

Derivatives for HPLC Analysis

Diploma Thesis

by

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1 Introduction

Liquid phase chromatography (HPLC) is a method of choice for the determination of inorganic and organic substances in various matrices.

Unfortunately, many substances of interest cannot be detected in HPLC because they do not contain the necessary chromophoric, fluorophoric or redox groups. However, this problem can be overcome by inducing derivatization reactions that add one of the above groups to the molecule investigated.

These reactions should produce stable derivatives rapidly and can be achieved in the following modes:

- ◆ In pre-column mode (before analytical separation), the reaction is generally performed manually in vials before HPLC injection but can also be automated. This mode allows more flexible working conditions (reaction time, solvent reaction, elimination of excess reagent, etc...). Separation of the molecules takes place after their modification by derivatization; derivative stability is necessary.
- ◆ In post-column mode, the reaction is performed automatically by adding a derivatization reagent after separation, and before detection, by means of a second HPLC pump. This approach requires heavier equipment but automation considerably reduces the number of manipulations. The derivatization reaction has to be fast and compatible with the mobile phase.

A derivatization reaction is very often required in order to increase sensitivity or selectivity and can be achieved by a specific detection, such as fluorescence or absorption in the visible light, at a high wavelength [1, 2]. Derivatization is particularly important for trace analyses of complex biological samples.

Derivatizations can be induced by organic or electrochemical reactions, such as oxidation and reduction, or by displacement or addition reactions. The latter result from an electrophilic or nucleophilic attack, which is determined by the reactivity of the substrate. Reduction and oxidation reactions are relatively non-specific and therefore less appropriate.

Derivatization reagents may be divided into four groups:

- ◆ non-fluorescent reagents, generally used in UV-VIS (benzoyl chlorides and sulfonyl benzene).
- ◆ fluorogenic reagents, generally non-fluorescent but which react with target compounds to form conjugated fluorescent cyclic molecules (fluorescamine).
- ◆ fluorescent reagents, which have a highly fluorescent aromatic group (fluophore) and a reactive group.
- ◆ reagents with redox properties, used in electrochemistry.

In clinical analyses of biological fluids, electrophilic reagents present the great disadvantage of reacting with all endogenous nucleophiles (numerous with regard to the substance to be analyzed). Therefore, method specificity decreases and electrophilic reagents degrade in water (the most abundant nucleophile in biological fluids). This is why a prior sample preparation is necessary in order to eliminate any interfering substances.

In contrast, nucleophilic reagents are more specific and more frequently employed: they do not have endogenous electrophilic compounds and they are stable in water.

Derivatization is also used for enantiomeric separation. Enantiomer quantification methods, such as chemical resolution or rotating power of polarized light, are not adequate to determine enantiomer traces in a biological environment.

Liquid phase chromatography (HPLC) has been found to be the most adequate method because many enantiomers present weak thermic stability and because gas phase chromatography requires high temperatures that can induce racemization.

In pre-column derivatization (indirect method), diastereoisomers are formed before injection by using a highly pure enantiomeric chiral reagent. Separation is then performed on an achiral column with an achiral mobile phase.

Amines constitute a large family of organic molecules and are found in amino acids but also in most pharmaceutical compounds (antibiotics, etc...), in agrochemistry (proteins, peptides, toxins, etc...) and in the environment (pollutants). It is therefore important to be able to quantify them in a sensitive and specific way.

More specifically, biogenic amines are organic bases with a low molecular weight, formed by the decarboxylation of amino acids and degraded by the metabolism, in animals, plants and micro-organisms [3, 4]. They present a particular interest as their formation is related to the protolytical process which takes place during the production of fermented foods (such as cheese, fish products, wine and beer) [5-8]. This phenomenon is problematic as the absorption of an important quantity of biogenic amines may cause headaches, nausea, hypo- or hypertension and even anaphylactic shocks [3, 9-13]. This is why research on their formation and their detection in food is of such importance.

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2 Detection methods

2.1 Spectrophotometric detection

2.1.1 UV-VIS Detection

UV detection is the most commonly used technique in HPLC but it sometimes lacks sensitivity or selectivity for trace analysis of medicinal substances (drugs). Chemical derivatization modifies substances with a low UV absorption into highly sensitive products.

2.1.1.1 Primary and secondary amines

2.1.1.1.1 NITROBENZENES

2.1.1.1.1.1 1-Fluoro-2,4-dinitrobenzene (FDNB ; 2,4-dinitro-1-fluorobenzene ; Sanger reagent)

FDNB reacts with amino acids and primary and secondary amines to form 2,4-dinitrophenyl (DNP) (Fig. 1), a stable derivative. It also reacts with aliphatic hydroxyl groups [8, 9].

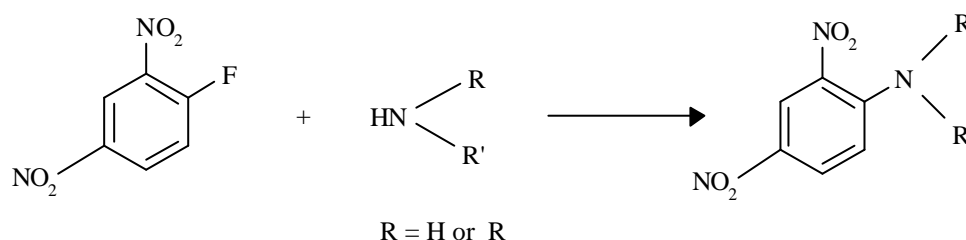


Fig. 1 Reaction of FDNB with primary and secondary amines [7]

The toxicity of this reagent is a major drawback.

Analytical applications

FDNB is used in the pre-column derivatization of aminoglycosides, such as amikacine [2, 3], tobramycine [4, 5], gentamycine [6], sisomycine [6], neamine, neomycine B and neomycine C [1]. Derivatization is performed in an alkaline medium.

- [42085](#) : 2,4-Dinitro-1-fluorobenzene purum p.a.; 98.0% (GC)

References

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2.1.1.1.2 4-Fluoro-3-nitrotrifluoromethylbenzene (4-fluoro-3-nitrobenzotrifluoride, FNBT)

FNBT reacts with primary amines (Fig. 2) but also with polyamines to form *N*-2'-nitro-4-trifluoromethylphenyl (NTP-polyamine), a UV absorbent polyamine (at 242 and 410 nm).

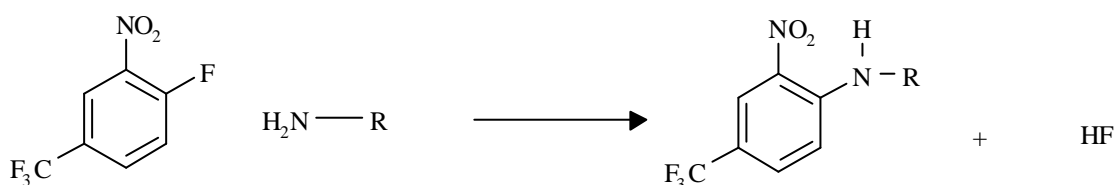


Fig. 2 Reaction of FNBT with primary amines [1]

NTP-polyamines can be extracted with organic solvents, the best being 2-methylbutane [2], which extracts a minimum amount of secondary products and evaporates easily.

FNBT does not react with secondary amines but with amino acids such as histidine.

Analytical applications

FNBT is employed to derivatize polyamines, such as putrescine, spermidine and spermine [2]. The reaction takes place at 60°C and is complete after 20 min. Limits of detection are 5 pmol for putrescine, 10 pmol for spermidine and 25 pmol for spermine.

Vessman and Strömberg used FNBT as first-step reagent in the two-step derivatization of tranexamic acid [3].

- [214337](#): 4-Fluoro-3-nitrobenzotrifluoride, 99%

References

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2.1.1.1.3 2,4,6-Trinitrobenzene-1-sulfonic acid (TNBS)

TNBS reacts with primary amines, and in particular with amino acids and peptides (Fig. 3)

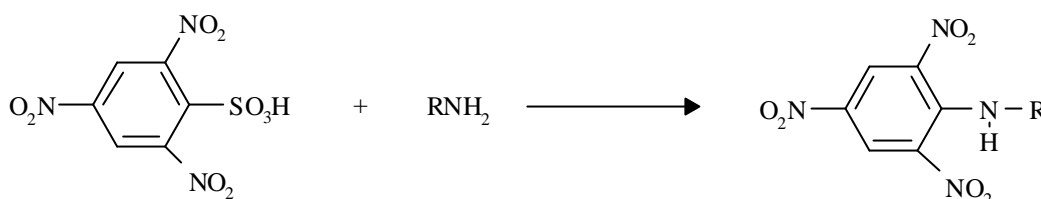


Fig. 3 Reaction of TNBS with primary amines [1]

The reaction takes place in an aqueous medium at pH 8, and at room temperature, without any undesirable secondary reactions. Nitrophenyl derivatives have a high molar absorption (340 nm).

Analytical applications

TNBS reacts with amikacine at a pH optimal between 9.5 and 10 [2]. Temperatures below 70°C and reaction times shorter than 30 min. induce an incomplete reaction which produces many derivatives.

- [92824](#): 2,4,6-Trinitrobenzenesulfonic acid Solution purum; ~1% in N,N-dimethylformamide
- [92822](#):: 2,4,6-Trinitrobenzenesulfonic acid Solution BioChemika; 1 M in water

References

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2.1.1.1.2 NINHYDRIN

Moore and Spackman [2, 3] developed a method which uses ninhydrin to determine amino acids. Despite low sensitivity, high instrumental cost and time consumption, this method has become classical because of its automation, reproducibility and precision.

Ninhydrin reacts with primary amines only (especially α -amino acids, except cysteine)

and affords blue-violet compounds (diketohydrindylidene-diketohydrindamine).

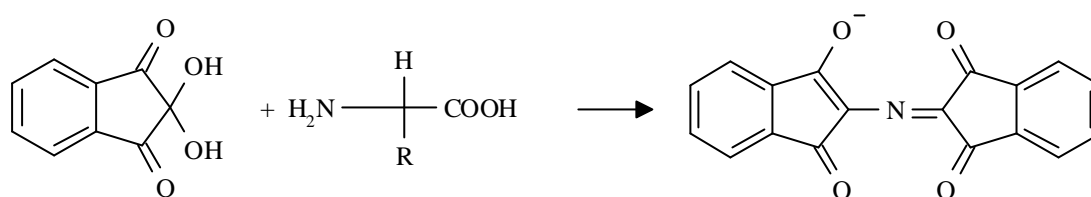


Fig. 5 Reaction of ninhydrin with amino acids [1]

Analytical applications

Ninhydrin is used in the post-column derivatization of aminoglycosides (streptomycin and dihydrostreptomycin). The reaction takes place in an alkaline medium with a reagent in the mobile phase. Introduction of NaOH ends the reaction. This method is applied to beef, chicken and pork meats [4].

Joosten *et al.* use ninhydrin in the automated on-line derivatization of amines. Pre-column derivatization is performed automatically after separation by HPLC, with ninhydrin in the mobile phase. Derivatization is ended by heating and detection in the VIS at 546 nm [5].

Glyphosate, a widely used herbicide, and aminomethylphosphonic acid (AMPA), are determined in biological fluids by derivatization with ninhydrin [6].

- [72489](#) Ninhydrin BioChemika ACS; ~99% (UV) (10g, 25g, 100g)
- [72490](#) Ninhydrin BioChemika ACS; ~99% (UV) (250g, 1 kg)

References

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2.1.1.1.3 DISUCCINIMIDO CARBONATE (DSC)

Activated carbamate reagents can be used for the HPLC determination of amino compounds. Succinimido phenyl- and *p*-bromophenylcarbamates (SIPC and SIBr-PC respectively) are produced by reaction of the disuccinimido carbonate (DSC) with aniline and *p*-bromoaniline, respectively. Carbamates produce a symmetrical urea derivative by reaction, in mild conditions, with both primary and secondary amines [1].

