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Review

Derivatization of thiol-containing compounds

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Abstract

The determination of thiol-containing compounds in biological fluids is important in biochemistry and clinical chemistry. In this paper, derivatization reagents for thiols are reviewed with respect to their reactivity, selectivity, spectroscopic characteristics and their applicability especially to high-performance liquid chromatography. Derivatization used in ultraviolet and electrochemical detection. The derivatization reagents contain a functional group, *e.g.* an N-substituted maleimide, active halogen or aziridine, which react with the thiol group. Derivatization for use in flow injection analysis, thin-layer chromatography or gas chromatography–mass spectrometry is also described.

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List of Abbreviations

ABD-F	4-Aminosulphonyl-7-fluoro-2,1,3-benzoxadiazole
AF	(2,3,4,6-Tetra-O-acetyl-1-thio-β-D-glucopyranosato-S) (triethylphosphine)gold(I)
ANM	Anilinonaphthylmaleimide
APM	Anilinophenylmaleimide
BIPM	N-[4-(2-Benzimidazolyl)phenyl]-maleimide
CoA	Coenzyme A
DACM	N-(7-Dimethylamino-4-methyl-2-oxo-3-chromenyl)maleimide
DAM	Dimethylaminophenylmaleimide
DBD-F	4-(N,N-Dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole
DBPM	N-[4-(6-Dimethylamino-2-benzofuranyl)phenyl]maleimide
GITC	2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate
GSH	Glutathione
HPLC	High-performance liquid chromatography
4-MSA	4-Maleimidylsalicylic acid
NAM	N-(9-Acridinyl)maleimide
NEM	N-Ethylmaleimide
OPA	*o*-Phthalaldehyde
SA 446	(2*R*, 4*R*)-2-(2-Hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid
SBD-F	Ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate
TGFα–PE40	Transforming growth factor-α–*Pseudomonas aeruginosa* exotoxin A 40

1. Introduction

The thiol group is widely distributed in biological materials and forms an important functional center in biological systems. Both low-molecular-mass compounds, such as cysteine, glutathione (GSH), lipoic acid or coenzyme A (CoA), and high-molecular-mass compounds, such as peptides, enzymes or membranes, contain the group. A wide range of biological phenomena is believed to be somehow dependent on the thiols contained in these compounds [1]. Redox-, methyl transfer-, carbon dioxide fixation reactions and reactions in which CoA participates are dependent on a thiol group, and also the so-called "thiol enzyme" plays an important role in biological systems. The determination of thiol amino acids, *e.g.* homocysteine, in plasma is significant for the diagnosis of some kinds of inborn errors of metabolism [2]. Some compounds having a thiol group are also used as a medicine, *e.g.* captopril is used as an antihypertensive agent.

The aliphatic thiols ($pK_a \sim 12$) are more acidic than alcohols (pK_a 16–19) and are more like the phenols (pK_a 9–11) in their acidity. Accordingly, they are easier to alkylate than alcohols and are chemically the most active group found in cells. This is caused by the large dipole moment, nucleophilicity and the vacancy in the d orbital of the thiol group. The following equation shows the acid–base equilibrium of the thiol group and the formation of a radical group, which represents the high reactivity of the thiol group to oxygen and its easy transformation into disulphide (RSSR) [3]. In order to retain the free form (RSH), a chelating agent such as ethylenediaminetetraacetic acid and bubbling nitrogen through the medium are recommended.

$$RSH \overset{-H^+}{\rightleftharpoons} RS^- \overset{-e}{\rightharpoonup} RS\cdot \overset{RS\cdot}{\rightharpoonup} RSSR$$

The determination of thiol-containing compounds in biological materials is important for studies on the active sites of enzymes, in pharmacodynamic studies of drugs having a thiol group or in diagnosis of some kinds of diseases. The disulphides are reduced with dithiothreitol or *n*-tributylphosphine to give the corresponding thiol-containing compounds, which are then subjected to derivatization and determination. The derivatization reactions are used to give more stable compounds having better detectabilities and separation properties.

In this paper, derivatization reagents for thiols are reviewed with respect to their reactivity,

selectivity, spectroscopic characteristics and especially their applicability to high-performance liquid chromatography (HPLC).

2. Derivatization for use in high-performance liquid chromatographic determination

In this chapter, attention is mainly focused on pre-column labelling in HPLC, which is more popular than post-column labelling since it is easier to perform in individual laboratories.

Promising derivatization reagents for thiol-containing compounds require two structural features: a functional group reactive towards the thiol group (reacting group) and a signalling group with a high detector response (Fig. 1). The former group contains N-substituted maleimides, active halogens and aziridine; the latter group contains chromophores, fluorophores and electrophores [4].

2.1. Derivatization reagents for ultraviolet or visible detection

Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)] and N-ethylmaleimide (NEM) are widely used as reagents for the determination of thiol-containing compounds. The former produces 2-nitro-5-sulfhydrylbenzoic acid showing visible absorption at *ca.* 412 nm (Fig. 2) and the latter loses its ultraviolet absorption (at *ca.* 300 nm) with the addition of thiol-containing compounds (Fig. 3). Several examples of the derivatization of thiol-containing compounds for use in HPLC with ultraviolet or visible detection have been reported [5–10] (Table 1).

