence reaction of luminol occurs in a basic solution to generate an energy-rich intermediate with subsequent light emission from 3-aminophthalic acid (Fig. 4). To obtain chemilumin-

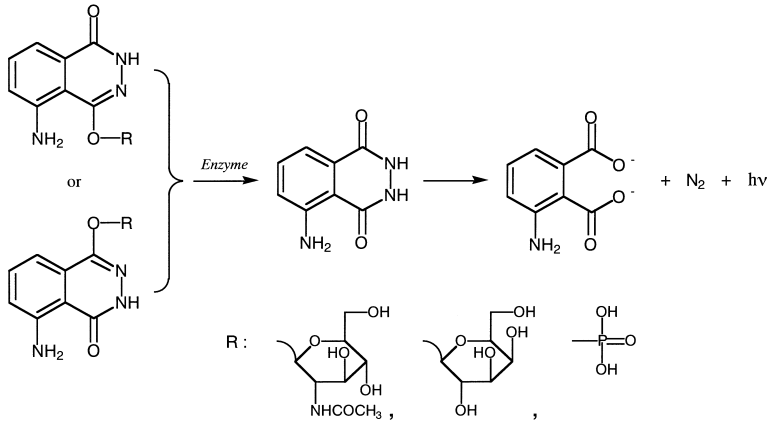


Fig. 2. Luminol-type enzyme substrates and their detection mechanism.

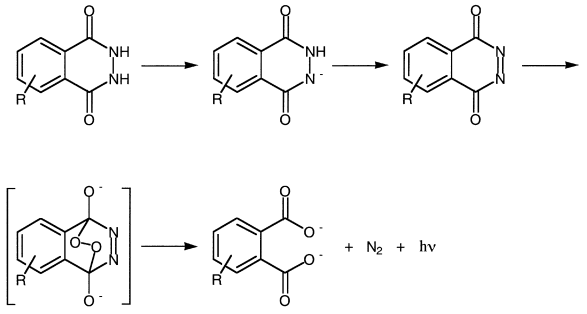


Fig. 4. Possible chemiluminescence reaction of luminol-type compounds.

escence from luminol in an aqueous solution, an oxidizing reagent, e.g. hydrogen peroxide, is needed. The oxidization reaction of luminol is catalyzed by metal ions such as Co(II), Cu(II), Fe(III), etc., or by an enzyme such as horseradish peroxidase, microperoxidase and so on [15–17]. Other cyclic acylhydrazides also show chemiluminescence in a similar way. Furthermore, some enhancers, i.e. *p*-iodophenol, 6-hydroxybenzothiazole and phenylboronic acid, of the chemiluminescence reaction of luminol-type compounds have been reported [26–29].

Since chemiluminescence of luminol is a radiation from the chemically excited 3-aminophthalic acid, the chemiluminescent emission spectrum of luminol and the fluorescent emission spectrum of 3-aminophthalate dianion closely resemble each other in shape. The same relationships are true for other luminol-type compounds [25,30–34]. Furthermore, the Φ_{CL} of luminol-type compounds depends partly on the fluorescence quantum yields of the aromatic *o*-dicarboxylate dianions [32–37]. Thus, the structures having higher fluorescent quantum yields are desirable for the skeletons of luminol-type chemiluminescence compounds, and this strategy has been applied to the basic design of some chemiluminescent compounds and reagents [25,32,33,38].

2.3. Apparatus for chemiluminescence detection in LC and CE

Fig. 5 illustrates the schematic flow diagram for the detection system of (iso)luminol derivatives in

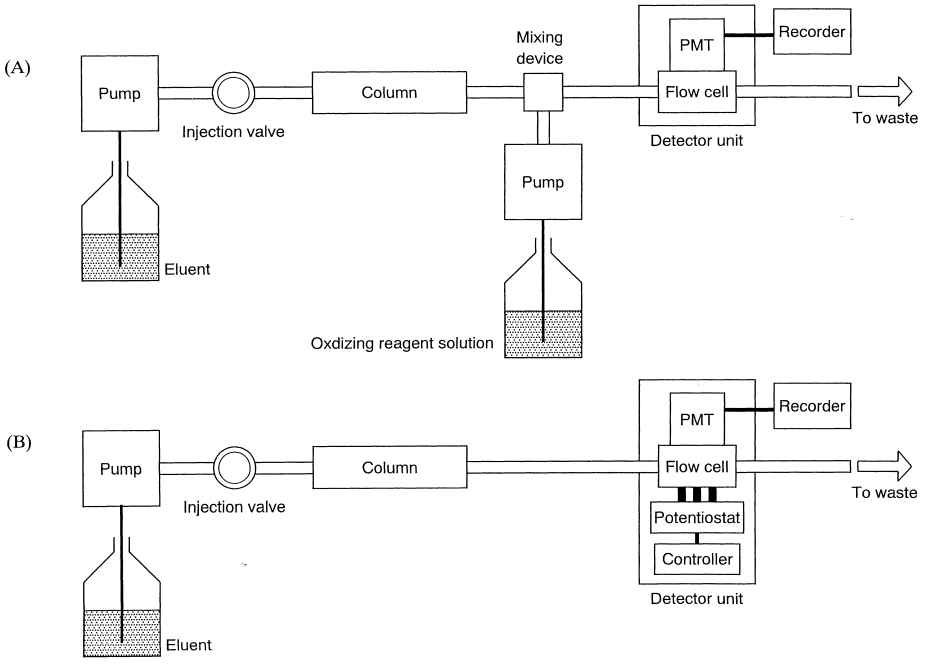


Fig. 5. Schematic flow diagrams for the detection system of (iso)luminol derivatives in LC. (A) Chemical oxidation device; (B) electrochemical oxidation device.

LC. For the chemiluminescence detection of luminol-type compounds, an on-line oxidizing device based on chemical or electrochemical reaction is indispensable before or in the chemiluminescence detector. In a chemical oxidation device, the oxidizing reagents, hydrogen peroxide and some catalysts, have been commonly used. The reagent solution(s) is introduced to the column effluent in a three-way union just before the chemiluminescence detector (Fig. 5A); the tube between the union and the detection cell should be minimized, because the chemiluminescence life-time of (iso)luminol derivatives is very short in many cases [15,16,25,32–38].

Electrogenerated chemiluminescence (ECL) methods of luminol-type compounds have also been reported in which an electrochemical oxidation of the compounds is utilized in place of chemical oxidation (Fig. 5B) [39,40]. A glassy carbon or platinum has been used as a working electrode to carry out the oxidation reaction. The ECL reaction can be controlled by the applied potential to the electrode. The ECL system is simpler because it does not require the postcolumn reaction apparatus consisting of pump(s), mixing union and reaction tube. The method has almost the same sensitivity as those in the chemical oxidation methods.

Not only LC but also CE are applied to the separation of luminol-related compounds in a flow system, and the principles and the applications of chemiluminescence in CE have been reviewed [13,41–43]. In the case of CE, there are some oxidation methods to carry out the chemiluminescence reaction. The off-column merging interface (Fig. 6A) [44], on-column coaxial flow interface (Fig. 6B) [45,46], off-column coaxial flow interface (Fig. 6C) [47,48], and end-column reservoir interface (Fig. 6D) [49,50] are the representative oxidation systems using oxidizing reagent solution, whereas ECL coupled with CE is also reported [51,52].

Recently, on-line solid-phase chemiluminescence techniques oxidized with barium peroxide powder were reported for LC and CE analyses of luminol derivatives [53,54].