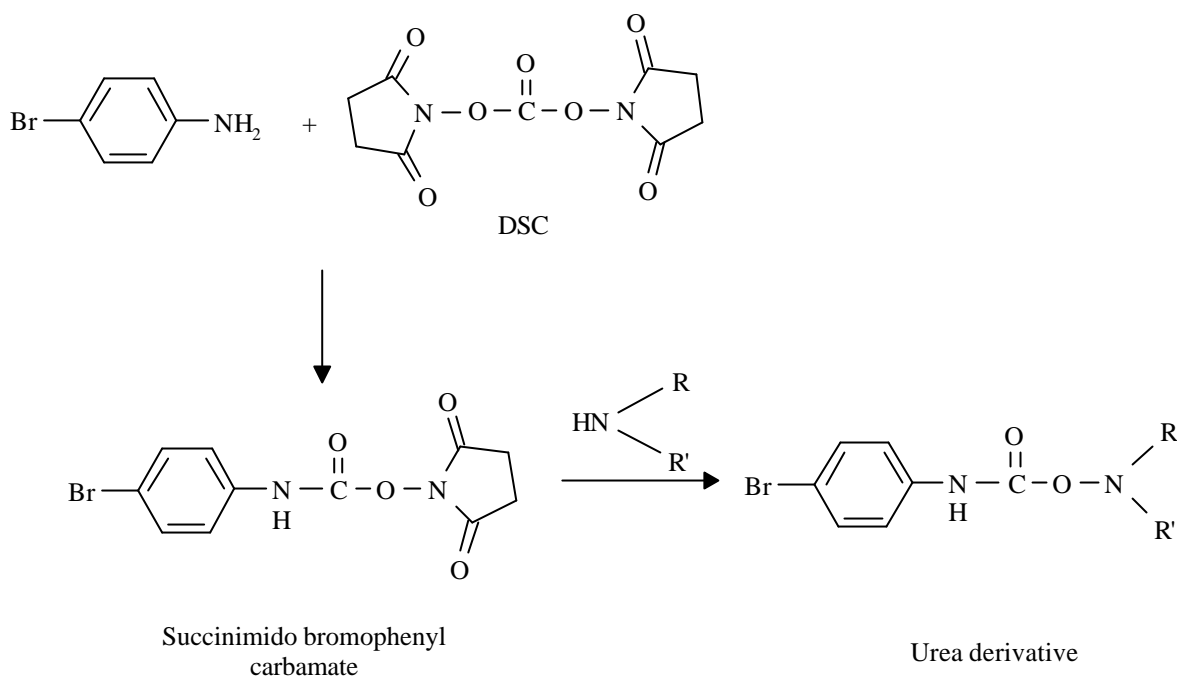


Fig. 6 Preparation of activated carbamate reagent and formation of urea derivative from activated succinimido bromophenyl reagent with amines [2]

SIPC and SIBr-PC urea derivatives are stable and have absorption maxima at 240 and 250 nm. Their molar absorption is 26'000 and 28'000, respectively. An excess amount of reagent will interfere with their detection but this can be avoided by adding an important amount of monoethanolamine.

In the same family, succinimido naphthylcarbamate (SINC) has been synthesized for fluorimetric detection (λ_{ex} . 305 nm, λ_{em} . 378 nm).

- [43720](#) Di(N-succinimidyl) carbonate purum; ~97% (NMR) ,

References

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2.1.1.1.4 4-N,N-DIMETHYLAMINOAZOBENZENE-4'-SULFONYL CHLORIDE (DABSYL-CL, DABS-CL)

Sulfonyl DABS-C1 reacts with primary and secondary amines, thiols, imidazoles, phenols and aliphatic hydroxyl groups. It is mainly used to derivatize amino acids [1-3]. Dabsyl derivatives of a number of amino acids have absorption maxima between 448 and 468 nm.

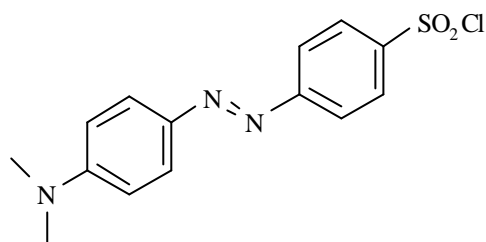


Fig. 7 Structure of DABS-Cl

Analytical applications

An automated derivatization with dabsyl chlorides combined to HPLC has been described for the determination of biogenic amines in complex matrices [5]. Derivatives are stable at room temperature and detection is performed in VIS ($\lambda = 436-460$ nm) with high specificity and sensitivity.

A HPLC method using dabsyl chloride and UV detection has been developed by Handley *et al.* for the analysis of histamine in human plasma and in the plasma and cortical tissues of the rat [6].

- [39068](#) **4-Dimethylaminoazobenzene-4'-sulfonylchloride** BioChemika for fluorescence; $\geq 98.0\%$ (AT)

References

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2.1.1.1.5 SOLID-PHASE REAGENTS WITH UV OR VIS LIGHT ABSORBING MOIETY

Until 1977-78, there were no publications on the use of immobilized reagents, known as solid-phase or polymeric reagents, for HPLC derivatization [8]. But in the last 15 years there has been a growing interest for the development and application of solid-phase reagents in analytical chemistry.

A majority of polymeric organic reagents are based on cross-linked polystyrene with small amounts of divinylbenzene (STY-DVB). Polymer chemistry has considerably improved and has made it possible to produce the polymeric support, attachment group

and different tags, which are then transferred to the investigated molecule. A number of procedures are now commercialized.

These reagents use an ionic or covalent attachment. The nature and physical parameters of these supports can be modified; the variations will affect derivatization efficiency, selectivity and reagent longevity. Further to modifying the nature of the support, it is also possible to change the reagent's attachment. These attachments can be disulfur, activated esters, carbonates or activated carbamates (Fig 8).

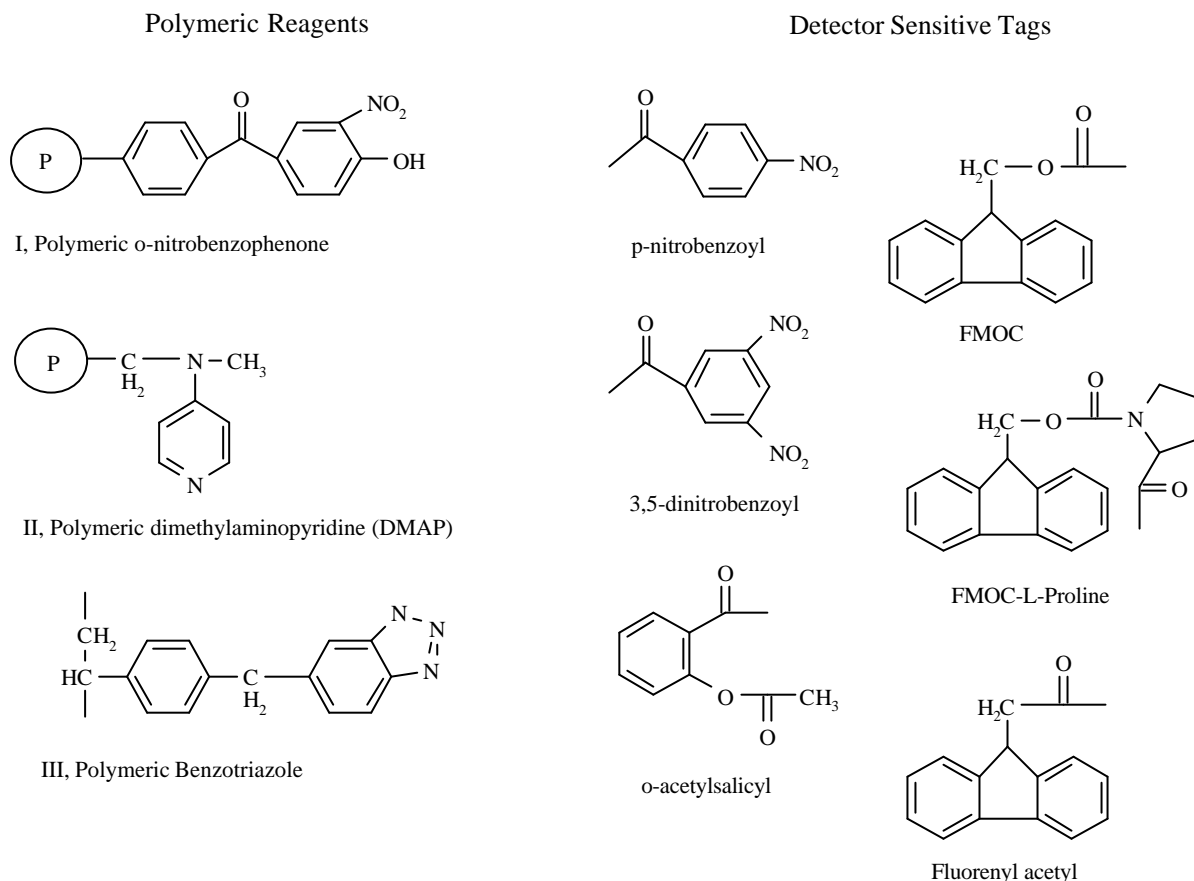


Fig. 8 Schematic illustration of the different parts of a tag reagent system that shows the base support, and the possible attachments and tags [2].

The use of solid-phase derivatizing agents, instead of a solution-phase reaction, presents certain advantages, including in some cases the ability to filter and re-use the derivatizing agents. Krull's group greatly contributed to this kind of approach [1, 2]. Previously, Chou *et al.* used a polymeric *o*-acetylsalicyl activated anhydride for improved UV detection at 196 nm, and electrochemical detection of primary and secondary amines [3]. Later, a polymeric benzotriazole *o*-acetylsalicyl [4] activated reagent was developed and used for the derivatization of primary and secondary amines, as well as polyamines such as cadaverine and putrescine.

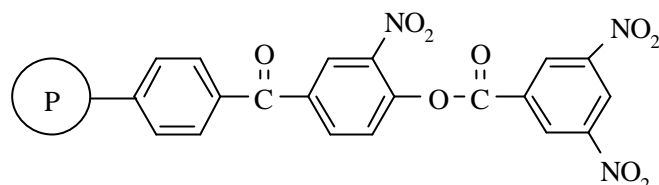
Polymeric 3,5-dinitrobenzoyl

Bourque and Krull described a solid-phase technique with 3,5-dinitrobenzoyl for improved derivatization of chiral and achiral aliphatic amines, amino alcohols and amino

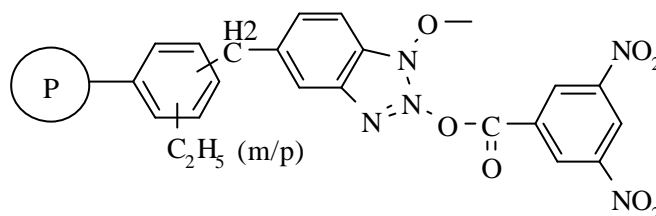
acids in HPLC with UV detection [5].

Analytical applications

Amphetamine in urine can be determined by using 3,5-dinitrobenzoyl (DNB) moiety in polymeric benzophenone-DNB and benzotriazole-DNB [6] (Fig. 9). For amphetamine derivatives, the limit of detection is 14 ng/ml and the limit of quantification 47 ng/ml.



Polymeric benzophenone-DNB



Polymeric benzotriazole-DNB

Fig. 9 Polymeric benzophenone-DNB [7]

- [39410](#) DMAP on polystyrene, 'Dimethylaminopyridine' crosslinked with 2% DVB; ~3.0 mmol "DMAP"/g resin ,

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2.1.1.2 Tertiary aliphatic amines

ACETIC ANHYDRIDE [1]

Tertiary aliphatic amines can be separated on a HPLC column and detected by post-column derivatization after reaction with a color reagent, an acetic anhydride solution of citric acid. Reaction temperature is 120°C and UV-VIS detection is performed at a wavelength of 550 nm (red wine color).

Primary and secondary amines do not react with this type of reagent. The lower limit of determination is 0.01% for aliphatic mono, di- and trialkylamines.

- [45830](#) Acetic anhydride puriss. p.a. ACS; ≥99.5% (NT)

References

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2.1.1.3 Polyamines

BENZOYL CHLORIDES [1]

Derivatization with tosyl-, dansyl or benzoyl chlorides, which derivatize di- and polyamines, affords stable reaction products. Benzoyl chloride is particularly advantageous because it implies a short derivatization procedure (contrary to tosyl chloride) and has a long elution time (advantage over dansyl chloride derivatives).

Redmond and Tseng describe a rapid and simple procedure using benzoyl chloride [2]. The sensitivity of this method is 100 pmol. Verkoelen *et al.* developed a 100 fold more sensitive method using RP-HPLC separation with isocratic elution and a UV detection at 229 nm [3]. Detection limits are about 1 pmol.