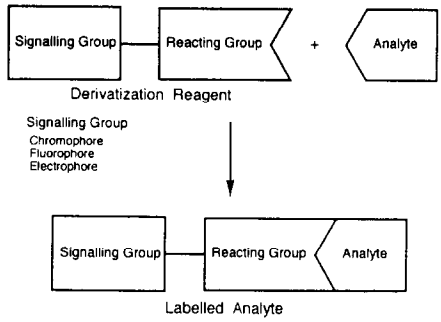


Fig. 1. Design of derivatization reagent.

One example is based on the combination of reversed-phase HPLC with a post-column reactions with 6,6′-dithiodinicotinic acid. Thiol-containing compounds, including GSH, cysteine, cysteamine, homocysteine and penicillamine, were separated with 33 m*M* KH_2PO_4 at pH 2.2 and quantitatively determined with detection limits of 0.1 nmol. The method was applied to the assay of GSH in human erythrocytes and in *E. coli*. 6,6′-Dithiodinicotinic acid has the great advantage of forming a stable product at a lower pH than Ellman's reagent [5].

Nika [6] described a post-column system suitable for the detection of primary amino groups and thiol groups in peptides. After HPLC separation, well-defined portions of the effluent are directed, by means of an automatic splitting device, into one of the two reactors either based on the ninhydrin assay or on Ellman's assay, respectively.

A sophisticated selective chromatographic detection system for CoA has been reported by Yamato *et al.* [7]. The short-chain acyl-CoA

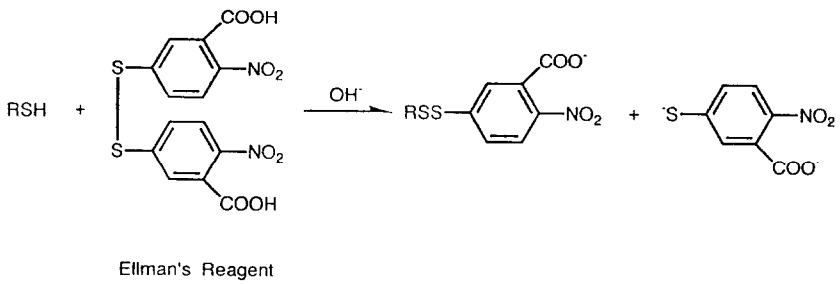


Fig. 2. Reaction of thiol with Ellman's reagent.

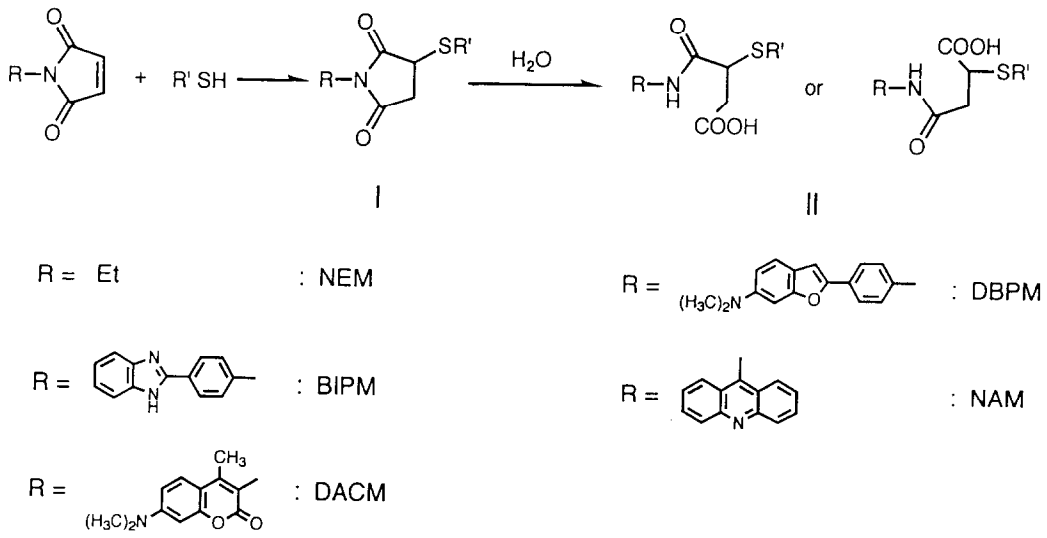


Fig. 3. N-Substituted maleimides and their reactions with thiols.

Table 1
Derivatization reagents for use in high-performance liquid chromatographic determination of thiol-containing compounds

Reagents	Representative compounds to be determined	Pre- or post-column	Detection (nm)	Ref.
6,6'-Dithiodinicotinic acid	GSH and the others	Post	UV (344)	5
Ellman's reagent	GSH and the others	Post	Visible (412)	5
Ellman's reagent	Thiol group in peptide	Post	Visible (420)	6
Ellman's reagent	GSH, GSSG	Post	Visible (412)	10
Ellman's reagent	Acetyl-CoA	Post	Visible (412)	7
Ellman's reagent	AF	Post	Visible (412)	8
GITC	Tiopronin, rentiapril, bucillamine	Pre	UV (250)	9
DBPM	GSH and the others	Pre	FL (Ex. 355, Em. 457)	19
DBPM	Penicillamine	Pre	FL (Ex. 360, Em. 455)	26
DBPM	GSH and the others	Pre	CL	20
4-MSA	GSH	Pre	Time-resolved luminescence	21
NAM	GSH and the others	Pre	FL (Ex. 360, Em. 435)	22
NAM	Cysteine, N-acetylcysteine	Pre	FL (Ex. 360, Em. 435)	23
NAM	Cysteine and the others	Post	FL (Ex. 365, Em. 435)	28
N-(1-Pyrene)maleimide	Mercaptoacetate, N-acetylcysteine	Pre	FL (Ex. 342, Em. 396)	24