2.4. Application of chemiluminescence

As described above, the most obvious use of the chemiluminescence reaction of luminol has been to

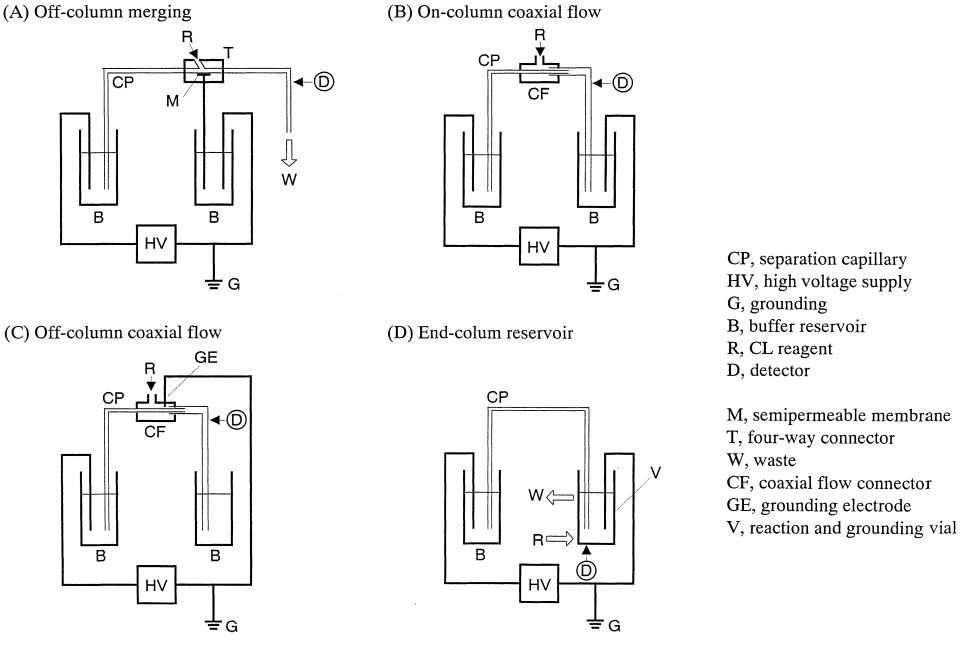


Fig. 6. Schematic flow diagrams for the detection system of (iso)luminol derivatives in CE. (A) Off-column merging interface; (B) on-column coaxial flow interface; (C) off-column coaxial flow interface; (D) end-column reservoir interface.

determine the oxidant or catalytic substance in the reaction. Determination of metal ions based on their catalytic activity has been reported on numerous occasions. The luminol-type compounds are most widely applied to the determination of a peroxidase activity, in which the enzyme itself, hydrogen peroxide or enzymatically-oxidizable substances is measured. Glucose, for example, could be determined by oxidation in the presence of glucose oxidase to produce hydrogen peroxide, followed by the peroxidase-mediated chemiluminescence reactions. Many biological compounds can be determined in a similar way using the corresponding oxidase.

The chemiluminescence detections of (iso)luminol derivatives have been also applied to enzyme immunoassays and chemiluminescence immunoassays. As a label, the former uses peroxidase and the latter uses a luminol-type compound (substrate for the peroxidase) or catalysis. Various assay modes (competitive or noncompetitive, homogeneous or heterogeneous) have been proposed. Some excellent reviews have classified them [9,55,56].

Recently, some chemiluminescence sensors based on the luminol reaction have been reported [57–60]. The first chemiluminescence sensor was reported in 1978 for the measurement of hydrogen peroxide [57], and since then various types of chemiluminescence sensors, such as enzyme-based sensors, non-enzyme-based sensors and immunosensors, have been developed for inorganic, organic and biological compounds.

The luminol-type chemiluminescence derivatization reagents have been utilized to the determination of many biological and pharmaceutical compounds in LC or CE. In the following sections, some derivatization reagents with luminol-type structures are reviewed. The chemiluminescence derivatization reagents have been classified into two groups by reaction type: ‘‘chemiluminogenic reagent’’ and ‘‘chemiluminescence labeling reagent’’. In the former, the chemiluminesce derivatization reagents themselves are generally weakly chemiluminescent, and react with target compounds to form the conjugated-ring molecules, resulting in production of chemiluminescence. In the latter, the reagents are composed of a highly chemiluminescent moiety and a reactive moiety, and the reactive moiety attaches to an analyte to form chemiluminescence-labeled derivatives.

3. Luminol-type chemiluminogenic reagents

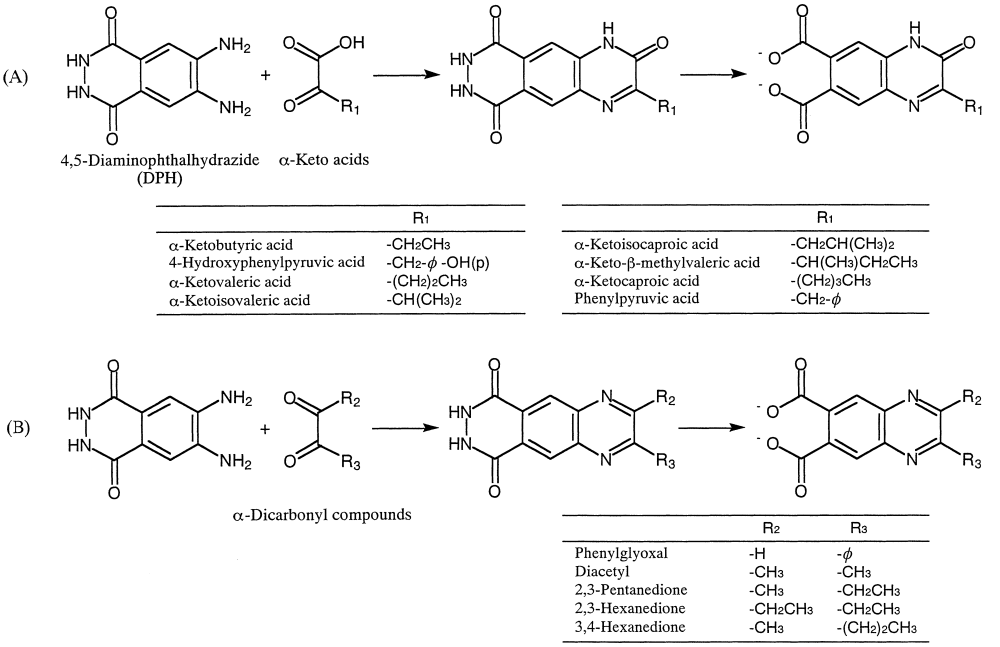
In this section, luminol-type chemiluminogenic reagents used in LC are described, and they are classified according to the structure of the analytes.

3.1. Reactions for α-keto acids and α-dicarbonyl compounds

4,5-Diaminophthalhydrazide (DPH) was reported as the derivatization reagent for α-keto acids, α-dicarbonyl compounds and their related compounds (Fig. 7) [61–67].

DPH reacts with α-keto acids at 100 °C for 45 min in diluted hydrochloric acid to give the highly chemiluminescent derivatives (Fig. 7A). These derivatives are separated by reversed-phase (RP) LC with isocratic elution, and detected chemiluminometrically after mixing with oxidizing reagents [hydrogen peroxide and alkaline potassium hexacyanoferrate(III)] (Fig. 8A). The detection limits for the acids are in the range 4–50 fmol per injection [61]. This method was applied to the determination of α-keto acids including phenylpyruvic acid in human plasma by using α-ketocaproic acid as an internal standard (I.S.) (Fig. 8B) [62], and it was found that the plasma concentration levels of some α-keto acids increased in patients with primary biliary cirrhosis [63]. In addition, *N*-acetylneuraminic acid (NANA) and *N*-glycolylneuraminic acid (NGNA), which have the α-keto acid structure, are also reacted with DPH to form chemiluminescent derivatives. This method is utilized in the measurement of NANA released by hydrochloric acid hydrolysis in human serum and urine by using NGNA as an I.S. (Fig. 9) [64].