However, Mei found that the absorbance of derivated polyamines increased ca. 50 times from 254 to 198 nm in CH₃CN [4]. After optimization of the conditions, this procedure allows a detection at 198 nm without significant background noise. Limits of detection are 0.8 pmol for putrescine, 1 pmol for spermidine and 1.3 pmol for spermine.

Analytical applications

Polyamines in canned fish are detected by UV absorption, using a pre-column derivatization procedure with benzoyl chloride [5].

Sato *et al.* developed a pre-column derivatization technique for the determination of histamine in fish with a diazonium coupling reagent, followed by VIS detection at 420 nm, [6].

- [89730](#) Toluene-4-sulfonyl chloride puriss.; ≥99.0% (AT) , Tosyl chloride
- [39220](#) 5-(Dimethylamino)naphthalene-1-sulfonylchloride BioChemika for fluorescence; ≥99.0% (HPLC) , DNSCl; Dansyl chloride
- [12930](#) Benzoyl chloride puriss. p.a. ACS; ≥99.5% (T)

References

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2.1.1.4 Aromatic amines

2.1.1.4.1 DIMETHYLAMINO BENZALDEHYDE

Dimethylaminobenzaldehyde reacts by condensation with primary aromatic amines and forms a Schiff base.

Analytical applications

This reagent is used to analyze sulfonamides with a post-column derivatization monitoring with VIS at 450 nm [1].

- [39070](#) **4-(Dimethylamino)benzaldehyde** puriss. p.a. for the determination of hydroxyproline; $\geq 99.0\%$ (HPLC) , Ehrlich's Reagent sicc

References

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2.1.1.4.2 PHENYLISOCYANATE (PHI) [1]

Phenylisocyanate is an excellent derivatization agent for compounds with active hydrogen atoms. Primary and secondary aromatic amines react with PHI to form very stable N-aryl-N'-phenylureas. UV detection is measured at 255 nm. Molar coefficient is higher than $25000 \text{ mol}^{-1} \text{ cm}^{-1}$ and allows a detection inferior to 1ng. Excess PHI is eliminated with *n*-propanol.

[78750](#) **Phenyl isocyanate** puriss. p.a. for the detection of alcohols and amines; $\geq 99.0\%$ (GC)

References

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2.1.2 UV-VIS and fluorimetric detection

In the 70's and 80's, different derivatization methods were developed for UV-VIS detection in HPLC [1-5]. In the 90's, fluorimetric detection was brought forward as a more sensitive and, above all, more selective method for HPLC and capillary electrophoresis [6-7]. A number of publications present HPLC methods which use different derivatization reagents for the fluorimetric detection of pharmaceutical substances [8-11].

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2.1.2.1 Primary amines

2.1.2.1.1 O-PHTHALALDEHYDE (OPA)

OPA is well known for the derivatization of amino acids [1, 2]. This fluorogenic agent reacts rapidly, but only with primary amines in an aqueous basic medium (pH 9-11) and in presence of a mercaptan (RSH such as 2-mercaptoethanol), to form a fluorescent isoindole derivative [3].

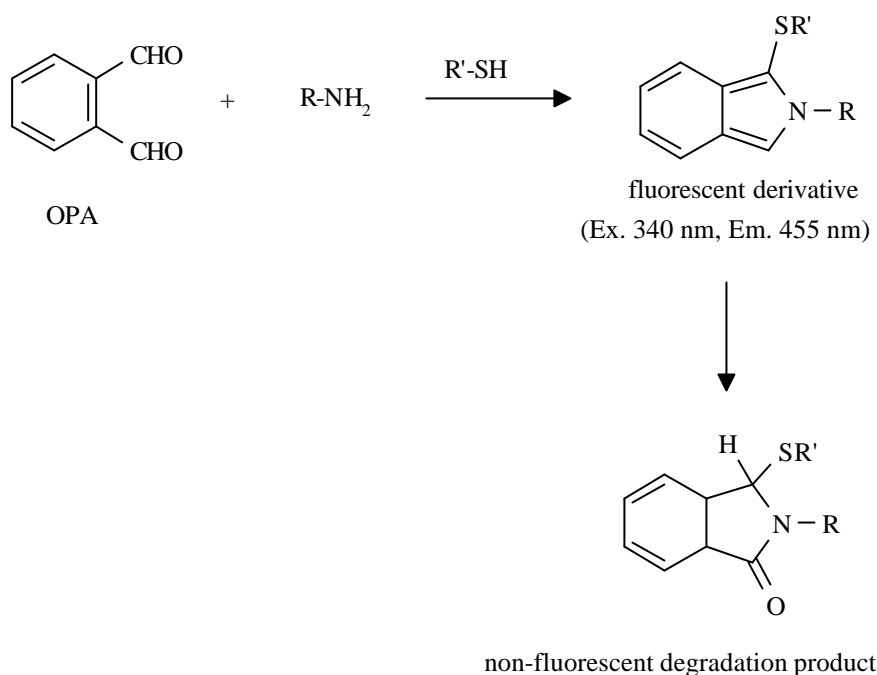


Fig. 10 Reaction of OPA with a primary amine.

The derivatization reaction is performed at room temperature in 2 minutes in a mixture of borate buffer (pH 6-8 for amines and pH 9.7-10 for amino acids) and methanol.

This reaction may be applied to pre- or post-column derivatizations. In pre-column mode, derivatization can be achieved either manually by controlling the reaction time and time interval before injection, or automatically, and allows a highly sensitive and reproducible analysis [5-8]. Moreover, OPA is also used in post-column mode because of its short reaction time and fluorogenic property (non-fluorescent) [4].

Addition of nitrioloacetic acid to the reaction mixture improves stability by a factor 4 [9].

OPA reacts with primary amines only, but addition of NaOCl allows to derivatize secondary amines. Derivatives are highly unstable and are rapidly degraded into non-fluorescent substances. Automation solves the problem of degradation. 2-Ethanolol [10], 3-mercaptopropionic acid [11, 12] or *N*-acetyl-L-cysteine [12, 14] afford more stable fluorescent derivatives than 2-mercaptoethanol.

This reagent may also be used for electrochemical detection (cf. § 2.2.2.1).

Analytical applications

This reagent is used in the pre-column derivatization of different biogenic amines in wine (histamine, methylamine, ethylamine, tyramine, tryptamine, 2-phenylethylamine, putrescine and cadaverine) [22].

Baek *et al.* combined OPA/3-mercaptopropionic acid reagent with Fmoc-Cl and *p*-nitrobenzyloxycarbonyl chloride to analyze biogenic amines in soya [23].

Vines *et al.* developed a post-column derivatization with fluorescence detection to determine ratline and its metabolites in biological fluids [24].

OPA is a derivatization agent for the determination of histamine and histidine in than [25].

Shaped *et al.* developed a reverse-phase HPLC method for the quantitative and simultaneous determination of B₁ and B₂ fumonisines [26].

- [79760 Phthaldialdehyde](#) BioChemika for fluorescence; ≥99.0% (HPLC) , OPA;

Reagents analogue to OPA

2,3-Naphtalenedialdehyde (NDA) [15-17], 3-(2-furoyl)quinoline-2-carbaldehyde (FQCA) [18], 3-benzoyl-2-quinoline-2-carbaldehyde (BQCA) [19] and 3-(4-carboxybenzoyl)-2-quinoline carbaldehyde (CBQCA) [20] are reagents analogue to OPA but the thiol compound is replaced by a cyanide ion (KCN or NaCN).

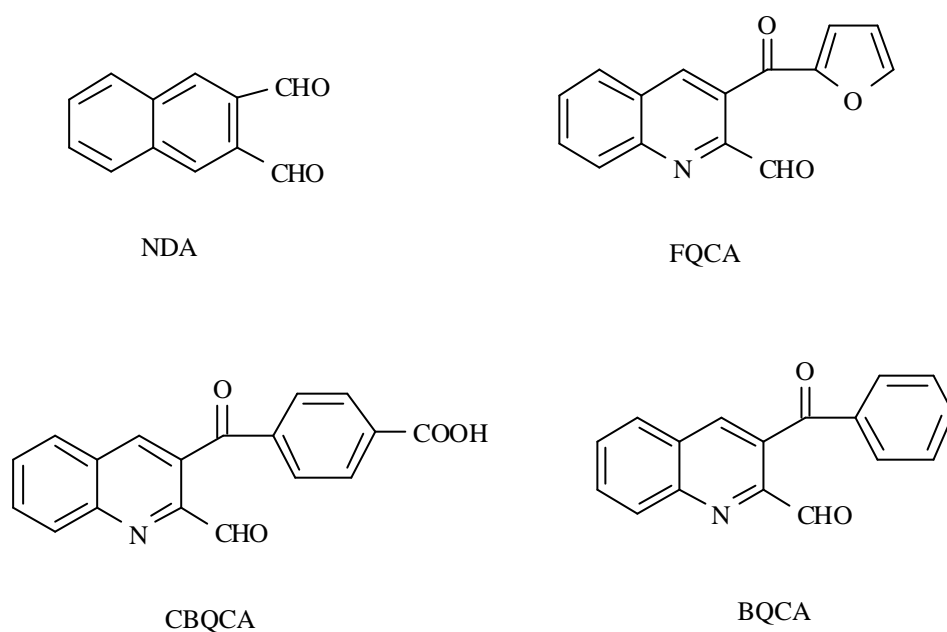


Fig. 11 Reagents analogue to OPA [21]

Reaction time (15-60 min at room temperature) is much longer than with OPA but the derivatives are more stable and are suitable for pre-column derivatization (NDA reaction, Fig. 12).

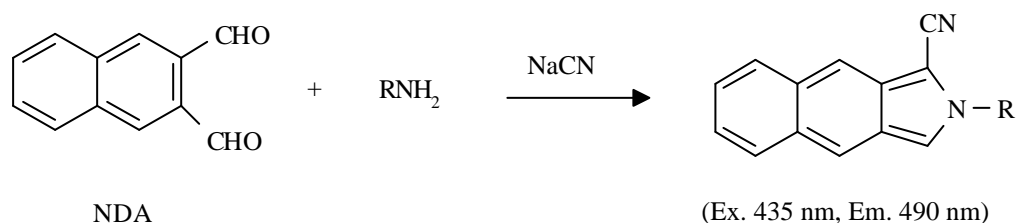


Fig.12 Reaction of NDA with a primary amine [21]

Derivatization with BQCA (Fig. 13) affords femtogram detection limits and a better

derivative stability than with OPA.

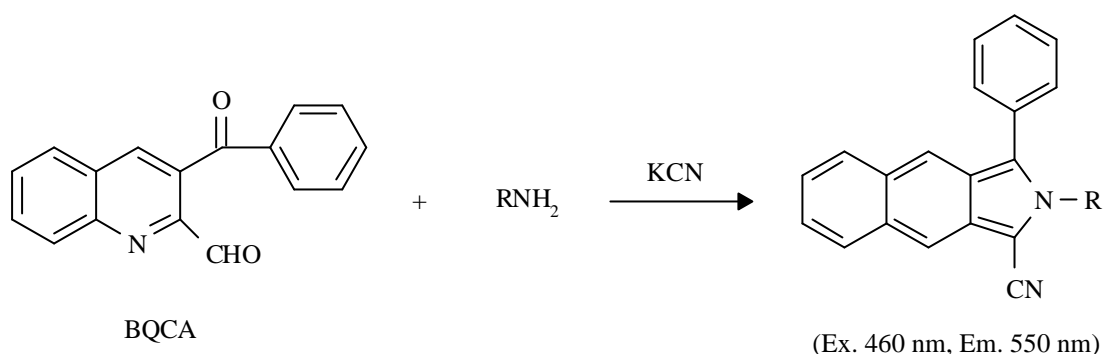


Fig. 13 Reaction of BQCA with a primary amine [21]

CBQCA allows fast chemistry at room temperature and no excess reagent problems.

Analytical applications

Frathni and Lionetti present different analyses of primary amines with NDA and cyanide ions [25].

Ware *et al.* describe a method for the determination of fumonisines in maize with NDA as derivatization agent [27].

Kilkenny *et al.* developed a procedure for the analysis of α -difluoromethylornithine (DFMO) [28] with a pre-column NDA derivatization in alkaline conditions.

70215 Naphthalene-2,3-dicarboxaldehyde BioChemika for fluorescence , NDA ,

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2.1.2.1.2 FLUORESCAMINE

This fluorogenic compound (with no inherent fluorescence propriety) reacts with primary amines and amino acids in a borate buffer (pH 9.5-10) at room temperature. The condensation reaction is complete in a few minutes [1, 2] and forms a fluorescent pyrrolidone (Fig. 14).