Table 1 *(continued)*

Reagents	Representative compounds to be determined	Pre- or post-column	Detection (nm)	Ref.
DACM	Mercaptoacetate, N-acetylcysteine	Pre	FL (Ex. 400, Em. 480)	24
N-[*p*-(2-Benzoxazolyl)-phenyl]maleimide	Penicillamine	Pre	FL (Ex. 319, Em. >360)	25
N-[4,5,6-Dimethoxy-2-phthalimidyl)phenyl]-maleimide	2-Mercaptoethanol and the others	Pre	FL (Ex. 312, Em. 422)	27
4-(6-Methylnaphthalen-2-yl)-4-oxobuten-2-oic acid	GSH and the others	Pre	FL (Ex. 300, Em. 445)	29
4-(6-Methoxynaphthalen-2-yl)-4-oxobuten-2-oic acid	GSH and the others	Pre	FL (Ex. 310, Em. 450)	30
Monobromobimane, monobromotrimethyl-aminobimane	N-Acetylcysteine and the others	Pre	FL (filter)	34
Monobromobimane	N-Acetylcysteine and the others	Pre	FL (filter)	35
Monobromobimane	TGFα–PE40	Pre	FL (Ex. 382, Em. 470)	36
Monobromobimane	CoA, acyl-CoA	Pre	FL (Ex. 400, Em. 475)	37
Dansylaziridine	GSH, cysteine	Pre	FL (Ex. 338, Em. 540)	38
Dansylaziridine	Penicillamine	Pre	FL (Ex. 338, Em. >430)	39
SBD-F	Cysteine and the others	Pre	FL (Ex. 385, Em. 515)	43
SBD-F	Captopril	Pre	FL (Ex. 385, Em. 515)	46, 47
SBD-F	Peptide	Pre	FL (Ex. 385, Em. 515)	45
ABD-F, SBD-F	Cysteine, cystine and the others	Pre	FL (Ex. 380, Em. 510)	46, 47
OPA	Cysteine and the others	Post	FL (Ex. 360, Em. >405)	53
OPA	Cysteine, GSSG	Post	FL (Ex. 360, Em. >405)	54
OPA	GSH and the others	Pre	FL (Ex. *ca.* 340, Em. *ca.* 450)	51
OPA	GSH, GSSG	Post	FL (Ex. 358, Em. 460)	55
OPA	GSH	Post	FL (Ex. 340, Em. 440)	56
APM	N-Acetylcysteine and the others	Pre	ED	60
APM	SA 446	Pre	ED	61
DAM	Captopril	Pre	ED	62
N-(Ferrocenyl)maleimide	GSH	Pre	ED	63
3,5-Di-*tert.*-butyl-1,2-benzoquinone	GSH and the others	Pre	ED	64
OPA	GSH, GSSG	Pre	ED	65

UV: ultraviolet, FL: fluorescence, CL: chemiluminescence, ED: electrochemical detection

thioesters were separated by reversed-phase ion-pairing HPLC, and then acetyl-CoA was selectively detected on-line with a post-column immobilized-enzyme reactor containing phosphotransacetylase. Thio-CoA liberated enzymatically from acetyl-CoA was determined spectrophotometrically after reaction with Ellman's reagent in the reagent stream. The calibration graph was linear between 0.2 and 10 nmol, with a detection limit of 0.05 nmol.

An HPLC method using Ellman's reagent has been developed for determining Auranofin (AF) (2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranosato-S) (triethylphosphine)gold (I) in urine. The method comprises initial chromatographic separation of AF followed by on-line decomposition by potassium iodide with a released thiol group undergoing a color-developing reaction with the reagent [8].

A derivatization procedure has been developed for converting enantiomeric thiol-containing compounds (tiopronin, rentiapril, bucillamine) into their diastereomers for resolution by reversed-phase HPLC. The thiol-containing compounds were derivatized with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) as a chiral derivatization reagent and triethylamine as a basic catalyst. The thiol group reacted smoothly with the isothiocyanate to form the dithiocarbamate derivative within 30 min at room temperature. The ultraviolet detection wavelength was set at 250 nm, based on the absorption of the thiocarbonyl group. The resulting diastereomers were well separated on an octadecyl-bonded silica column (Fig. 4) [9].

The other derivatization reagents for ultraviolet detection have been reviewed by Imai and Toyo'oka [4].

2.2. Derivatization reagents for fluorescence or chemiluminescence detection

Many fluorigenic reagents for thiols have been developed and used as pre- or post-column derivatization reagents in HPLC with fluorescence or chemiluminescence detection. Several excellent reviews have been published [4,11–14].