DPH also reacted with α-dicarbonyl compounds including phenylglyoxal at 100 °C for 45 min in diluted hydrochloric acid in the presence of β-mercaptoethanol to give the highly chemiluminescent derivatives (Fig. 7B). These derivatives are separated by RPLC with isocratic elution, followed by chemiluminescence detection (Fig. 10). The detection



	R_1
α-Ketobutyric acid	$-CH_2CH_3$
4-Hydroxyphenylpyruvic acid	$-CH_2$-ϕ-OH(p)
α-Ketovaleric acid	$-(CH_2)_2CH_3$
α-Ketoisovaleric acid	$-CH(CH_3)_2$

	R_1
α-Ketoisocaproic acid	$-CH_2CH(CH_3)_2$
α-Keto-β-methylvaleric acid	$-CH(CH_3)CH_2CH_3$
α-Ketocaproic acid	$-(CH_2)_3CH_3$
Phenylpyruvic acid	$-CH_2$-ϕ

	R_2	R_3
Phenylglyoxal	-H	-ϕ
Diacetyl	$-CH_3$	$-CH_3$
2,3-Pentanedione	$-CH_3$	$-CH_2CH_3$
2,3-Hexanedione	$-CH_2CH_3$	$-CH_2CH_3$
3,4-Hexanedione	$-CH_3$	$-(CH_2)_2CH_3$

Fig. 7. Chemiluminescence derivatization reactions of (A) α-keto acids and (B) α-dicarbonyl compounds with DPH.

limits for them are in the range 1.1–300 fmol per injection [65]. This method is applicable to the determination of 3α,5β-tetrahydroaldosterone in human urine [66] and dexamethasone in human plasma after oral administration of the drug by using beclomethasone as an I.S. [67]. 3α,5β-Tetrahydroaldosterone and dexamethasone, respectively, are converted into the corresponding glyoxal compounds by oxidation of the respective α-ketol moieties with copper(II) acetate, and the resulting glyoxal compounds are derivatized with DPH to form the corresponded chemiluminescence products (Fig. 11).

The DPH derivatives of α-keto acids and α-dicarbonyl compounds were identified to have quinoxalinone and quinoxaline structures, respectively (Fig. 7) [32,35], as in the case with the fluorometric methods using 1,2-diaminobenzene-type compounds [68]. The DPH derivatives of α-keto acids and α-dicarbonyl compounds had at least 100 times more intense chemiluminescence emission than DPH itself [35]. These results indicate that DPH is a chemiluminogenic derivatization reagent for them and the intense chemiluminescence is attributable to the highly fluorescent derivatives, quinoxalinone and quinoxaline. Furthermore, the isolated DPH derivative of α-ketovaleric acid has been successfully applied to serum glucose assay as a chemiluminogenic compound in place of luminol [38].

3.2. Reactions for 5-hydroxyindoles and catecholamines

6 - Aminomethylphthalazine - 1, 4(2*H*, 3*H*) - dione (AMP) was reported as the derivatization reagent for 5-hydroxyindoles and catecholamines (Fig. 12) [69–73].

AMP reacts selectively with 5-hydroxyindoles including serotonin at room temperature for 2 min in the presence of slightly alkaline potassium hexacyanoferrate(III) to give the highly chemiluminescent derivatives (Fig. 12A). These derivatives are separated by RPLC with isocratic elution, followed by chemiluminescence detection. The detection limits for the indoles are in the range 0.7–4.0 fmol per injection [69]. This method was applied to the determination of 5-hydroxyindoles in rat brain tissues by using 5-hydroxyindole-3-acetonitrile as an I.S. [70], and of 5-hydroxyindole-3-acetic acid in

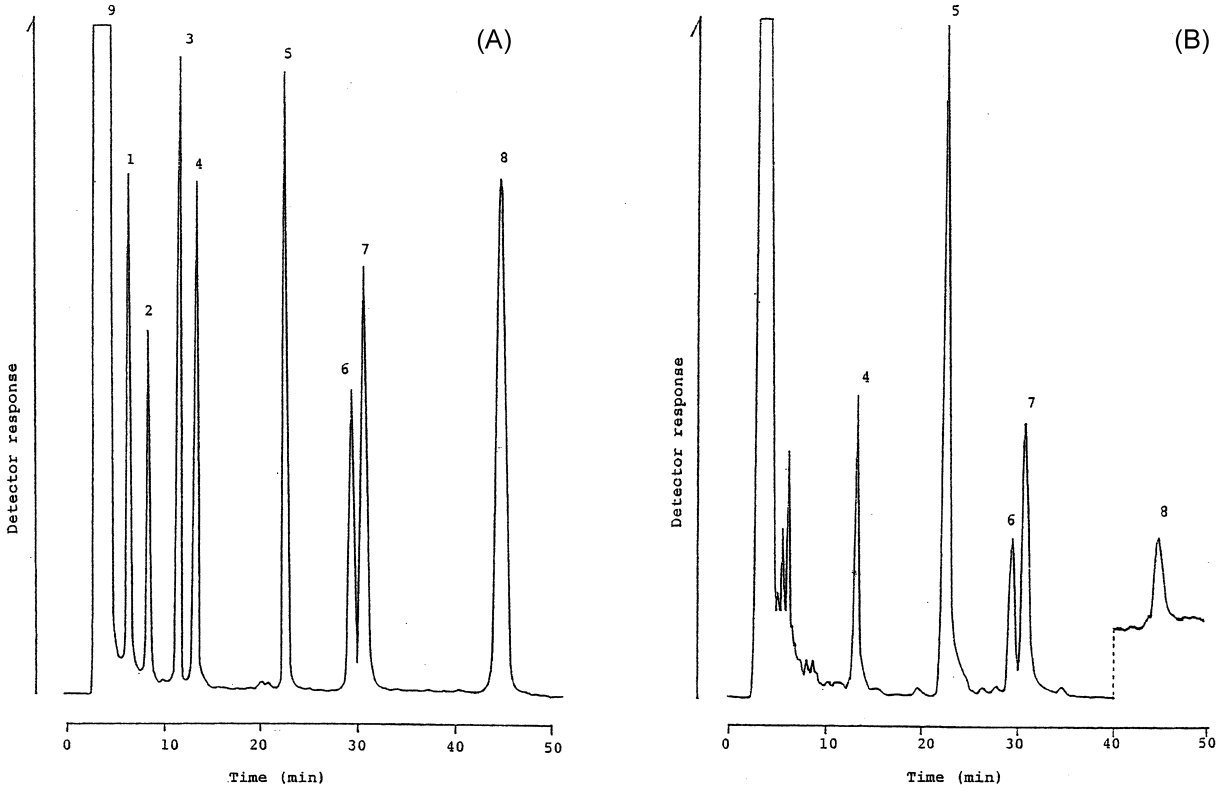


Fig. 8. Chromatograms of the DPH derivatives of α-keto acids obtained with (A) a standard solution and (B) a normal human plasma. Peaks: 1=α-ketobutyric acid; 2=4-hydroxyphenylpyruvic acid; 3=α-ketovaleric acid; 4=α-ketoisovaleric acid; 5=α-ketoisocaproic acid; 6=α-keto-β-methylvaleric acid; 7=α-ketocaproic acid; 8=β-phenylpyruvic acid; 9=reagent blank. From Ref. [62].

human urine by using α-methylserotonin as an I.S. (Fig. 13) [71].

AMP also reacts with catecholamines (epinephrine, dopamine and isoprenaline) under the different conditions (50 °C for 40 min) from those for 5-hydroxyindoles to afford the chemiluminescent derivatives (Fig. 12B). The derivatives are separated by RPLC with isocratic elution, followed by chemiluminescence detection. The detection limits for the amines are in the range 2–5600 fmol per injection [72]. Norepinephrine, however, is not detected under the derivatization, separation and chemiluminescence conditions. This method was applied to the determination of isoprenaline in human plasma after an oral administration of the drug by using *N*-methyldopamine as an I.S. [73].

The structures of AMP derivatives with 5-hydroxyindoles and catecholamines were unidentified except for their molecular masses. However, it is estimated that the structures of AMP derivatives with 5-hydroxyindoles and catecholamines have 2-(phthalazinyl)benzoxazole skeleton (Fig. 12), because it was proven that the fluorometric derivatizing methods of them using benzylamine formed the corresponding 2-phenylbenzoxazole derivatives [74,75]. The AMP derivatives of 5-hydroxyindoles had at least 100 times more intense chemiluminescence emission than AMP itself [76], but the derivatives of catecholamines have not been examined yet. The derivatives of catecholamines must have almost the same chemiluminescence intensity as that of 5-hydroxyindoles because the structures for each derivative and the detection limits for each analyte are pretty similar to each other.