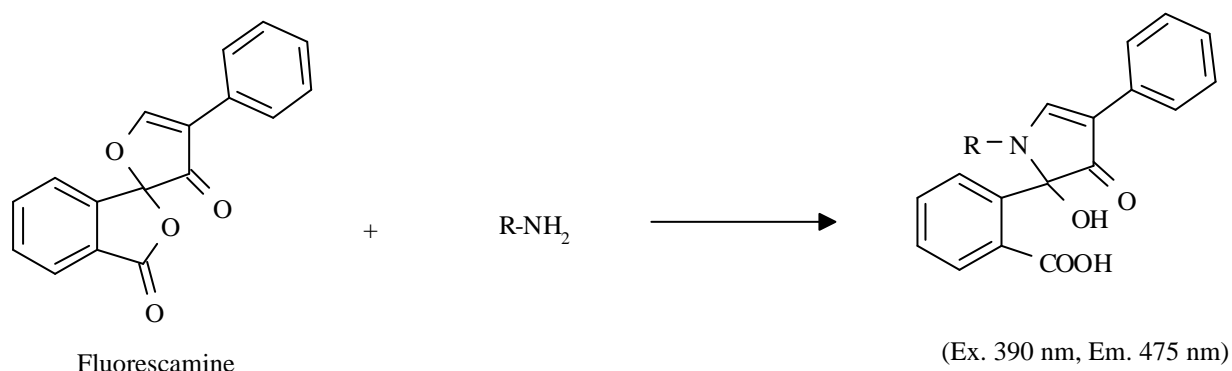


Fig. 14 Reaction of fluorescamine with a primary amine [3].

Fluorescamine offers enhanced fluorescence detection of primary amines and is useful

to determine labile substances.

Fluorescamine is quickly hydrolyzed in water; degradation products are non-fluorescent.

Fluorescent derivatives are not very stable in aqueous eluents and sensitivity is 2-5 times lower than in the OPA reaction.

This reagent is specific for compounds with a primary amine group. Therefore, in presence of secondary amines, it produces non-fluorescent derivatives.

Analytical applications

Fluorescamine is widely used to derivatize sulfonamides (pH 3, 20 min) in foods [5, 6, 9], but also to derivatize fumonisines [7].

Lanbeck-Vallen *et al.* apply a post-column derivatization with fluorescamine, and liquid phase chromatography with coupled columns, to determine ampicilline in biological fluids [8].

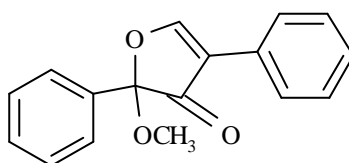
Mascher and Kikuta developed a post-column derivatization with fluorimetric detection for the determination of amoxicilline (λ_{ex} . 395 nm, λ_{em} . 485 nm) [10].

Djozan and Farajzadeh propose a selective and sensitive method for the determination of nitroaromatics in water. This procedure uses a solid-phase extraction followed by reverse-phase HPLC analysis with fluorimetric detection (395-495 nm) [11].

- [47614 Floram](#)[®] BioChemika for fluorescence; $\geq 99.0\%$ (UV) , Fluorescamine

Reagent analogue to fluorescamine

2-Methoxy-2,4-diphenyl-3[2H]-furanone (MDF) (Fig. 15) reacts with primary amines in the same way as fluorescamine does and, although the reagent has a long reaction time (about 30 min. at room temperature), derivatives are stable.



MDF

Fig. 15 Structure of MDF [3]

- [64958 2-Methoxy-2,4-diphenyl-3\(2H\)-furanone](#) BioChemika for fluorescence; $\geq 98.0\%$ (HPLC) , MDPF

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2.1.2.2 Primary and secondary amines

Fluorescence labeling agents (Fig. 16) with a reactive group, such as (A) sulfonyl chloride, (B) carbonyl chloride and fluoride, (C) isothiocyanate, and (D) succinimidyl moiety have been developed for HPLC or CE determination of primary and secondary amines. Reagents react with the amines to give corresponding fluorescent derivatives.

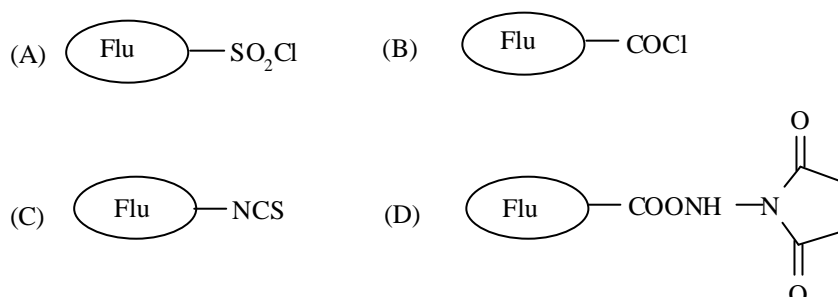
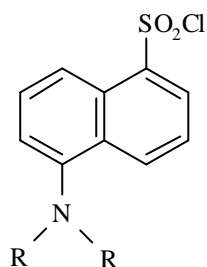


Fig. 16 Fluorescent labeling agents for amines [1]

2.1.2.2.1 SULFONYL CHLORIDES

In the same way as 1-dimethylaminonaphtalene-5-sulfonyl (DNS-Cl), sulfonyl chlorides (Fig. 17) react with primary and secondary amines in weakly alkaline conditions to form fluorescent sulfamides (Fig. 18) with emission wavelengths between 470 and 510 nm.



- a, R = CH₃
- b, R = C₂H₅
- c, R = *n*-C₃H₇
- d, R = *n*-C₄H₉
- e, R = *n*-C₅H₁₁

Fig. 17 Sulfonyl chlorides

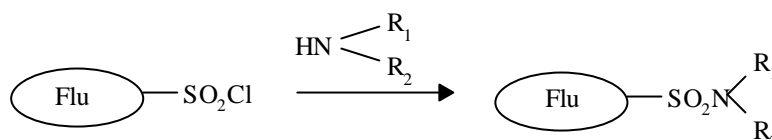


Fig. 18 Reaction of sulfonyl chlorides with amines [1]

2.1.2.2.1.1 1-Dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl or dansyl chloride)

Dansyl chloride is usually used when primary and secondary amines are derivatized with fluorescence detection [2]. UV detection of dansylated compounds is not a common procedure except for amino acids [3, 4].

DNS-Cl reacts with primary and secondary amines by nucleophilic substitution and forms a fluorescent dansyl derivative.

The optimal reaction time (30-120 minutes) depends on the amino compounds [5, 6]. DNS derivatives are stable. The reagent is hydrolyzed during derivatization in order to produce 1-dimethylaminonaphthalene-5-sulfonic acid, a highly fluorescent compound widely employed in pre-column derivatization. As DNS-Cl acts on the phenol hydroxyl, amino compounds which contain this group, such as catecholamine, tyramine and tyrosine, produce multiple fluorescent derivatives [7].

DNS-Cl also acts on primary and secondary alcohols in aprotic solvents such as benzene and chloroform to form the corresponding fluorescent ethers.

Analytical applications

DNS-Cl allows to determine biogenic amines such as putrescine, cadaverine, spermidine and spermine [12, 13, 14, 15]; derivatization takes place at 70°C in 10 min.

- [39220](#) 5-(Dimethylamino)naphthalene-1-sulfonylchloride BioChemika for fluorescence; ≥99.0% (HPLC) , DNSCl; Dansyl chloride

2.1.2.2.1.2 Reagents analogue to sulfonyl chlorides

In the same way as DNS-Cl, dabsyl-Cl (1-di-*n*-butylaminonaphthalene-5-sulfonyl chloride)

[8], mansyl-Cl (2-methylanilino-naphthalene-6-sulfonyl chloride) [9], phisyl-Cl (4-(N-phtalimidyl)-benzene-sulfonyl chloride) [10] and BHBT-SOC1 (2-(5',6'-dimethoxybenzothiazolyl)-benzene sulfonyl) [11] react with primary and secondary amines (Fig. 19). Derivatization of amines with BHBT-SOC1 allows fluorimetric detection (λ_{em} : 450 nm and λ_{ex} : 330 nm). Limits of detection for primary and secondary amines are around 3 and 300 fmol, respectively.

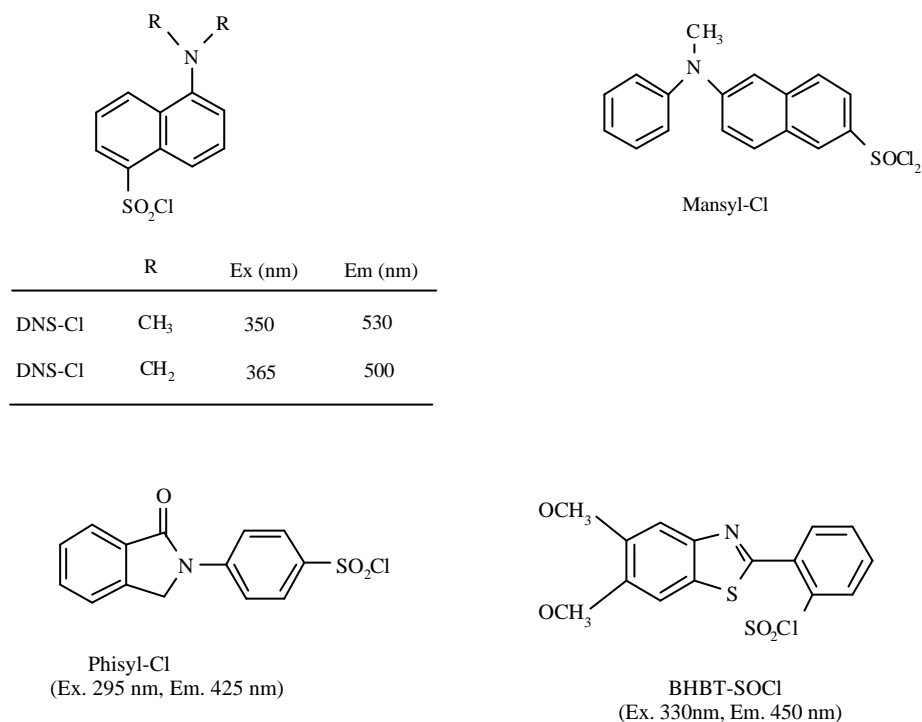


Fig. 19 Sulfonyl chlorides [1]

- [D6303](#) (1,3-Dihydro-1-oxo-2H-isoindol-2-yl)-4-benzenesulfonyl chloride Phisyl chloride

References

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2.1.2.2.2 CARBONYL CHLORIDES AND FLUORIDES

Derivatization with these reagents (Fig. 20) transforms amines into amides.

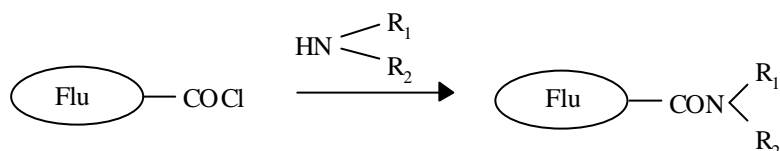


Fig. 20 Reaction of carbonyl chlorides with amines [1].

Carbonyl chlorides react with primary, secondary and tertiary amines, and with alcohols. The reactivity of carbonyl chlorides is higher than that of sulfonyl chlorides.

2.1.2.2.2.1 9-Fluorenylmethyl chloroformate (Fmoc-Cl)

This reagent is used in the pre-column derivatization of primary and secondary amines [2] (Fig. 21). It reacts by nucleophilic substitution in a borate buffer (pH 8) in 2 min and gives stable and fluorescent derivatives. Excess reagent is necessary but can be eliminated through reaction with an amino acid. This reagent allows fluorimetric (λ Ex. 265 nm et λ Em. 315 nm) and UV-VIS detection of amines.