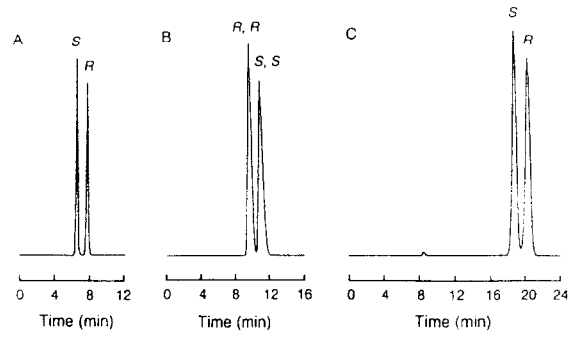


Fig. 4. Resolution of optical isomeric tiopronin (A), rentiapril (B), and bucillamine (C) by HPLC after reaction with GITC [9]. Conditions: column: TSKgel ODS-80TM (5 μm, 15 × 0.46 cm I.D.); mobile phase: CH_3OH–0.01 *M* phosphate buffer (KH_2PO_4–H_3PO_4, pH 2.8) (A; 50:50, C; 53:47); CH_3OH–0.01 *M* phosphate buffer (KH_2PO_4–Na_2HPO_4, pH 5.0) (B; 58:42); flow-rate: 1 ml/min; detection: ultraviolet (250 nm).

2.2.1. N-Substituted maleimides

Maleimides generally undergo facile addition to thiols as described above.

Kanaoka has found that certain N-substituted maleimides which are themselves nonfluorescent react readily with various thiol-containing compounds to form fluorescent addition products [15,16]. Among the initially examined maleimides, N-[4-(2-benzimidazolyl)phenyl]-maleimide (BIPM) turned out to be the first practical reagent, and a very sensitive fluorimetric determination of thiol-containing compounds has been developed with BIPM [17]. Further systematic search for fluorophores led ultimately to N-(7-dimethylamino-4-methyl-2-oxo-3-chromenyl)maleimide (DACM) as a promising reagent of this type which fluoresces at longer wavelengths and is more soluble in water than the others. In the design of DACM, solubility was enhanced by using the polar auxochrome dimethylamino residue [18]. These reagents (Kanaoka reagents) were used as fluorigenic probes which bind covalently to certain sites of proteins.

Recently several N-substituted maleimides having a fluorophore have been developed, and these reagents together with Kanaoka reagents have been used as fluorigenic derivatization

reagents for the determination of thiol-containing compounds with HPLC [19–28]. The structures of the representative reagents and their application are summarized in Fig. 3 and Table 1, respectively. Most of these reagents themselves do not show significant fluorescence and react readily with various thiol-containing compounds to form fluorescent addition products having high quantum yields as described above, although these quantum yields are highly dependent on the solvent. The reaction of these reagents with the thiol group proceeds for a few minutes around room temperature at pH 5–8. The fluorescent adducts (I) are unstable and are converted into two ring-cleaved fluorophores (II) at the N–C = O position of maleimide. When the reagents are used for pre-column derivatization, a prolonged reaction time is required to completely convert fluorophore I into II; otherwise, two peaks for fluorophores I and II are observed in the chromatogram. The other item to be noticed is that the formation of the diastereomeric adducts occurs with an optically active thiol-containing compound. Although the reacting group has the above described disadvantages, this reaction is expected to be preferential if not specific to thiols. The derivatization is performed directly in the biological fluid (*i.e.* plasma, serum or urine), using a 5–10 fold excess of the appropriate reagent. The sample generally is subjected directly to HPLC after dilution with a suitable solvent. The sensitivity depends on the fluorophore used but is usually in the order of picomoles. Following are examples of the application of these kinds of reagents.

The pre-column labelling of six kinds of thiol-containing compounds (GSH, cysteine, N-acetylcysteine, cysteamine, homocysteine, and CoA) in biological fluids (rat tissues and human serum) with N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM) followed by HPLC with fluorescence detection has been reported by Nakashima *et al.* [19]. The lower limits of detection at a signal-to-noise ratio of 2 were from 17 fmol (GSH) to 700 fmol (N-acetylcysteine). The peroxyoxalate chemiluminescence detection of these DBPM-derivatized thiol-containing compounds combined with HPLC has also been done. The lower detection limits were from 7 fmol (cysteamine) to 113 fmol (GSH) per 100 μl at a signal-to-noise ratio of 2 [20].

Time-resolved luminescence detection of derivatized thiol-containing compounds has been done by Schreurs *et al.* [21]. GSH was derivatized with 4-maleimidylsalicylic acid (4-MSA) and the obtained derivative was subjected to HPLC. A Tb^{3+} solution was added post-column to achieve complexation, and sensitized Tb^{3+} luminescence was detected. Compared to direct fluorescence detection, sensitized Tb^{3+} luminescence detection gave better results with respect to sensitivity (1 pmol on column) and selectivity as demonstrated for the spiked urine samples (Fig. 5).