3.3. Reactions for aldehydes

DPH, described above as the reagent for α-keto

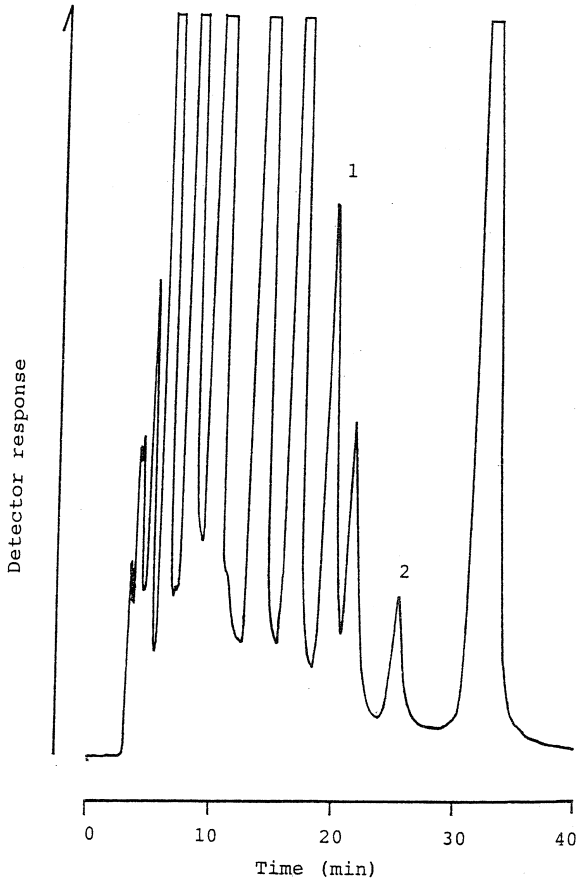


Fig. 9. Chromatogram of the DPH derivatives obtained with human serum from a healthy subject. Peaks: 1=NGNA; 2= NANA; other peaks are endogenous substances and reagent blank. From Ref. [64].

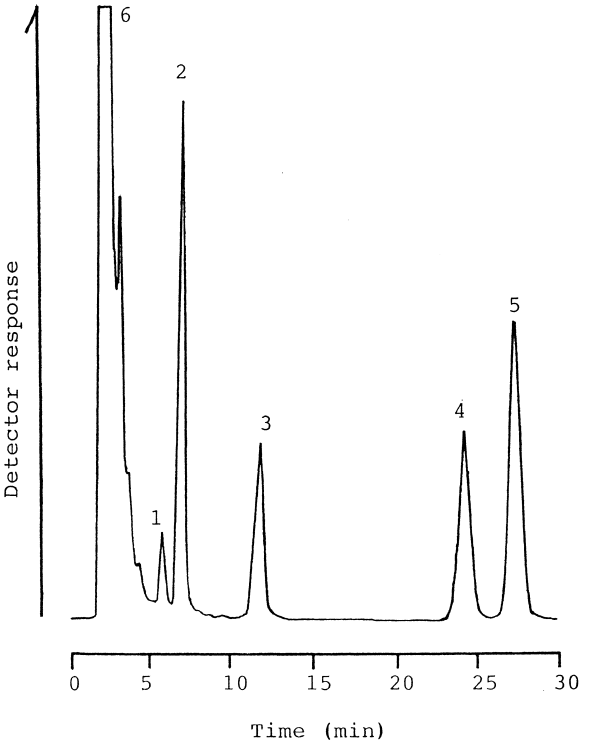


Fig. 10. Chromatogram of the DPH derivatives of five α-dicarbonyl compounds. Peaks: 1=phenylglyoxal; 2=diacetyl; 3= 2,3-pentanedione; 4=3,4-hexanedione; 5=2,3-hexanedione; 6= reagent blank. From Ref. [65].

acids and α-dicarbonyl compounds, and 5-amino-4-sulfanylphthalhydrazide (ASP) were reported as the derivatization reagents for aldehydes (Fig. 14) [77,78].

DPH reacts with aromatic and aliphatic aldehydes at 100 °C for 30 min in diluted hydrochloric acid to give the highly chemiluminescent derivatives (Fig. 14A). These derivatives are separated by RPLC with isocratic elution and detected by the addition with hydrogen peroxide and alkaline potassium hexacyanoferrate(III). The detection limits for aromatic and aliphatic aldehydes are in the ranges 1.0–150 and 50–2800 fmol, respectively, per injection [77].

ASP reacts selectively with aromatic aldehydes at 100 °C for 20 min in sulfuric acid to form the highly chemiluminescent derivatives (Fig. 14B). These derivatives are separated by RP–LC with isocratic elution, followed by chemiluminescence detection (Fig. 15). The detection limits for aromatic aldehydes are in the ranges 0.2–4.0 fmol per injection [78].

The structures of the chemiluminescent products from DPH and ASP with aromatic aldehydes were identified to have the corresponding 2-arylbenzimidazole and 2-arylbenzothiazole skeletons, respectively, as in the case with fluorogenic reagents, 1,2-diaminobenzenes [79] and *o*-aminothiophenols [80], respectively. The chemiluminescent products from both DPH and ASP had at least 200 times more intense chemiluminescence emission than those of the corresponding reagents themselves [35,37]. These results indicate that DPH and ASP are the chemiluminogenic derivatization reagent for aromatic aldehydes, and the intense chemiluminescence is

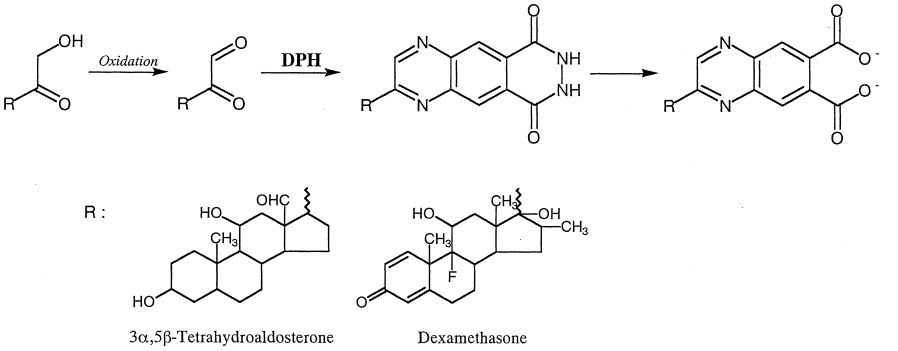


Fig. 11. Chemiluminescence derivatization reactions of 3α,5β-tetrahydroaldosterone and dexamethasone.

attributable to the highly fluorescent structures, 2-arylbenzimidazole and 2-arylbenzothiazole, respectively. One of the ASP derivatives could be induced to a high chemiluminescence labeling reagent for amines as described in the following sections.

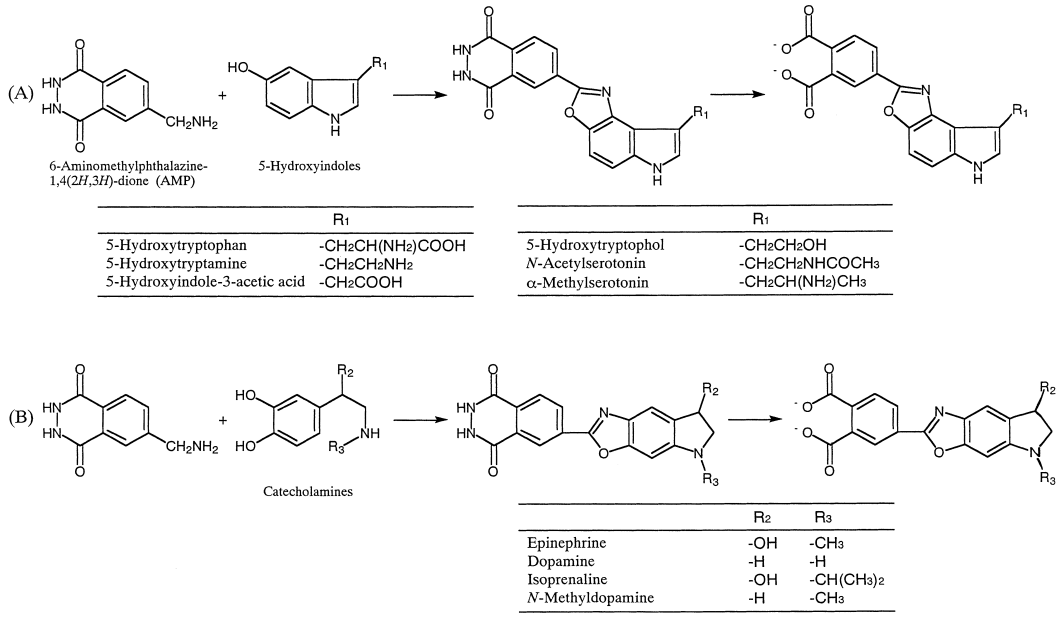
4. Luminol-type chemiluminescence labeling reagents

In this section, luminol-type chemiluminescence labeling reagents used in LC and CE are gathered. The chemiluminescence labeling reagents are defined as: the reagent is composed of a highly chemiluminescent moiety and a reactive moiety, and the reactive moiety attaches to an analyte to form the chemiluminescence-labeled derivatives. We classify them according to the functional groups of analytes.