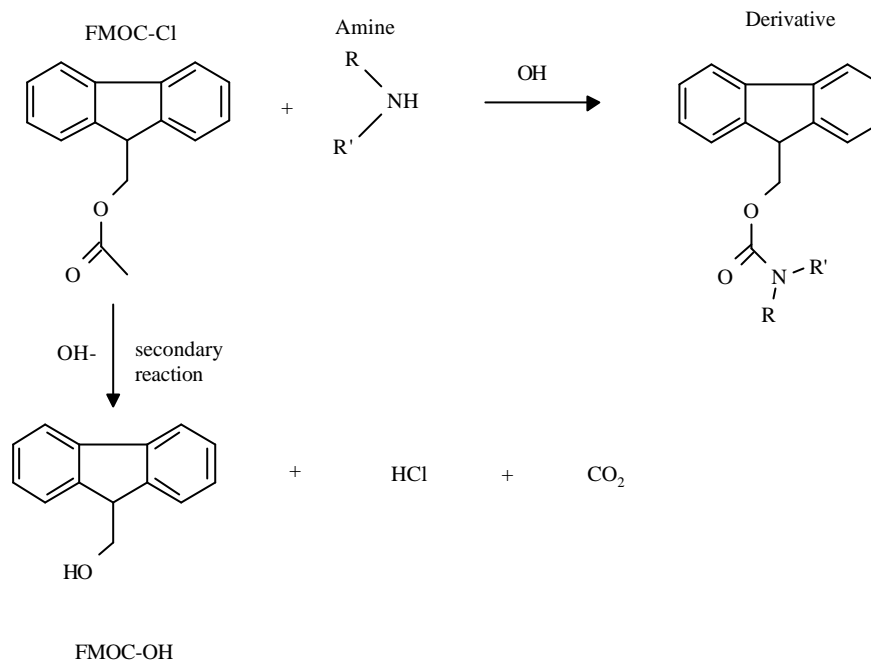


Fig 21 Reaction of Fmoc-Cl with amines [1].

Fluorescence constitutes the major drawback of Fmoc-OH but it can be overcome by extracting the derivative with an organic solvent, such as pentane. As with DNS-C1, Fmoc-C1 is also reactive towards phenolic hydroxyl moieties. Further, the reagent combines with the imidazole ring of amino compounds. In this reaction, tyrosine and histidine form corresponding mono- and di-substituted derivatives.

Despite its disadvantages, this reaction has become popular after simplification [3, 4] and automation [3, 5] of the derivatization procedure. Sensitivity is comparable to that of the OPA method. The limitation of the reaction between OPA and primary amines has led to developing a mixed system of OPA and Fmoc-Cl [6, 7].

Analytical applications

Fmoc-C1 is used in different analyses as a derivatization reagent to determine biogenic amines [16, 17]. This reagent is also applied to the derivatization of aminoglycosides, such as gentamycin [18] and neomycin [18].

Maeder developed a pre-column on-line derivatization which uses Fmoc-C1 for the determination of amphetamines with UV detection [21].

- [23186](#) Fmoc-Cl, 9-Fluorenylmethyl chloroformate puriss. p.a. derivatization reagent for HPLC; $\geq 99.0\%$ (HPLC)

2.1.2.2.2 Reagents analogue to Fmoc-Cl

The following reagents (Fig. 22) are analogue to Fmoc-C1: 2-naphthyl chloroformate (NT-COCl) [8], 2-(1-pyrenyl)ethyl chloroformate (PE-COCl) [9], 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl chloride (DMEQ-COCl) [10], 6-methoxy-2-

methylsulfonylquinoline-4-carbonyl chloride (MMSQ-COCl) [11], 7-dimethylaminocoumarin-3-carbonyl fluoride (DAM-F) [12] and 4-(5',6'-dimethoxybenzothiazolyl) benzoyl fluoride (BHBT-COF) [2].

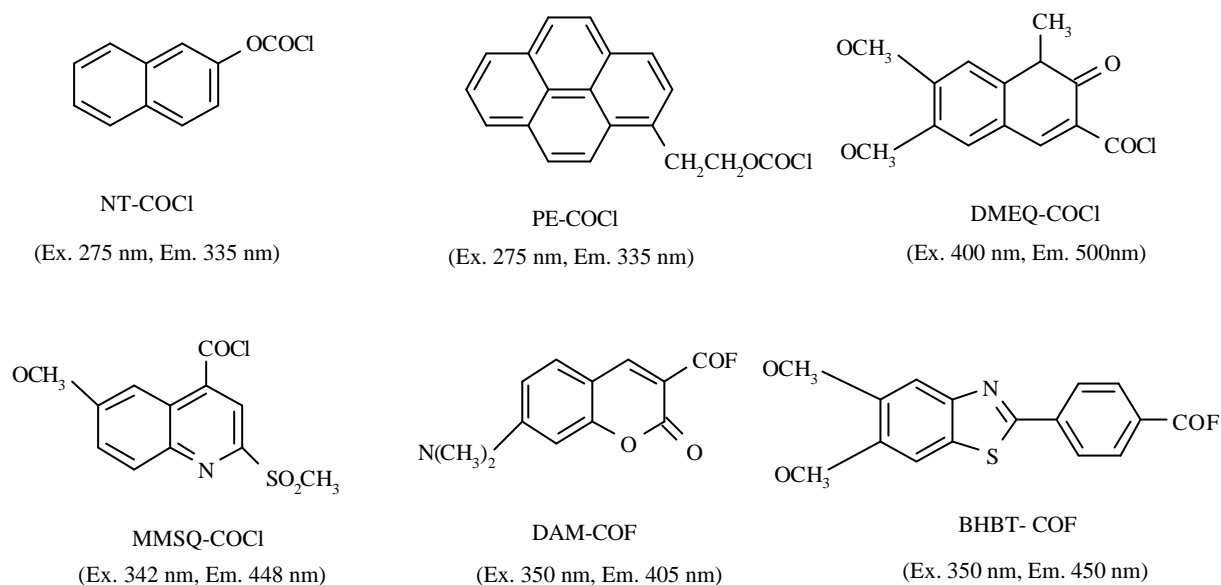


Fig. 22 Structure of carbonyl chlorides and fluorides [1]

In general, they react in an aprotic solvent, such as benzene or acetonitrile, and reactions are complete within 5 min. at room temperature, except for NT-COCl (100°C, 1 h). Detection limits (S/N = 3) of DMEQ-COCl and BHBT-COF are 2-3 fmol on column. The fluorimetric detection of amines is monitored with excitation at 350 nm and emission at 450 nm. These reagents are widely used in the pre-column derivatization of hydrophobic amines.

Analytical applications

DMEQ-COCl has been applied to determine β -phenylethylamine, a neuromodulator of the central nervous system in human plasma [13], 1,2,3,4-tetrahydroisiquinoline in rat brain [14], and amantadine, a brain metabolic stimulant in human plasma [15].

Although not mentioned above, two new chlorides, 2-naphthylloxycarbonyl chloride and *p*-nitrobenzylloxycarbonyl chloride, are used to derivatize biogenic amines [20].

References

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2.1.2.2.3 1,2-NAPHTHOQUINONE-4-SULFONATE (NQS)

NQS reacts with primary and secondary amines, by a Michael addition, in relatively mild conditions. The derivative can be detected by UV-VIS and by fluorimetry (Ex. 260 nm, Em. 435 nm). This reagent is soluble in water.

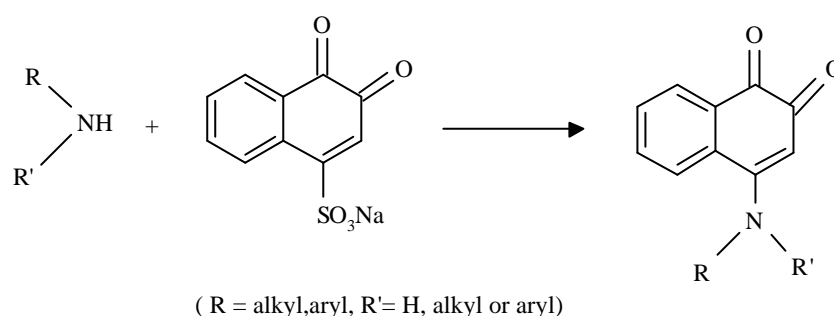


Fig. 23 Reaction of NQS with amines [1]

Based on the reaction with NQS, a flow injection analysis method (FIA) coupled to spectrophotometric detection [2, 3] was developed for the determination of amines. The instability of this reagent in alkaline medium (pH 10) can be overcome by the flow injection technique.

Analytical applications

NQS is a derivatization agent for the determination of aminoglycosides. It reacts with the guanidino moieties of certain aminoglycosides and forms fluorescent derivatives. Several research groups [4-6, 8] have quantified streptomycin in foods by using post-

column derivatization with fluorescence detection (λ_{ex} : 347 nm, λ_{em} : 418 nm). However, aminoglycosides, which only contain primary and secondary amines, do not form fluorescent derivatives with NQS.

NQS is also used to determine amphetamine and metamphetamine. Campins-Falco *et al.* have analyzed these substances in urine by reverse-phase HPLC with simultaneous purification of the samples. Derivatization with NQS takes place on solid-phase [7].

- [70382](#) **3,4-Dihydro-3,4-dioxo-1-naphthalenesulfonic acid Sodium salt** purum p.a. for spectrophotometric det. of isonicotic hydrazide, amines and amino acids; $\geq 97.0\%$ (T) , Folin's Reagent;

References

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- [2] Saurina, J. and Hernández-Cassou, S. *Anal. Chim. Acta.* **1993**, 283, 414.
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2.1.2.2.4 ISOCYANATES ET ISOTHIOCYANATES

2.1.2.2.4.1 Isocyanates

Phenyl isocyanate (PIC) reacts with primary and secondary amines and forms *N, N'*-disubstituted ureas but also reacts with alcohols, water, phenols and carboxylic acids [2].

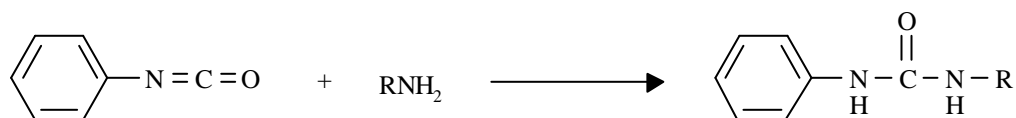


Fig. 24 Reaction of PIC with primary amines [1]

This reaction requires excess reagent and, with monoamines, is complete in a few seconds at room temperature. *N*-alkyl-*N'*-phenylureas are detected at 240 nm. The molar coefficient is $20'000 \text{ mol}^{-1}\text{cm}^{-1}$, which allows a limit of detection between 1 and 5 ng.

As isocyanates have a fast reaction with water and alcohols and form urethanes, they are usually replaced by isothiocyanates, which are less reactive.

NIC (isocyanate 1-naphtyl) can be used as a pre-column derivatization reagent for amino acids [3].

- [78750 Phenyl isocyanate, PIC](#), puriss. p.a. for the detection of alcohols and amines; $\geq 99.0\%$ (GC)
- [170518 1-Naphthyl isocyanate, NIC, 98%](#)

References

- [1] Toshimasa Toyo'oka (édité) *Modern Derivatization Methods for Separation Sciences*, **1999**, John Wiley and sons, New York
- [2] Bjorkqvist, B. *J. Chromatogr.* **1981**, 204, 109.
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2.1.2.2.4.2 Phenyl isothiocyanate (PITC, Edman reagent)

In 1949, Edman introduced PITC for amino acid [2] and peptide sequencing [3]. PITC is also used to derivatize a wide range of primary and secondary amines (Fig. 25). Cyclization and rearrangement of derivatives is usually achieved in acidic conditions and produces 3-phenyl-2-thiohydantoin (PTH) derivatives.

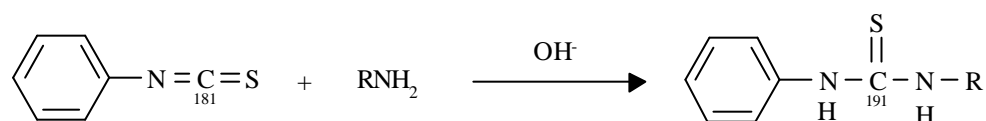


Fig. 25 Reaction of PITC with primary amines [1]

Analytical applications

Supercritical fluid chromatography with UV detection (SFC-UV), and high performance liquid chromatography with diode array UV-VIS detection (HPLC-DAD), were compared for the analysis of amphetamines derivatized with PITC [4].

- [78779 Phenyl isothiocyanate](#) BioChemika for protein sequence analysis; ampoule; $\geq 99.5\%$ (GC) , PITC
- [78781 Phenyl isothiocyanate](#) BioChemika for protein sequence analysis; $\geq 99.5\%$ (GC) , PITC

References

- [1] Toshimasa Toyo'oka (édité) *Modern Derivatization Methods for Separation Sciences*, **1999**, John Wiley and sons, New York
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[4] McAvoy, Y. ; Cole, M. and Gueniat, O. *Forensic Sci. Int.* **1999**, 102 (1), 13.