Recently, naphthoylacrylic compounds have also been shown to be useful fluorigenic reagents for HPLC with fluorescence determinations of bioactive aliphatic thiols, providing good selectivity for the thiol function under mild reaction conditions [29,30]. These compounds are structurally related to N-substituted maleimides bearing similar unsaturated systems and are highly

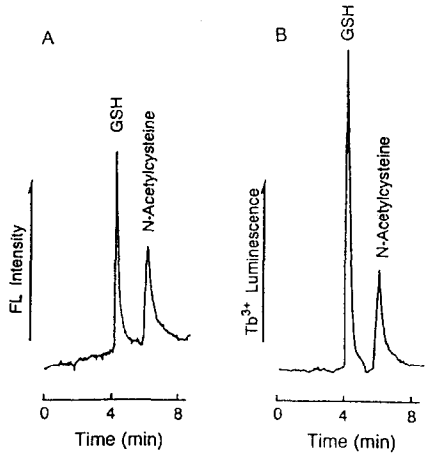


Fig. 5. Time-resolved luminescence detection of derivatized thiol-containing compounds in HPLC [21]. Conditions: column: RoSil C_{18} HL (5 μm, 14 × 0.31 cm I.D.); mobile phase: CH_3CN–H_2O (1:4) containing $5 \cdot 10^{-3}$ *M* Tris buffer (pH 7.2) and $1 \cdot 10^{-3}$ *M* tetrabutylammonium bromide; flow-rate: 0.5 ml/min; detection: (A) fluorescence (λ_{ex} 302 nm, λ_{em} 408 nm); (B) time-resolved luminescence (λ_{ex} 322 nm, λ_{em} 545 nm).

reactive towards nucleophiles (thiols), but they differ in the presence of a carbon–carbon linkage between the aromatic substrate and the reactive side chain. Such a structural feature is favourable for providing stable thiol adducts [31].

One trial of the post-column derivatization of cysteine and the other thiol-containing compounds with N-(9-acridinyl)maleimide (NAM) followed by fluorescence detection has been performed, but the detection limit was 50 pmol owing to the high background level [28].

2.2.2. Bimanes

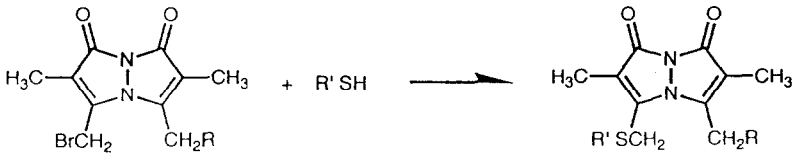
Monobromobimane and monobromotrimethylaminobimane couple with thiol-containing compounds at pH 8.0 at room temperature in 5 min to produce fluorescent thioethers (λ_{ex} *ca.* 380 nm, λ_{em} *ca.* 450 nm) (Fig. 6) [32–37]. The reaction has been used for the pre-column labelling of biological thiol-containing compounds; the resultant derivatives were subjected to cation-exchange or reversed-phase column separation and detected at the picomole level. However, care should be taken not to use excess amounts of the reagents; otherwise they have to be removed by some method after the reaction [34]. The following are recent examples of the application of these kinds of reagents.

A reversed-phase HPLC method with fluorescence detection useful for fermentation and downstream process development was developed for monitoring transforming growth factor-α–*Pseudomonas aeruginosa* exotoxin A 40 (TGFα–PE40). This protein is a chimeric recombinant protein synthesized in *E. coli*. In the fermentation, full-length TGFα–PE40 is present along with PE40 which is a C-terminal fragment of TGFα–PE40. A new technique based on the sulfhydryl specificity of the fluorescent probe monobromobimane has been used, in which treatment of in-process samples with dithiothreitol followed by the probe produces fluorescently-labelled TGFα–PE40 but does not label PE40 due to the lack of cysteine residues in this fragment. Thus, reversed-phase HPLC analysis using fluorescence detection provides the selectivity necessary to discriminate between TGFα–PE40 and PE40 [36].

A method for the determination of tissue levels of free CoA and long-chain acyl-CoA was developed by derivatization with monobromobimane followed by reversed-phase HPLC. The separation of the CoA–bimane adducts was achieved with a 3-μm Hypersil ODS C_{18} column using gradient elution. The detection limit was lower than 3 pmol [37].

2.2.3. Dansylaziridine

Replacement of the chloride in 5-dimethylaminonaphthalene-1-sulphonylchloride (dansylchloride) by an aziridine function led to the introduction of dansylaziridine as a fluorescence label. The reagent reacts selectively with thiols at pH 8.2 at 60°C in 1 h to give fluorescent compounds (λ_{ex} 338 nm, λ_{em} 540 nm), whereby the aziridine ring is opened (Fig. 7). Other functional groups having weaker nucleophilic properties, such as phenols, amines and alcohols, do not react. The detection limits are in the picomole range and the reagent is fluorescent, so it is used only for pre-column derivatization.



R = H : Monobromobimane

R = $N^+(CH_3)_3$: Monobromotrimethylaminobimane

Fig. 6. Reactions of bimanes with thiols.

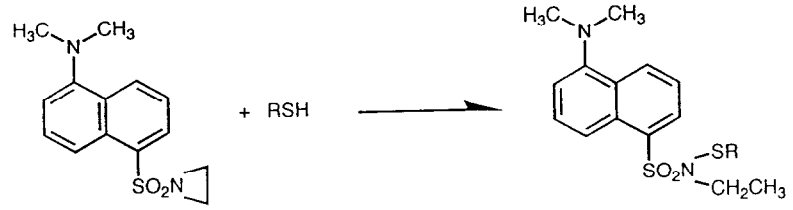


Fig. 7. Reaction of dansylaziridine with thiol.

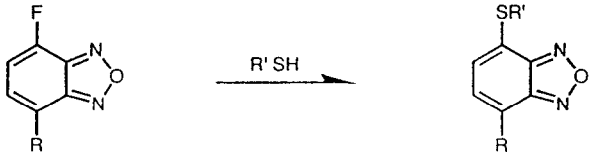
Amino acids containing a thiol group [38] as well as other thiol-containing compounds such as penicillamine [39] can be analyzed with this method.