4.1. Reactions for amines

The luminol-type chemiluminescence labeling reagents used for amines are illustrated in Fig. 16.

N-(4-Aminobutyl)-*N*-ethylisoluminol (ABEI) that



	R_1
5-Hydroxytryptophan	$-CH_2CH(NH_2)COOH$
5-Hydroxytryptamine	$-CH_2CH_2NH_2$
5-Hydroxyindole-3-acetic acid	$-CH_2COOH$

	R_1
5-Hydroxytryptophol	$-CH_2CH_2OH$
N-Acetylserotonin	$-CH_2CH_2NHCOCH_3$
α-Methylserotonin	$-CH_2CH(NH_2)CH_3$

	R_2	R_3
Epinephrine	-OH	$-CH_3$
Dopamine	-H	-H
Isoprenaline	-OH	$-CH(CH_3)_2$
N-Methyldopamine	-H	$-CH_3$

Fig. 12. Chemiluminescence derivatization reactions of (A) 5-hydroxyindoles and (B) catecholamines with AMP.

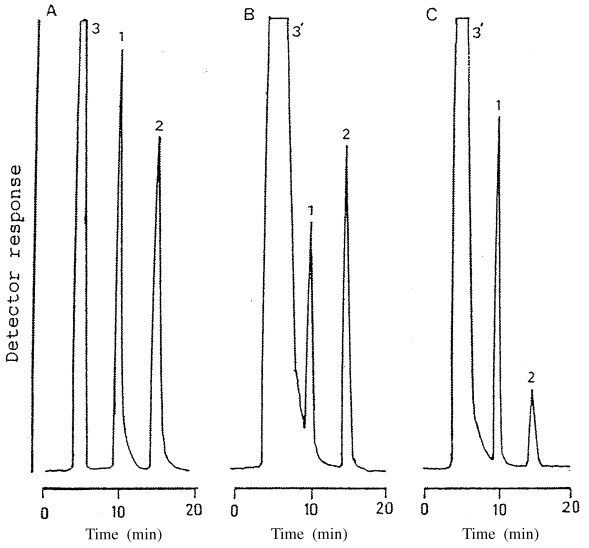


Fig. 13. Chromatograms of the AMP derivatives obtained with (A) a standard solution, and (B) a normal human and (C) a carcinoma patient urine sample. Peaks: 1=5-hydroxyindole-3-acetic acid; 2=a-methylserotonin; 3=reagent blank; 3′=reagent blank and urinary endogenous substances. From Ref. [71].

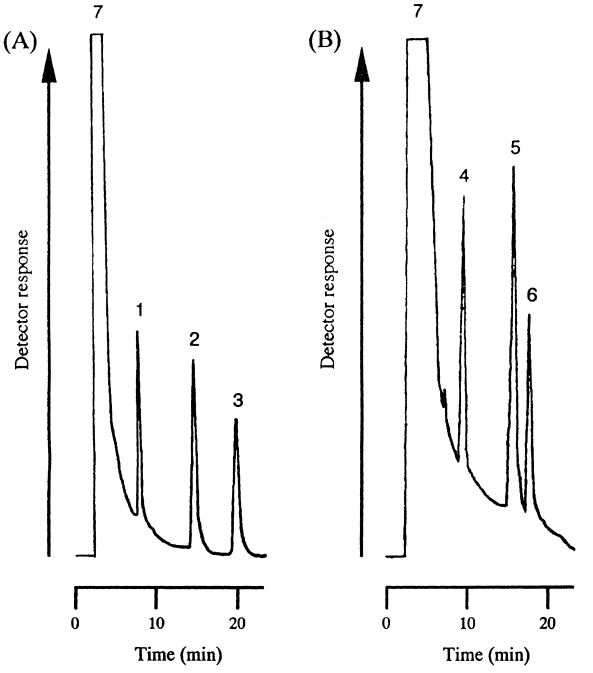


Fig. 15. Chromatograms of the ASP derivatives of aromatic aldehydes. Peaks: 1=benzaldehyde; 2=4-tolualdehyde; 3=4-chlorobenzaldehyde; 4=4-formylbenzoic acid; 5=4-hydroxybenzaldehyde; 6=vanillin; 7=reagent blank. From Ref. [78].

was used in immunoassays and so on from the 1970s is the first chemiluminescence labeling reagent in LC. ABEI, after coupling to *N*,*N*′-disuccinimidyl carbonate (DSC), reacts with primary and secondary amines for 2 h at room temperature or for 30 min at 80 °C in the presence of organic bases to give the chemiluminescent ABEI derivatives [81]. This ABEI–DSC method was applied to the determination of methamphetamine and amphetamine in human serum [82] and urine (Fig. 17) [83], and their detection limits were 20 pmol/ml serum or urine (about 20 fmol on column). ABEI-labeled alkylamines are also detected with ECL in CE, and highly sensitive analysis could be achieved in the range 1–6 fmol per injection [51]. On the other hand,

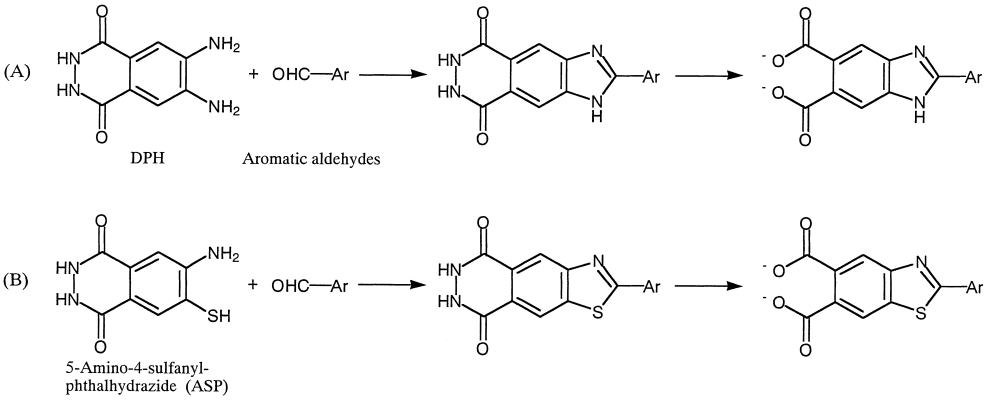


Fig. 14. Chemiluminescence derivatization reactions of aldehydes with (A) DPH and (B) ASP.

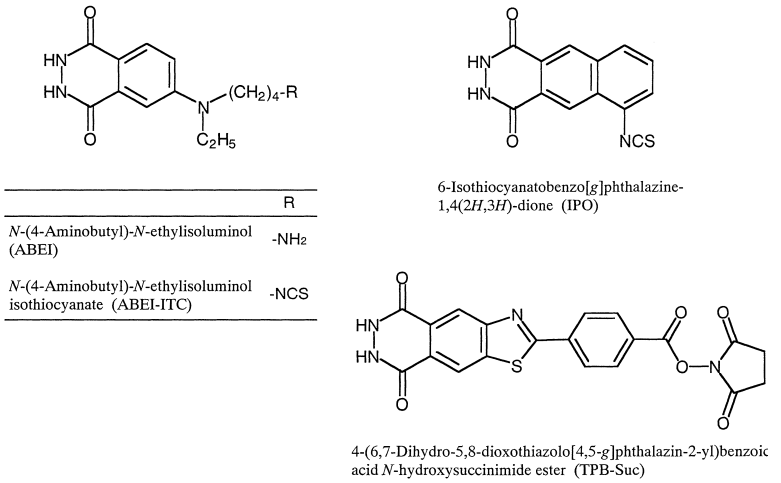


Fig. 16. Chemiluminescence labeling reagents for amines.