2.1.2.2.5 SUCCINIMIDYLS

N-succinimidyl-1-naphtylcarbamate (SINC) (Fig. 26) reacts with amino acids in a borate buffer 0.5 M (pH 9.5) in 1 min. at room temperature and forms naphtylcarbamoyl derivatives [1].

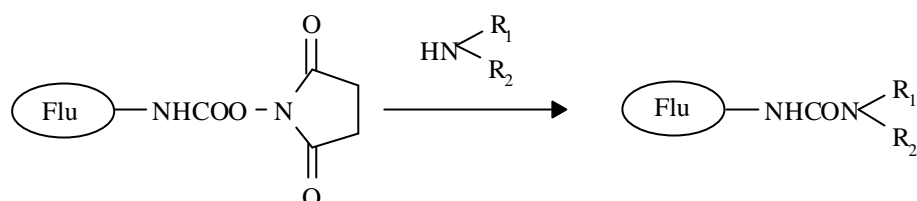


Fig. 26 Reaction of succinimidylys with amines [2]

Fast reaction and elimination of excess reagent by hydrolysis makes SINC very appropriate for pre-column automated derivatization [3]. Very intense fluorescence allows determination of subpicomole quantities.

N-succinimidyl-1-fluorenylcarbamate (SIPC) [4] and 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC) [5] have also been developed (Fig. 27).

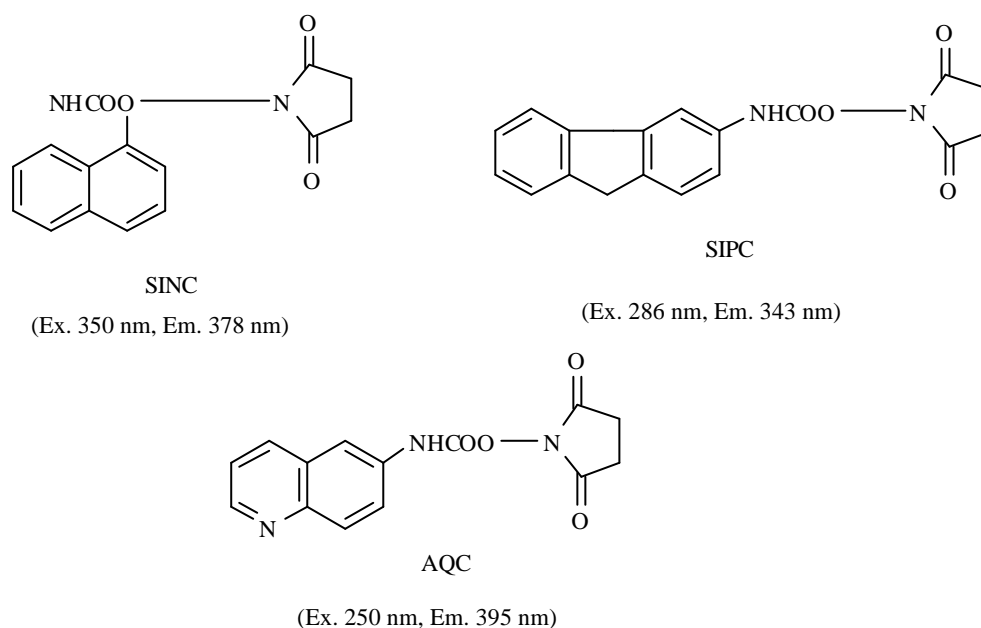


Fig. 27 Structure of succinimidyl reagents [2]

Analytical applications

Velazquez *et al.* use AQC as a reagent for the derivatization of fumonisines [6].

References

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2.1.2.2.6 BENZOFURAZANS

Halogenobenzofurazan reagents, such as NBD-C1 (4-chloro-7-nitro-2,1,3-benzoxadiazole) and NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) [1-3], react with both primary and secondary amines at 50-60°C under alkaline conditions (pH 8-9) (Fig. 28). The reaction with NBD-F is 10 times faster than with NBD-C1 and is complete in 1 min. As the hydrolyzed product, NBD-OH, is intensely fluorescent, both reagents are used for post-column derivatization.

DBD-F is a fluorescent reagent for amines with a higher sensitivity than NBD-F but a lower reactivity [4].

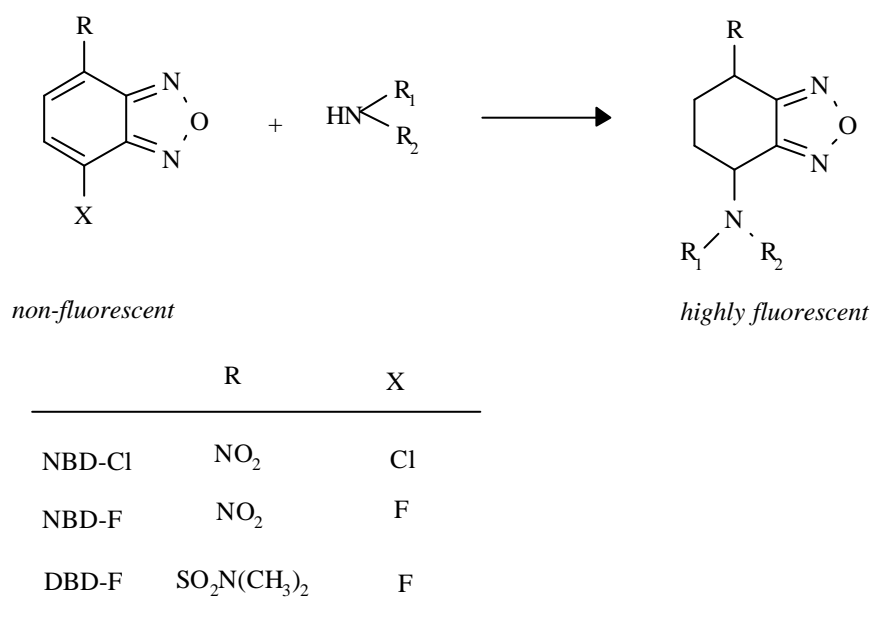


Fig. 28 Benzofurazans [6]

DBD-COCl (4-(*N*-chloroformylmethyl-*N*-methyl)-amino-7-*N,N*-dimethylaminosulfonyl benzofurazan) has been synthesized to act as an electrophilic reagent for primary and aromatic amines [5]. The reaction takes place in a benzene solution at room temperature and gives fluorescent products (λ_{ex} about 440 nm, λ_{em} about 550 nm). This compound also reacts with alcohols, thiols and phenols.

Benzofurazan derivatives, with an excitation wavelength close to 480 nm, can be determined in HPLC with Ar ion fluorimetric laser detection (LIF).

Analytical applications

NBD-F [7] and DBD-F [8] are used to derivatize fumonisines.

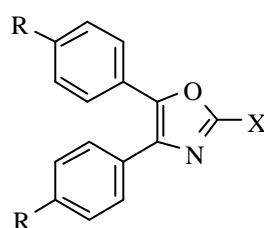
- [47140](#) **4-Fluoro-7-nitrobenzofurazan** BioChemika for fluorescence; $\geq 99.0\%$ (TLC), NBD-F
- [25455](#) **4-Chloro-7-nitrobenzofurazan** BioChemika for fluorescence; $\geq 99.0\%$ (TLC), NBD-chloride;

References

- [1] Watanabe, Y. and Imai, K. *Anal. Chim. Acta* **1981**, 130, 377.
- [2] Watanabe, Y. and Imai, K. *Anal. Chim. Acta* **1981**, 116, 471.
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2.1.2.2.7 OXAZOLES

Oxazole reagents, DIFOX and SAOX-Cl (Fig. 29), have been used to derivatize amines, thiols and alcohols.



	R	X	Ex (nm)	Em (nm)
DIFOX	H	F	320	420
SAOX-Cl	SO ₂ N(CH ₃) ₂	Cl	361	475

Fig. 29 Oxazoles [1]

References

- [1] Toshimasa Toyooka (édité) *Modern Derivatization Methods for Separation Sciences*, 1999, John Wiley and sons, New York.

2.1.2.2.8 SOLID PHASE REAGENTS

Compared to liquid-phase derivatization, solid-phase derivatization has become increasingly attractive due to some unique features such as good selectivity and simple procedure. Polymeric reagents have been proposed for solid-phase derivatization (Fig. 30) [1-3].

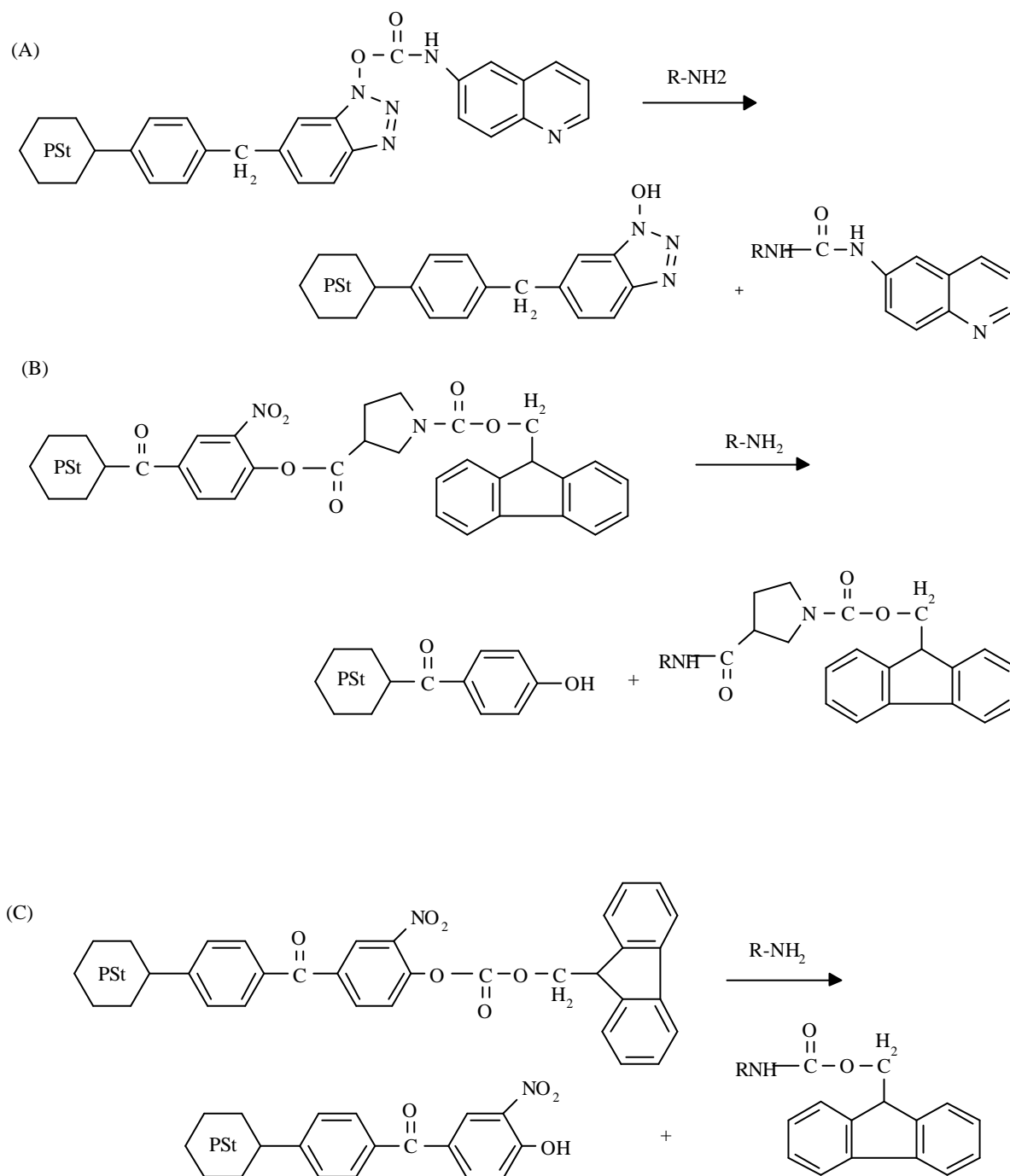


Fig. 30 Polymeric reagents for amines [4]

2.1.2.2.8.1 Polymeric 6-aminoquinoline (6-AQ) tagged to a derivatization reagent

An activated carbamate, 6-AQ, was developed and applied to the on-line derivatization of amines in HPLC. Derivatized amines are separated by conventional RP-HPLC with UV and fluorimetric detection (Fig. 31).