2.2.4. *Halogenosulphonylbenzofurazans*

Ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulphonate (SBD-F) [40] and 4-aminosulphonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [41], having the reactive fluorine moiety at the *para* position, show no native fluorescence (Fig. 8). However, these reagents react with thiols within 1 h at pH 9.5 and 60°C to give fluorophores having a fluorescence at longer wavelengths, which is preferable because there are many substances that fluoresce at 300–400 nm in biological samples and interfere in the type of analyses being discussed. These reagents are soluble in water, and the resultant fluorophores are stable at pH 9.5 in a refrigerator for 1 week. Recently developed 4-(N,N-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) has properties nearly similar to those of SBD-F and ABD-F, but the reaction rates are several times faster than those with ABD-F. The DBD-F reacts quantitatively with thiols after 10 min at 50°C and pH 8.0. Their order of reactivities (DBD-F > ABD-F > SBD-F) reflects the intensities of electron withdrawal at the 7-position of the 4-fluorobenzofurazan. It is suggested that the electron-withdrawing effect of the dimethylsulphonamide group (SO_2NMe_2) is greater than that of the sulphonamide group (SO_2NH_2) [42].

The derivatives of cysteine, GSH and other thiol-containing compounds with SBD-, ABD- or DBD-F have been separated on a reversed-phase column and detected at the picomole or sub-picomole level [43,44]. An antihypertensive drug, captopril, has also been derivatized with SBD-F and determined by HPLC [45].

The simultaneous determination of thiol- and disulphide-containing compound has been done using two kinds of these reagents [46,47]. Initially, the thiols in the sample were labelled with ABD-F in an alkaline medium (pH 9.3) containing disodium ethylenediaminetetraacetate. After the extraction of both unreacted and hydrolyzed ABD-F with ethyl acetate, the remaining disulphide-containing compounds were derivatized with SBD-F in the presence of a



R = $SO_3^-NH_4^+$	: SBD-F	SBD-SR' (λex 395 nm, λem 520 nm)
R = SO_2NH_2	: ABD-F	ABD-SR' (λex 380 nm, λem 520 nm)
R = $SO_2N(CH_3)_2$	: DBD-F	DBD-SR' (λex 380 nm, λem 515 nm)

Fig. 8. Reactions of halogenosulphonylbenzofurazans with thiols.

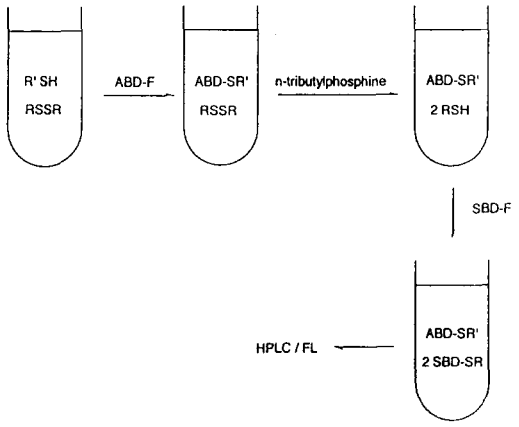


Fig. 9. Procedure for simultaneous determination of thiol- and disulphide-containing compounds [47].

reducing agent, *n*-tributylphosphine (Fig. 9). The ABD-thiols derived from the thiol-containing compounds and SBD-thiols derived from the disulphide-containing compounds were separated on a reversed-phase column and detected fluorometrically.

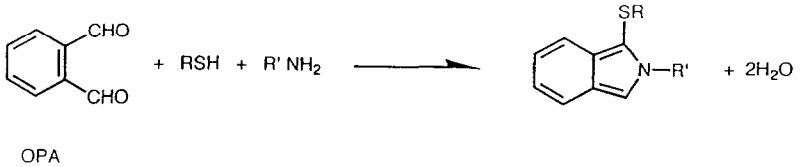
These newly synthesized fluorescent reagents having a benzofurazan structure have been reviewed in terms of their reactivity, fluorescence characteristics, stability, selectivity, and their applicability to analysis by Imai and co-workers [48–50].

2.2.5. *o*-Phthalaldehyde

o-Phthalaldehyde (OPA) is a reagent with no native fluorescence, but together with thiol- and primary amino-containing compounds it produces a highly fluorescent isoindole fluorophore (λ_{ex} *ca.* 350 nm, λ_{em} *ca.* 405–450 nm) (Fig. 10). It is developed for primary amino functions, such as amino acids and polyamines [11,14,48], but the method was also used in both pre- [51,52] and post- [53–56] column derivatizations for use in HPLC determination of thiol-containing compounds. The former is highly sensitive and can detect 25 fmol of thiol-containing compounds on column. In contrast, the detection limit of the latter method is in the picomole range. The isoindole derivative is also responsive to electrochemical detection which is described in the following section.