N-(4-aminobutyl)–*N*-ethylisoluminol isothiocyanate (ABEI–ITC), an activated ABEI derivative for amines, was isolated synthetically and was used for the determination of histamine with electrochemical oxidation. The derivatization reaction is performed at 80 °C for 1 h, and the detection limit for histamine is 1.5 pmol per injection [84].

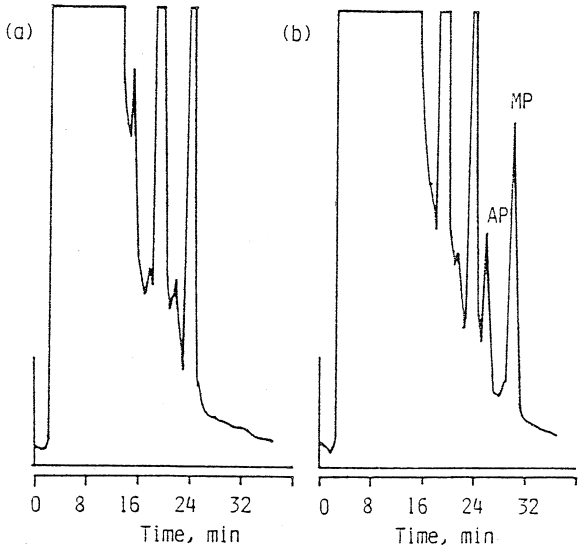


Fig. 17. Chromatograms of the ABEI derivatives with methamphetamine and amphetamine in urine sample. Peaks: AP= amphetamine; MP=methamphetamine; other peaks are endogenous substances and reagent blank. From Ref. [83].

6-Isothiocyanatobenzo[*g*]phthalazine-1,4(2*H*,3*H*)-dione (IPO) containing an isothiocyanate group was reported as the chemiluminescence labeling reagents for amines. Amines are labeled with IPO at 80 °C for 10 min in the presence of triethylamine and detected highly sensitively. The detection limits for primary and secondary amines are in the ranges 30–120 and 0.8–3 fmol per injection, respectively [85]. This IPO method was applied to the measurement of maprotiline in human plasma by using desipramine as an I.S. because the IPO-labeled secondary amines were detected highly sensitively (Fig. 18). The detection limit for the antidepressant is 0.36 pmol/ml plasma (1.5 fmol on column) [86].

4-(6,7-Dihydro-5,8-dioxothiazolo[4,5-*g*]phthalazin-2-yl)benzoic acid *N*-hydroxysuccinimide ester (TPB-Suc) that is derived from a highly chemiluminescent ASP derivative of 4-formylbenzoic acid has been reported as the chemiluminescence labeling reagent for amines. TPB-Suc reacts with both primary and secondary amines at 80 °C for 20 min in the presence of triethylamine. The detection limits for the amines are at sub-fmol levels per injection [87]. Furthermore, this method was applicable to the determination of amantadine in human plasma.

4.2. Reactions for carboxylic acids

The luminol-type chemiluminescence labeling re-

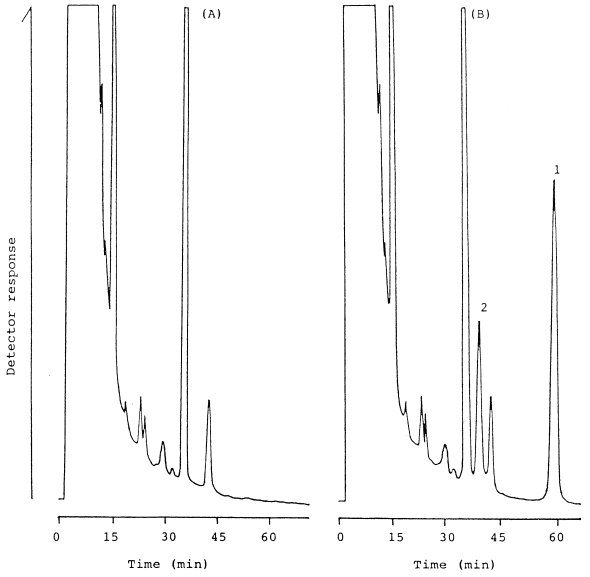


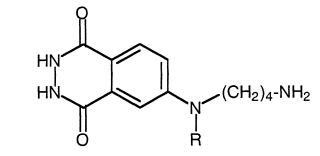
Fig. 18. Chromatograms of the IPO derivatives obtained with drug-free and plasma spiked with maprotiline and desipramine. Peaks: 1=maprotiline; 2=desipramine; other peaks are endogenous substances and reagent blank. From Ref. [86].

agents used for carboxylic acids are illustrated in Fig. 19.

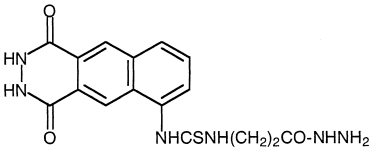
ABEI reported above as the derivatization reagent for amines has been also used for the chemiluminescence labeling reagent of carboxylic acids. ABEI reacts with carboxylic acids at 60 °C for 2 h in the presence of suitable condensing agents to give the chemiluminescent derivatives. The detection limit of cholic acid was about 20 fmol per injection [81]. This ABEI method was applied to the determination of eicosapentanoic acid in serum after single-step liquid–liquid extraction as a deproteinization, and the detection limits were 200 fmol per injection [88]. Ibuprofen and naproxen, both the drugs containing carboxyl group, were also chemiluminescence-labeled with ABEI. The ABEI-labeled ibuprofen and naproxen using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and some bases as condensing agents, and incubating at 50 °C for 10 min are also detected with ECL in LC, and the detection limits are 150 and 450 pmol, respectively, per injection [89]. Ibuprofen in human saliva is detected after derivatized with ABEI by using flubiprofen as an I.S. (Fig. 20). The optimum derivatization conditions were determined by using factorial design analysis, and the detection limit of ibuprofen is 3.4 pmol per 0.5 ml saliva [90]. The chemiluminescence reaction of ABEI-labeled bile acids was performed on the barium peroxide particle after CE separation using neither postcolumn oxidation reagents' delivery nor electrochemical oxidation. The ABEI-labeled acids were detected at levels as low as 10 pmol per injection [54].

N-(4-Aminobutyl)-*N*-methylisoluminol (ABMI), an ABEI-like labeling reagent, has been used as the derivatization reagent for the carboxylic acid analyses in LC. Polyunsaturated fatty acids are reacted with ABMI in the presence of EDC to form chemiluminescent derivatives. The detection limit for each acid is about 2.5 pmol per injection [91]. This ABMI method was applicable to the serum sample because the reaction proceeded under mild conditions.

6 - [*N* - (3 - Propionohydrazino)thioureido]benzo[*g*] phthalazine-1,4(2*H*,3*H*)-dione (PROB), an IPO derivative of β-alanine and hydrazine, was reported as the labeling reagent for carboxylic acids. PROB react



	R
ABEI	-C_2H_5
N-(4-Aminobutyl)-*N*-methylisoluminol (ABMI)	-CH_3



6-[*N*-(3-Propionohydrazino)thioureido]benzo-[*g*]phthalazine-1,4(2*H*,3*H*)-dione (PROB)

Fig. 19. Chemiluminescence labeling reagents for carboxylic acids.