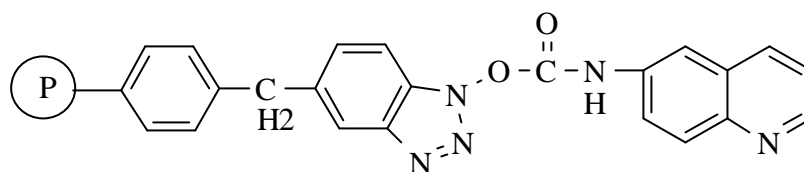


Fig. 31 Polymeric benzotriazole-6-AQ [4]

2.1.2.2.8.2 Polymeric benzotriazole activated reagent containing Fmoc group

A polymeric benzotriazole containing a Fmoc group was developed for UV and fluorimetric detection (Fig. 32).

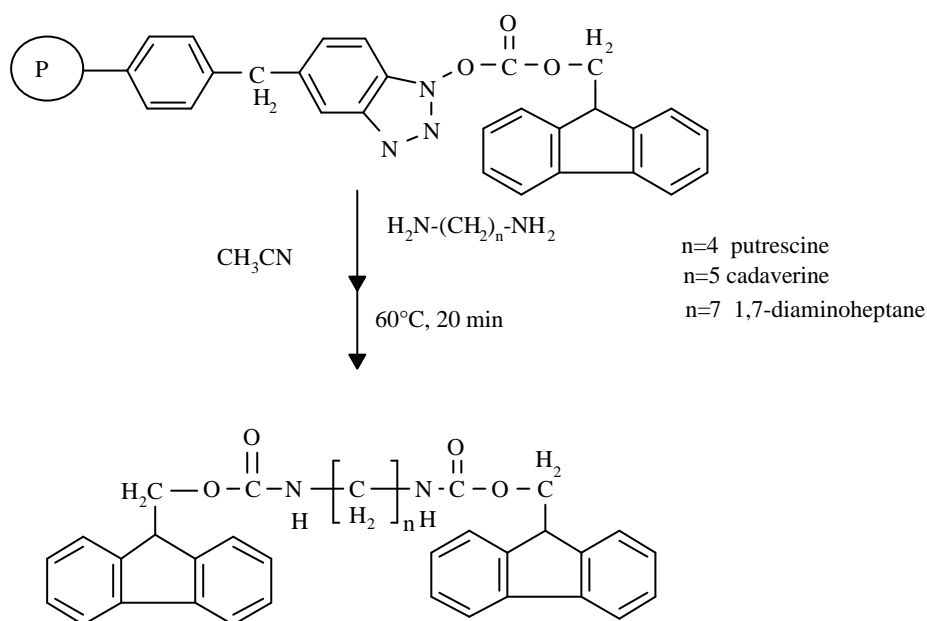


Fig. 32 Derivatization of polyamines with polymeric benzotriazole-Fmoc reagent [4]

This reagent is sensitive to moisture and high temperatures. This is why Gao *et al* developed an improved polymeric reagent, the fluorenyl-attached polymeric *o*-nitrobenzophenone (Fig. 33) [6].

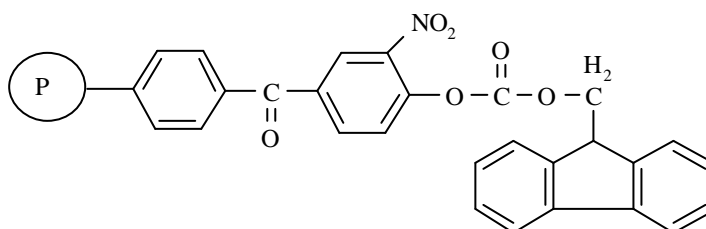


Fig. 33 Polymeric 3-nitro-4-[[9-fluorenylmethoxy-carbonyl]oxy]benzophenone [4].

Analytical applications

This reagent allows to determine cadaverine and putrescine, which are polyamines found in human urine [5].

References

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2.1.2.2.9 IONS AL³⁺

Aluminum chloride reacts with amines to form a highly fluorescent complex.

Analytical applications

Tetracyclines are derivatized with Al³⁺ ions at 60 °C to form a highly fluorescent derivative (λ_{ex} . 390 nm, λ_{em} . 490 nm). Limits of detection are 20-230 mg/g, depending on the tissue in which they are quantified [1].

- [06220](#) Aluminum chloride anhydrous puriss. p.a.; crystallized; $\geq 99.0\%$ (AT)

References

- [1] McCracken, R.J. ; Blanchflower, W.J. ; Haggan, S.A. and Kennedy, D.G. *Analyst* **1995**, 120, 1763.

2.1.2.2.10 POTASSIUM FERRICYANIDE (K₃FE(CN)₆)

Ferricyanide is normally used to determine thiamine, which forms a fluorescent thiochrome after oxidation.

Analytical applications

A post-column derivatization method has been proposed to determine amprolium in muscle tissues (chicken) [1, 2].

- [60299](#) Potassium hexacyanoferrate(III) BioChemika MicroSelect; $\geq 99.0\%$ (RT) , Potassium ferricyanide
- [60300](#) Potassium hexacyanoferrate(III) puriss. p.a. ACS; $\geq 99.0\%$ (RT) , Potassium ferricyanide

References

- [1] Van Leeuwen, W. and van Gend, H.W. *Z.Lebensm. Unters. Forsch.* **1998**, 186, 500.
[2] Nagata, T. and Saeki, M. *J. AOAC*, **1996**, 69 (6), 941.

2.1.2.3 Particular amines

2.1.2.3.1 CATECHOLAMINES

Catecholamines are found in extremely small amounts in biological samples in which many precursors and metabolites co-exist. A highly selective and sensitive reagent is required for their determination. However, traditional reagents for amines are not sufficiently selective. Methods using trihydroxyindole (THI) [2, 3] and ethylenediamine (ED) [4, 5] afford highly selective reactions (Fig. 34). The THI method is the most selective but does not allow fluorescence detection with dopamine. Furthermore, these methods have a limited sensitivity. Condensation reactions between catecholamines and ED can only be applied to post-column HPLC analysis of urine catecholamines.

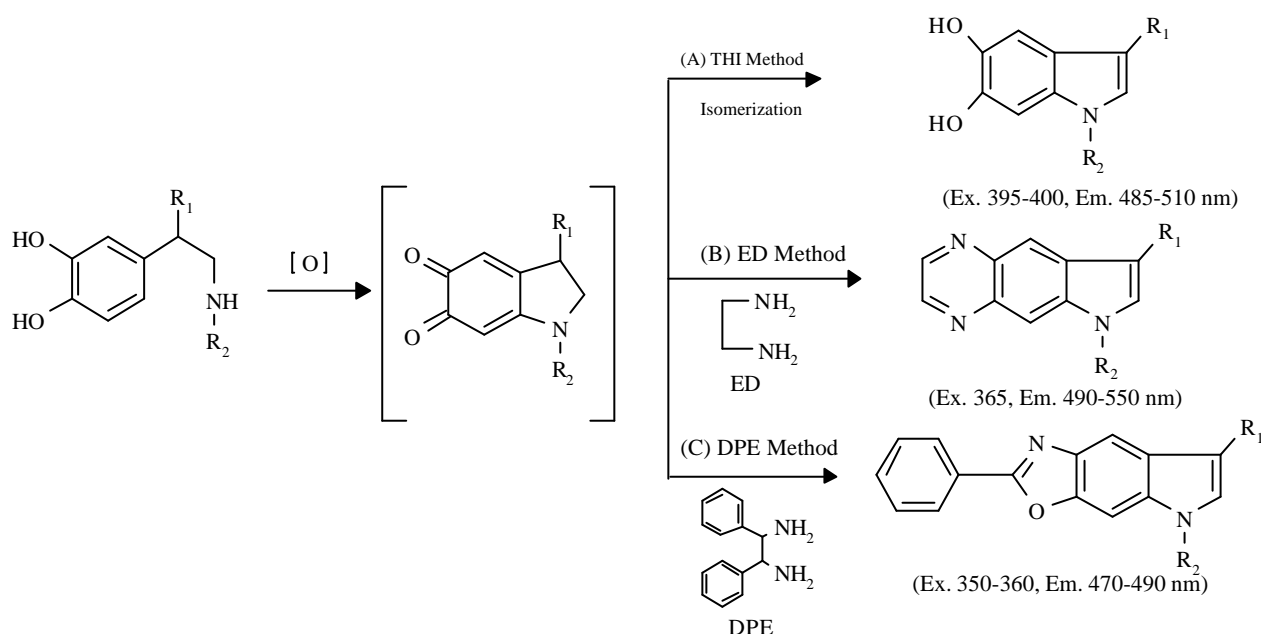


Fig. 34 Fluorogenic reactions of catecholamines by (A) THI, (B) ED and (C) DPE method [1].

1,2-Diphenylethylenediamine (DPE) [6] has been reported as a highly selective and sensitive reagent for catecholamines. DPE reacts selectively towards catecholamines under mild conditions (pH 6.5-6.8, 37-50°C) in the presence of potassium hexacyanoferrate (III), to yield a single fluorescent derivative from each catecholamine; this method is therefore applicable to both pre- and post-column derivatization. The DPE method is very sensitive and selective and requires only a simple clean-up of plasma by solid-phase extraction, using a cation-exchange cartridge. Clean-up by liquid-liquid extraction is also effective [7, 8]. Automated pre-column derivatization has

been proposed by Kamahori *et al.* [9].

- [03550](#) Ethylenediamine puriss. p.a.; absolute; ≥99.5% (GC) , 1,2-Diaminoethane
- [42747](#) N,N'-Diphenylethylenediamine purum; ≥97.0% (NT)

References

- [1] Toshimasa Toyo'oka (édité) *Modern Derivatization Methods for Separation Sciences*, **1999**, John Wiley and sons, New York.
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2.1.2.3.2 INDOLAMINES

Indolamines react with formaldehyde [1], chloroacetaldehyde [1] and methoxyaldehyde [2] to form fluorescent derivatives. Reaction conditions are fairly drastic (acidic medium, 80-100°C, 15-60 min) in presence of an oxidizing agent. These reagents are used in pre-column derivatization.

References

- [1] Iiszuka, H. and Yajima, T. *Chem. Pharm. Bull.* **1981**, 33, 2591.
- [2] Iiszuka, H. and Yajima, T. *Chem. Pharm. Bull.* **1983**, 16, 103.

2.1.2.3.3 5-HYDROXYINDOLES (SEROTONINE RELATED COMPOUNDS)

5-Hydroxyindoles are tryptophane metabolites which play an important physiological role in the human body. In fact, determining amines in biological samples is useful to elucidate the metabolism of tryptophan and diagnose a number of mental disorders such as schizophrenia and migraine.

Benzylamine reacts with 5-hydroxyindoles such as serotonin and 5-hydroxyindol-3-ylacetic acid, under fairly mild conditions (pH 9.0, 37°C, 20 min.) (Fig. 35 A), and in the presence of potassium hexacyanoferrate (III), to give a highly fluorescent oxazole compound [2]. This reaction can be applied to pre- and post-column derivatization in HPLC [3].