2.3. Derivatization reagents for electrochemical detection

Among the various detection systems in HPLC, electrochemical detection, which has been developed by Riggin *et al.*, appears to be most promising for the analysis of electrochemically active compounds, because of its excellent sensitivity and selectivity [57]. Thiol-containing compounds are inherently electroactive and suitable for electrochemical detection [58,59], but they are usually unstable in biological fluids and so must be converted to stable derivatives. The utility of dimethylaminophenylmaleimide (DAM), anilinophenylmaleimide (APM), and anilinonaphthylmaleimide (ANM) as derivatization reagents was tested for thiol-containing compounds (Fig. 11). These reagents have both aromatic amino and maleimide groups as electrophore and reacting groups towards thiol, respectively. APM was the most favourable in terms of sensitivity and reactivity. N-Acetylcysteine, GSH, cysteine and penicillamine were readily converted into the adducts with APM. Picomole to femtomole levels of these thiol-containing compounds were separated and quantified [60]. The determination of a new antihypertensive agent, (2*R*,4*R*)-2-(2-hydroxyphenyl)- 3 -(3-mercaptopropionyl)- 4 -thiazolidinecarboxylic acid (SA 446), in human blood was done with APM. Because the drug is un-



OPA

Fig. 10. Reaction of OPA with thiol.

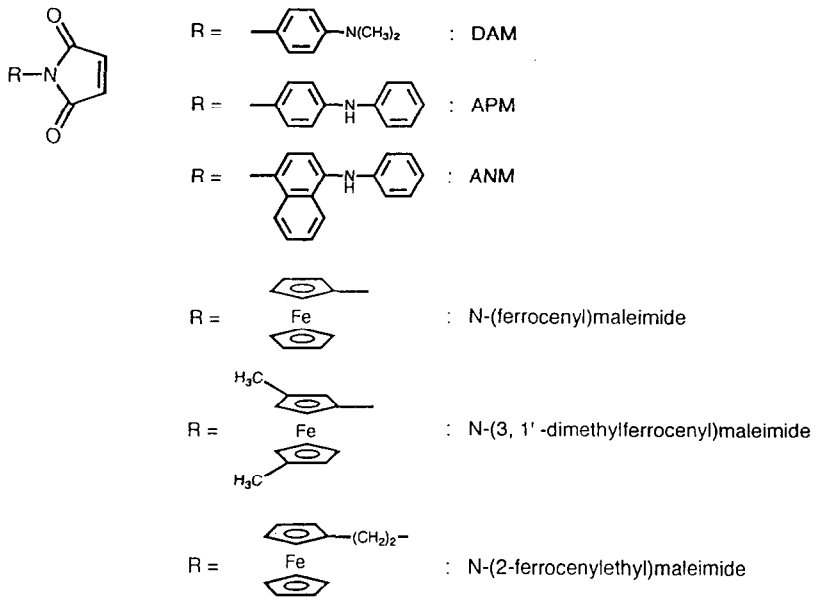


Fig. 11. Derivatization reagents for electrochemical detection.

stable in biological fluids, it was immediately derivatized by treating the freshly drawn blood specimens with APM. The adduct was separated and determined by HPLC with electrochemical detection on a reversed-phase column. The assay method was satisfactory with respect to the sensitivity and precision, providing a quantitation limit of 6.4 pmol/ml and a coefficient of variation of 3% [61]. The determination of captopril in human blood has been done with DAM. In this case, the separation of excess and/or decomposed derivatization reagents from the desired peaks was easy compared with the APM derivatization. The blood levels of captopril in patients who received an oral dose of 50 mg of the drug were measured by this method. The detection limit was 46.1 pmol/ml (Fig. 12) [62].

It is well known that ferrocene, an organometallic compound, is easily oxidized and reduced on the electrode. With respect to sensitivity and reversibility, ferrocene is one of the most suitable electrophores for incorporation into the derivatization reagent in HPLC with electrochemical detection. Three N-substituted maleimides, [N-(ferrocenyl)maleimide, N-(3,1'-dimethylferrocenyl)maleimide, and N-(2-ferrocenylethyl)maleimide (Fig. 11)] having ferrocene as an electrophore, were prepared and evaluated for pre-column derivatization of thiol-containing compounds in HPLC with electro-

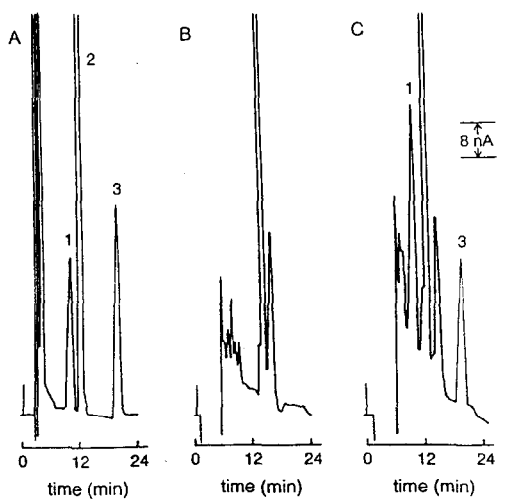


Fig. 12. High-performance liquid chromatograms obtained with (A) standard samples, (B) blank blood sample, (C) blood sample of patient orally given 50 mg of captopril [62]. Peaks: 1 = Captopril-DAM, 2 = DAM, 3 = SA 446-DAM (internal standard). Conditions: column: μBondapak C_{18} (8–10 μm, 30 × 0.39 cm I.D.); mobile phase: CH_3CN–0.8% $NH_4H_2PO_4$ pH 3.0 (1:2); flow-rate: 1 ml/min; detection: electrochemical detection.

chemical detection. The utility of these reagents was investigated by employing N-acetylcysteine as a model compound. Among the three, N-(ferrocenyl)maleimide was the most favourable reagent with respect to reactivity, stability and electrochemical properties. In this case, a dual-electrode coulometric detector was used and the method showed high selectivity and sensitivity with a detection limit of 0.06 pmol. The proposed method is applicable to the determination of GSH in biological specimens [63].