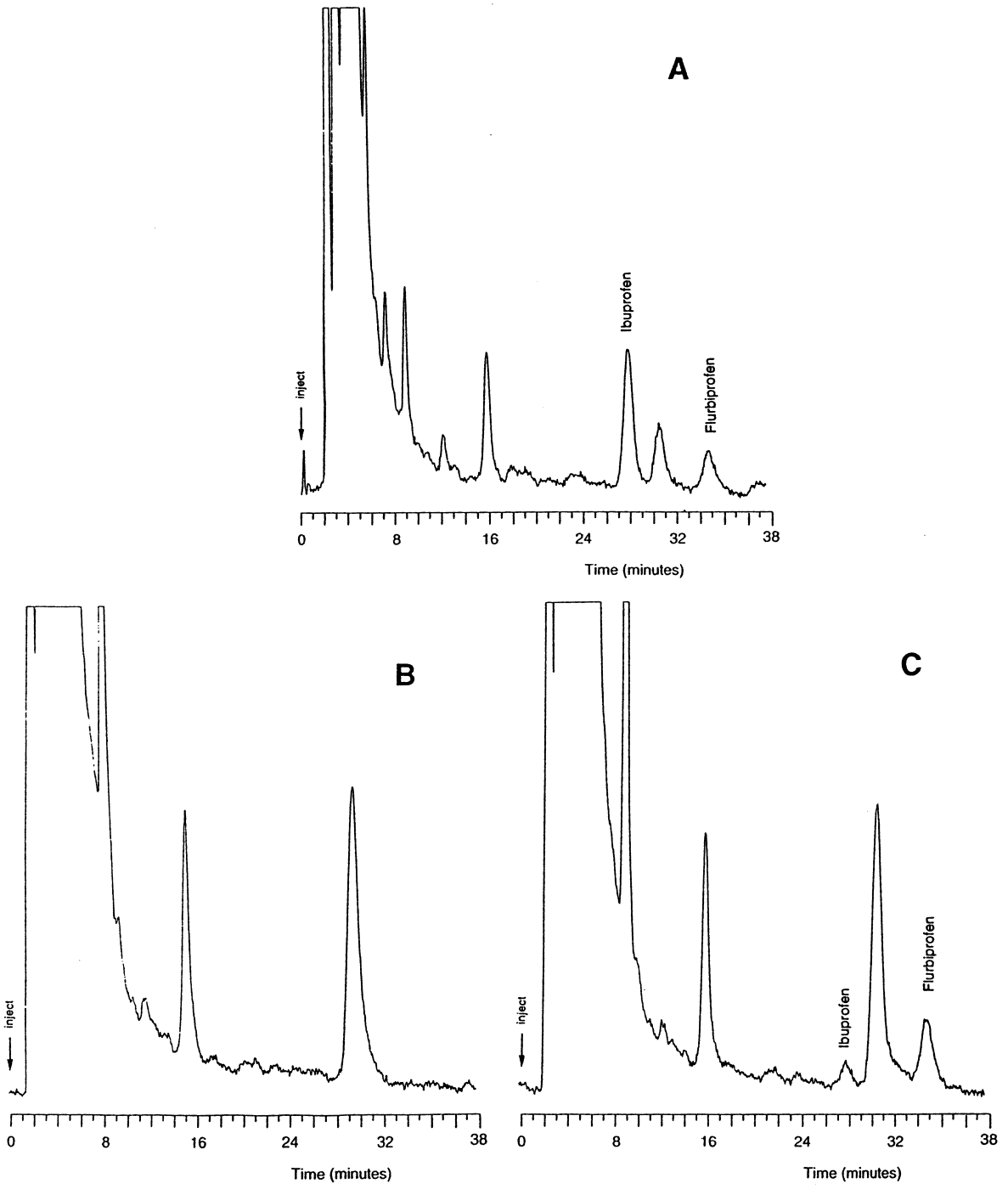


Fig. 20. Chromatograms of the ABEI derivatives obtained after derivatization of ibuprofen in saliva. From Ref. [90].

with fatty acids in the presence of EDC at room temperature for 40 min to give the chemiluminescence-labeled derivatives, which generate chemiluminescence by the usual chemical oxidizing agents in LC. The detection limits for the acids are in the range 10–70 fmol per injection [92]. This PROB method was successfully applied to the determination of some free fatty acids in human plasma.

4.3. Reactions for amino acids and peptides

The luminol-type chemiluminescence labeling reagents used for amino acids and peptides are illustrated in Fig. 21.

Isoluminol isothiocyanate (4-isothiocyanatophthalhydrazide; ILITC) is the first reported chemiluminescence labeling reagent for amino acids analyses. ILITC coupled with amino acids including proline and hydroxyproline within 10 min at room temperature in the presence of triethylamine, and the derivatives have been separated by RPLC. The averaged detection limits are about 10 fmol per injection [93]. This amino acid analysis method was applied to CE analysis coupled with the sheath flow cuvette (Fig. 6B), and the detection limits for the amino acids were about 0.5 fmol [94]. ILITC can also react with peptides. Glycylglycine and glycylglycylglycine are analyzed by the use of ILITC in CE, and the detection limits for them are sub-fmol levels [95].

ABEI is used for the determination not only of amines and carboxylic acids but also of amino acids and peptides in similar conditions to those for amines. ABEI-labeled amino acids formed in the presence of DSC were separated in CE and detected at end-column chemiluminescence detector at several fmol levels [49]. The ABEI–DSC method in CE with ECL detection was also applied to analysis of tripeptides at about 100 fmol levels [51]. In addition, the chemiluminescence reaction of ABEI-labeled amino acids was carried out in the flow-cell packed with barium peroxide powder after CE separation. ABEI-labeled amino acids were detected approximately 0.1 fmol per injection level [53].

ABEI-related compounds, *N*-[4-(*N*-α-ethoxycarbonyldiazoacetyl)aminobutyl] – *N* - ethylisoluminol (EDA–ABEI) [96] and *N*-(4-aminohexyl)-*N*-ethylisoluminol (AHEI) [97], are developed as the chemiluminescence labeling reagents for peptides. The labeling method using EDA–ABEI is highly selective and simple for peptide analysis. Since the derivatization reaction between ABEI and amino acids (peptides) proceeds under rather drastic conditions in the presence of condensing agents such as DSC or EDC, many side reactions occur to give many artifact peaks in the chromatogram. To minimize the side reactions, EDA–ABEI, an activated ABEI-like reagent that does not require any condensing agent is useful, but the reagent has not been utilized in flow-analysis yet. The reaction of peptides with EDA–ABEI is proceeded at room temperature for 1 h without using any condensing agent, and the detection limits are almost the same as ABEI–DSC method [96]. Whereas, the derivatization method of peptides with AHEI linked with Edman degradation reaction is reported. Peptides are converted with phenylisothiocyanate to the corresponded 2-anilino-5-thiazolinone amino acids at 50 °C for 10 min, and the anilinothiazolinone-amino acids are labeled with AHEI at 50 °C for 30 min in the presence of

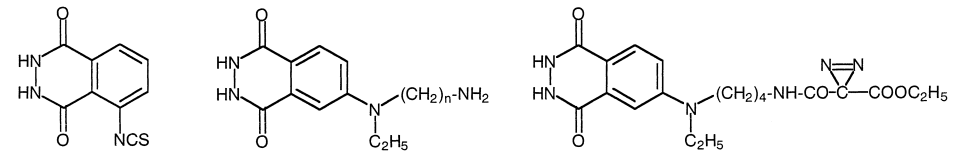


Fig. 21. Chemiluminescence labeling reagents for amino acids and peptides.

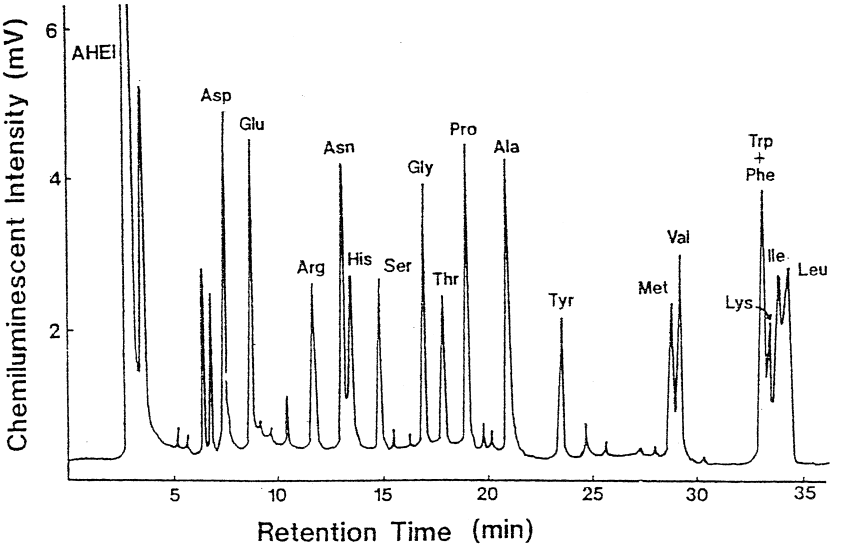


Fig. 22. Chromatogram obtained with eighteen phenylisothiocyanate-AHEI-amino acids. From Ref. [97].

pyridine. The AHEI-labeled 18 amino acids were separated with RPLC and detected at 0.5 fmol levels (Fig. 22) [97].