The reaction of thiols with 3,5-di-*tert.*-butyl-1,2-benzoquinone forms catechols which are responsive to electrochemical detection. GSH, cysteine, homocysteine and ergothionine from rat liver extract were determined by HPLC with electrochemical detection [64].

Two chromatographic methods for the assay of oxidized and reduced GSH in cells have been compared in which reduced GSH is either derivatized with OPA in an electrochemically active isoindole compound or directly measured using gold–mercury amalgam electrodes specific for thiol. The first method requires a derivatization procedure but provides efficient HPLC separation of the so-called isoindole derivative, while on the other hand, separation of the highly polar compounds requires an ion-pairing eluent. Both methods allow measurement of the reduced/oxidized GSH ratio in tumor cells. NEM was added to the sample, in order to avoid possible oxidation of reduced GSH during sample preparation, and then the sample was treated with dithiothreitol to determine the oxidized GSH [65].

3. Other methods

In this chapter, methods other than HPLC are summarized.

A method for the assay of chloramphenicol using an enzymatic reaction coupled with a *fluorescence detection system* has been developed [66]. Chloramphenicol was enzymatically acetylated by chloramphenicol acetyltransferase in the presence of acetyl-CoA as the acetyl donor, after which the liberated CoA-SH was derivatized with ABD-F. The assay was linear over the range 2.5–40 μg/ml.

Determination of thiol-containing compounds by *flow-injection analysis* has been reported by two groups [67–69]. Phosphotransacetylase is immobilized and used on-line in a stainless-steel column. CoA liberated enzymatically from acetyl-CoA is reacted with Ellman's reagent in the carrier stream. The immobilized enzyme can be employed for over 4 months without any significant decrease in activity [67]. A flow-injection analysis procedure with chemiluminescence detection has been developed for the determination of both thiol-containing drugs and the amino acid, cysteine [68,69]. Procedures are based on inhibition by the drugs of the chemiluminescence generated in the copper-catalyzed oxidation of luminol by hydrogen peroxide. The proposed methods were applied to the determination of cysteine, N-acetylcysteine, penicillamine, 2-mercaptopropionylglycine and thiouracil in pharmaceuticals.

Thin-layer chromatographic separation with fluorescence detection of derivatized thiol-containing compounds has been reported by several groups [70–73]. Bromobimanes [70] and halogenobenzofurazans [71,72] have been applied successfully in thin-layer chromatography of thiol-containing compounds with fluorimetric detection. Recently, thin-layer chromatographic separation of aliphatic thiols labelled with methyl 4-(6-methoxynaphthalen-2-yl)-4-oxo-2-butenoate has been done [73]. The fluorescent spots are visualized on irradiation at 254 and 366 nm.

A *gas chromatographic–mass spectrometric* method for determining tiopronin in human blood has been reported [74]. To prevent the oxidative degradation of tiopronin in the blood, its thiol group was immediately protected by treatment with isobutyl acrylate. The reaction was quantitative within 30 min. The blood sample was deproteinized and purified by a combination of liquid–liquid extraction and solid-phase extraction. Finally, the carboxyl moiety of the ester adduct was derivatized to the pentafluorobenzyl ester, which was then subjected to gas chromatography–mass spectrometry. The detection limit was estimated to be *ca.* 1 ng/ml.

Recently *capillary electrophoresis* has become a major tool in the field of analytical chemistry [75]. Terabe *et al.* reported the optical resolutions of derivatized amino acids by *micellar electrokinetic chromatography* [76,77]. The method is promising for the separation of not only amino acids but also peptides including thiol- or disulphide-containing ones.

4. Conclusions

The determination of thiol-containing compounds in biological fluids is important in biochemistry and clinical chemistry. Derivatization for use in HPLC with fluorescence detection is widely used for this purpose. Although many fluorophores have been used, the development of new reagents having the following fluorophores is required. The reagents should preferably have fluorescence characteristics at longer wavelengths (λ_{ex} and λ_{em}) as described above and used for laser technique. Also, for more sensitive detection, chemiluminescence reactions such as those described above or a time-resolved laser technique might be considered. For these techniques, suitable fluorophore skeletons for each technique are required. For example, polycyclic aromatic hydrocarbons such as perylene, rubrene and rhodamine will be effectively excited by the former technique, and pyrene, 2-phenylnaphthalene and benzopyrene, having longer fluorescence life times, are advantageous for use in the latter technique [48].

The various N-substituted maleimides react with thiol under mild conditions (room temperature or under ice-cooling) to produce the adducts, but sometimes multiple peaks are observed, as described above. Active halogens have also been used as the reacting group for this purpose. Although the conditions are more drastic, the obtained derivative shows a single peak on the chromatogram. However, these reagents are not selective for thiol because they also react with various nucleophiles (*e.g.* alcohols, phenols, and/or amino groups).

Electrochemical detection is also used for this purpose and thiol-containing compounds are inherently electroactive at high applied voltage; however, they are usually unstable in biological fluids and so must be converted to stable derivatives having a suitable electrophore.

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