4.4. Reactions for other compounds

The luminol-type chemiluminescence labeling reactions used for the determination of glycosides and thiols are illustrated in Fig. 23.

Ribonucleosides and digoxin glycosides, the examples for glycosides, were examined by chemiluminescence derivatization. Glycosides are oxidized with sodium periodate at 30 °C for 1 h in diluted hydrochloric acid to the corresponded dialdehydes, and the aldehydes are condensed with amino group of ABEI at room temperature for 15 min in alkaline carbonate buffer to form the respective chemiluminescence labeled derivatives (Fig. 23A). The detection limits for both ribonucleosides and digoxin glycosides are about 50 fmol per injection [98].

ABEI is also used to the determination of thiols by LC. The method is based on the derivatization of

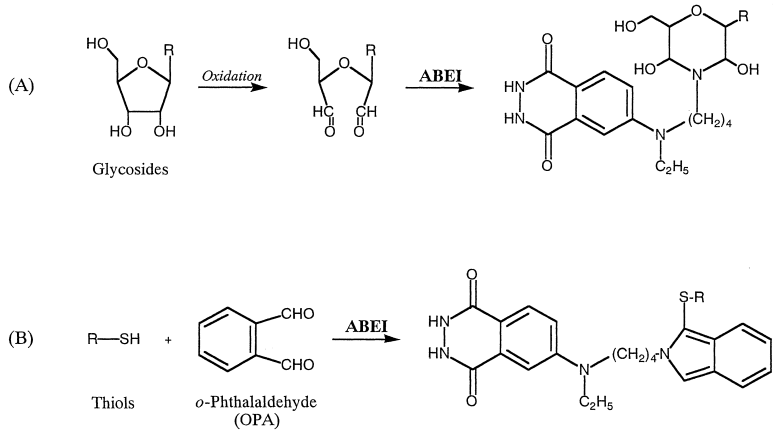


Fig. 23. Chemiluminescence derivatization reactions of (A) glycosides and (B) thiols with ABEI.

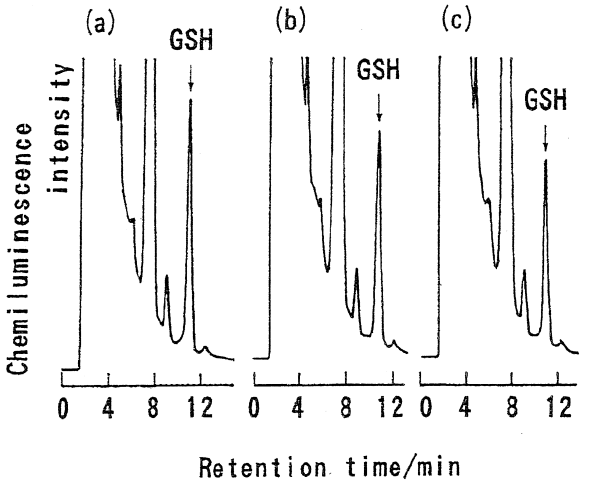


Fig. 24. Chromatograms of ABEI derivative of glutathione of blood samples from healthy male persons. From Ref. [99].

thiols with *o*-phthalaldehyde (OPA) and amino group of ABEI to form isoindole derivatives (Fig. 23B), and the RPLC separation with isocratic elution. The derivatization reaction is proceeded even at room temperature for 2 min in slightly alkaline conditions, and the detection limits for glutathione and cysteine are 15 and 35 fmol, respectively, per injection [99]. This method was applied to the determination of glutathione in human blood samples (Fig. 24). Although the OPA reaction is well known as a fluorogenic reaction for amines and thiols, this chemiluminescence method should be classified as ''chemiluminescence labeling reaction'' using ABEI (chemiluminescent itself) as a reagent [100].

5. Conclusions

The chemiluminescence method is a powerful analytical tool because of the following advantages: high sensitivity, high selectivity, wide linear range and simple instrumentation without a light source. The primary reason for high sensitivity and selectivity is that only a few compounds are chemiluminescent. But this rarity simultaneously means that most of analytes should be derivatized in chemiluminescence flow-analyses. This review has dealt with the principles of chemiluminescence of luminol-type compounds and its analytical use for derivatization reagents.

Luminol-type chemiluminescence derivatization reagents have been classified into two groups by reaction type: ''chemiluminogenic reagent'' and ''chemiluminescence labeling reagent''. In the former, the chemiluminesce derivatization reagents themselves are generally weakly chemiluminescent, and react with target compounds to form the conjugated-ring molecules, resulting in production of intense chemiluminescence. Furthermore, they have the possibility to improve the sensitivity and selectivity by introduction (use) of spectrochemiluminometry. Because the conjugated-ring derivatives luminesce in much longer wavelengths than the reagents and their degradative compounds (dagradation products), so they should be detected highly selectively and efficiently by spectrometry.

AMP, ASP and DPH are examples of chemiluminogenic derivatization reagents for 5-hydroxyindoles, aromatic aldehydes and α-keto acids, respectively. Although this type reagents reported so far are not so many and applied to limited analytes, they are so selective as to allow simple procedure and instrumentation. In the latter, the reagents are composed of a highly chemiluminescent moiety and a reactive moiety, and the reactive moiety attaches to an analyte to form chemiluminescence-labeled derivatives. ABEI and ILITC and their related compounds are examples of chemiluminescence-labeling derivatization reagents for amines, carboxylic acids and so on. They can be applied to a wide variety of analytes having a certain functional group, but they sometimes react with the concomitants having the same functional groups to cause interfering signals and background noise. They are apt to require more complicated clean-up procedures and/or sophisticated separation conditions.

Reliable and validated chemiluminescence methods have been proposed for many analytes in clinical, environmental and industrial matrices, and the advent of highly sensitive chemiluminescence detectors and/or efficient spectrochemiluminometer in LC and CE should stimulate further interest and advance in LC and CE. Of course, chemiluminescence has already become an essential tool in clinical, medical, pharmaceutical and environmental analyses.

6. Nomenclature

ABEI	*N*-(4-aminobutyl)-*N*-ethylisoluminol
ABEI–ITC	*N*-(4-aminobutyl)–*N*-ethylisoluminol isothiocyanate
ABMI	*N*-(4-aminobutyl)-*N*-methylisoluminol
AHEI	*N*-(4-aminohexyl)-*N*-ethylisoluminol
AMP	6-aminomethylphthalazine-1,4(2*H*, 3*H*)-dione
ASP	5-amino-4-sulfanylphthalhydrazide
CE	capillary electrophoresis
DMSO	dimethylsulfoxide
DPH	4,5-diaminophthalhydrazide
DSC	*N*,*N*′-disuccinimidyl carbonate
ECL	electrogenerated chemiluminescence
EDA–ABEI	*N*-[4-(*N*-α-ethoxycarbonyldiazoacetyl)-aminobutyl]–*N*-ethylisoluminol
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
GC	gas chromatography
ILITC	isoluminol isothiocyanate
IPO	6-isothiocyanatobenzo[*g*]phthalazine-1,4(2*H*,3*H*)-dione
I.S.	internal standard
LC	liquid chromatography
NANA	*N*-acetylneuraminic acid
NGNA	*N*-glycolylneuraminic acid
OPA	*o*-phthalaldehyde
PROB	6-[*N*-(3-propionohydrazino)thioureido]-benzo[*g*]phthalazine-1,4(2*H*,3*H*)-dione
RP	reversed-phase
TPB-Suc	4-(6,7-dihydro-5,8-dioxothiazolo-[4,5-*g*]-phthalazin-2-yl)benzoic acid *N*-hydroxysuccinimide ester
Φ_{CL}	chemiluminescent quantum yield

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