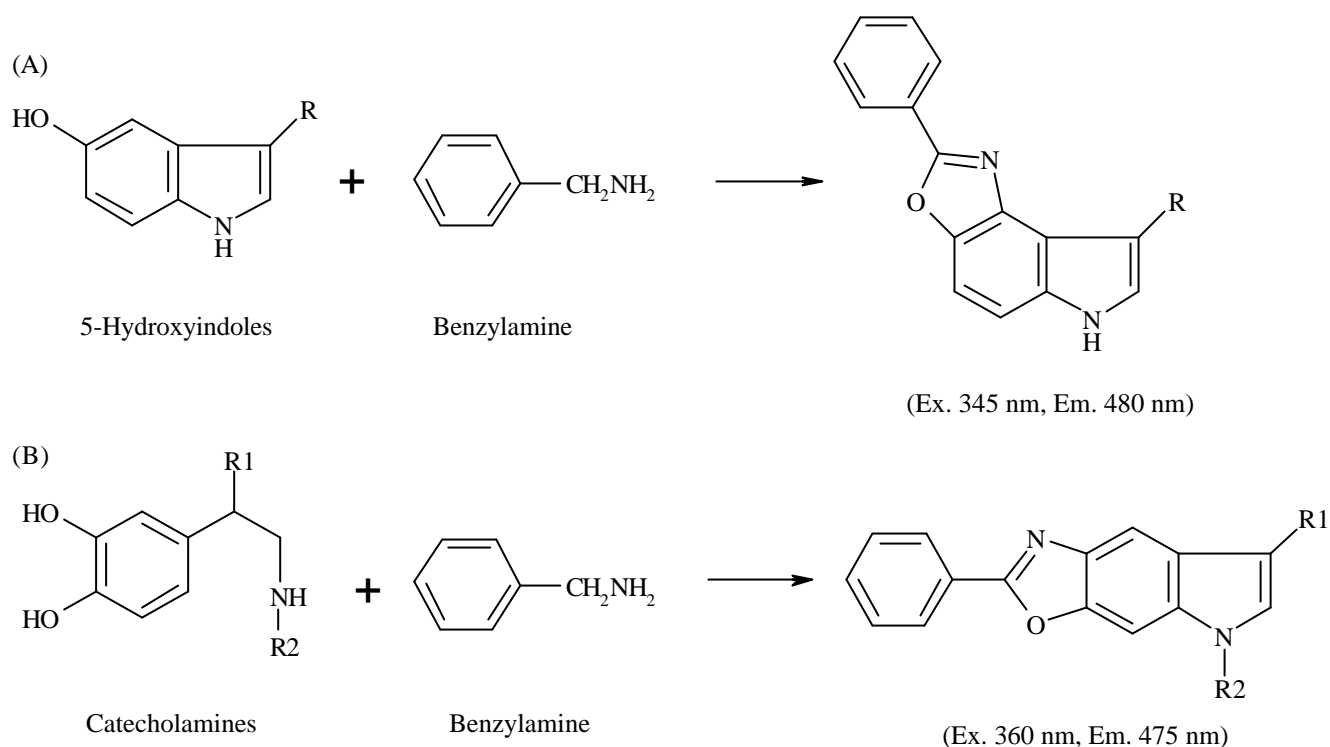


Fig. 35 Fluorogenic reactions of: (A) 5-hydroxyindoles and (B) catecholamines with benzylamine [1].

Analytical applications

5-Hydroxyindoles in urine, plasma and rat brain [4] as well as tryptophane hydroxylase [5] are quantified by post-column derivatization. Pre-column derivatization determines indoles in the microdialysates of rat brain [6].

Benzylamine also reacts with catecholamines but under slightly different conditions to those of indoles [7] (fig. 35 B). Thus, a pre-column HPLC derivatization method was developed for their quantification in urine [8].

- [13180](#) Benzylamine puriss. p.a. derivatization reagent for GC; $\geq 99.0\%$ (GC) ,

References

- [1] Toshimasa Toyo'oka (édité) *Modern Derivatization Methods for Separation Sciences*, **1999**, John Wiley and sons, New York.
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- [8] Yamaguchi, M. ; Ishida, J. and Yoshimura, M. *Analyst* **1998**, 123, 307.

2.1.2.3.4 GUANIDINO COMPOUNDS

Guanidino compounds can be converted selectively into fluorescent derivatives by reaction with 9,10-phenanthraquinone (PQ) [2] or benzoin [3, 4] (Fig. 36). They also react with ninhydrin. Although the structure of this product still remains unprecise, the reaction with compounds containing a guanidino group is highly specific.

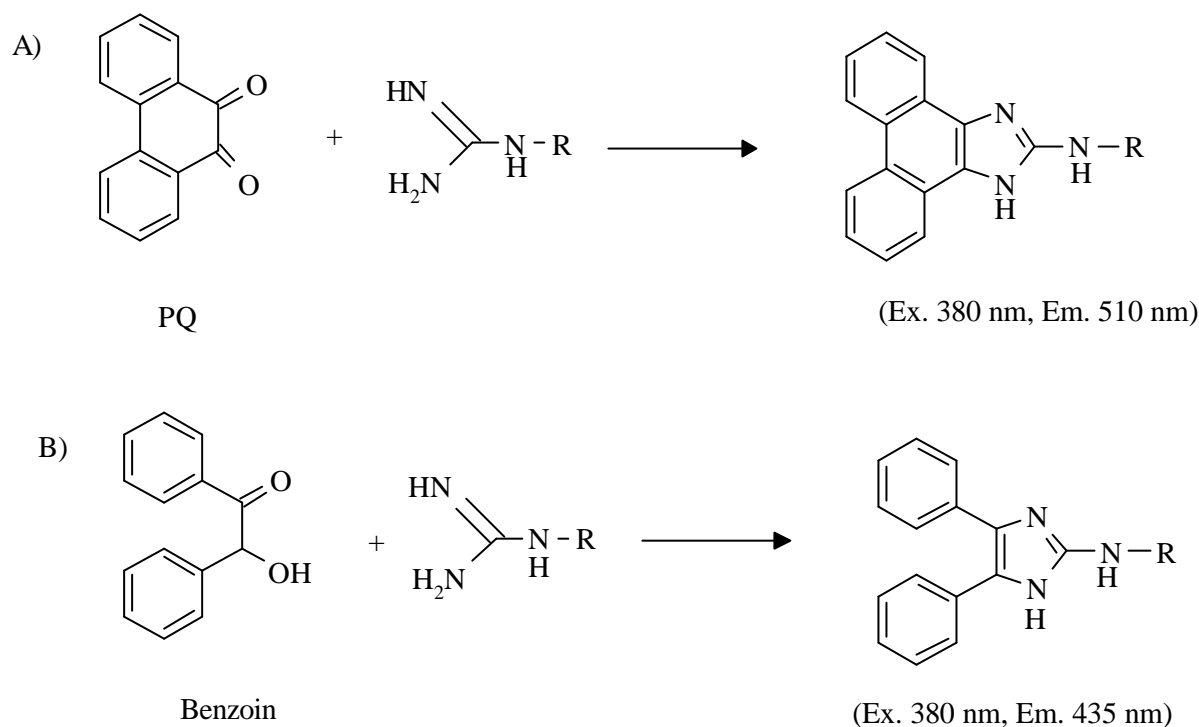


Fig. 36 Fluorogenic reactions of guanidino compounds with (A) PQ and (B) benzoin [1].

PQ reacts with guanidino compounds under alkaline conditions, and subsequent acidic conditions, to give fluorescent derivatives. This reagent can only be used for post-column HPLC derivatization.

Benzoin, however, yields a single fluorescent derivative, 2-amine substituted-4,5-diphenylimidazole, in basic medium and is thus used for both post- and pre-column HPLC derivatization [5].

Analytical applications

Benzoin was initially employed to quantify endogenous guanidino compounds in uremic patients with pre- or post-column derivatization [6, 7].

Kai *et al.* describe a method for the quantification of leupeptine in mouse plasma with a pre-column derivatization [8]. Limits of detection of leupeptine in serum and muscles are 250 pmol/ml and 500 pmol/g, respectively.

- [12510](#) Benzoin purum; ≥99.0% (GC)

References

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2.2 Electrochemical detection (ECD)

2.2.1 Introduction

Electrochemical methods provide sensitivity and precision both for the quantification and specificity of chemical substances which can be reduced or oxidized on the surface of the electrode. However, many important compounds with no redox properties are found within the available potential range. Redox attachment is therefore interesting for ECD. A number of publications describe electrochemical derivatization [1-4].

References

- [1] Johnson, D.C. ; Weber, S.G. ; Bond, A.M. ; Wightman, R.W. ; Shoup, R.E. and Krull, I.S. *Anal. Chim. Acta* **1986**, 180, 187.
- [2] Lisman, J.A. ; Underberg, W.J.M. and Lingeman, H. *Electochemical derivatization in Chromatographic Science Series Vol. 48 Detection-oriented derivatization techniques in liquidChromatography*, **1990** Ligeman, H. and Underberg, L.J.M. (Eds) Marcel Dekker, New York, pp. 283-322.
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2.2.2 Primary and secondary amines

2.2.2.1 O-phthalaldehyde (OPA)

OPA, which is presented in § 2.1.1.1.1, is the most widely used labeling reagent in ECD. Isoindoles, formed from amines, present favorable electroactive properties and allow a great number of applications [1].

Analytical applications

Direct oxidation of histamine requires a high potential (peak potential + 1.05 V with Pd electrode). HPLC with ECD has been adopted for the determination of histamine, following pre-column derivatization with OPA and 2-mercaptoethanol. The isoindole derivative obtained is electroactive at moderate potentials (+ 0.4 V with a PD electrode) [2].

In order to assay sympathomimetic drugs (heptaminol, amphetamine, phenylpropanolamine, β -phenethylamine (PEA) and 2-heptylamine), a derivatization procedure was developed using OPA and various thiols (2-mercaptoethanol, ethanethiol or *t*-butanethiol). PEA is an endogenous amine found in several mammalian tissues. PEA lacks hydroxy groups in the aromatic ring but gives no signal in the ECD system, even at high oxidation potentials. The electroactive properties of these substituted isoindolic products were investigated by amperometry (+0.9 V vs SCE) and allowed picomole limits of detection [3].

Millerioux *et al.* developed a pre-column OPA derivatization coupled to *t*-butanethiol to determine baclofen in human plasma [4].

Achilli *et al.* reported a pre-column derivatization with OPA/2-ME for the analysis of amines such as histamine, tyramine and wine polyamines [5].

- [79760](#) Phthaldialdehyde BioChemika for fluorescence; $\geq 99.0\%$ (HPLC) , OPA;
- [63690](#) 2-Mercaptoethanol BioChemika; $\geq 99.0\%$ (GC)
- [04290](#) Ethanethiol purum; $\geq 97.0\%$ (GC) , Ethyl mercaptan;
- [B3027](#) *t*-Butanethiol Sodium Salt Approx. 97% (titration) , 2-Methyl-2-propanethiol

References

- [1] Johnson, D.C ., Weber, S.G. ; Bond, A.M. ; Wightman, R.W. ; Schoup, R.E. and Krull, I.S. *Anal. Chim. Acta*, **1986**, 180, 187.
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2.2.2.2 Naphthalene-2,3-dicarbaldehyde

Naphthalene-2,3-dicarbaldehyde (NDA) reacts in presence of cyanide ions (CN⁻), with primary amines to produce a cyano[*f*]benzoisindole (CBI) derivative which fluoresces in a similar way as the derivative produced with OPA (Fig. 37).

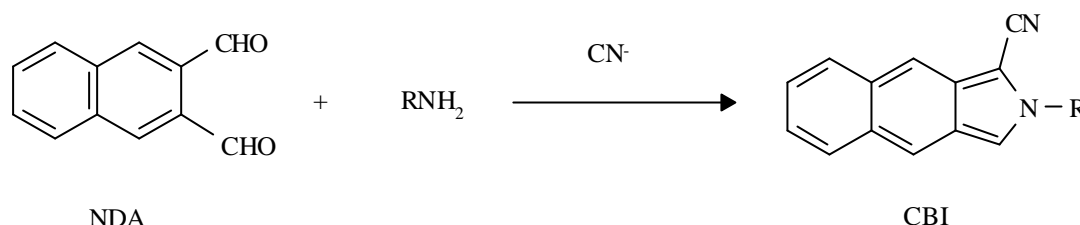


Fig. 37 Reaction of NDA with primary amines [1]

CBI derivatives have been found to be electroactive and are oxidized at a relatively low oxidation potential (+0.75 V vs Ag/AgCl). ECD is especially useful when analyzing compounds with more than one primary amine group. Derivatization combined with NDA/CN and ECD was linear over three orders of magnitude.

Analytical applications

Alendronic acid (4-amino-1-hydroxybutane-1-1-bisphosphonic acid) was developed as a potent inhibitor of bone resorption and is determined in urine and plasma by HPLC/EC. The assay is based on the pre-column derivatization of alendronate with NDA, in the presence of cyanide ions, to produce an *N*-substituted CBI derivative. The method was validated for a concentration range of 2.5-50.0 ng/ml [2, 3].

- [70215](#) Naphthalene-2,3-dicarboxaldehyde BioChemika for fluorescence , NDA

References

- [1] Toshimasa Toyo'oka (édité) *Modern Derivatization Methods for Separation Sciences*, 1999, John Wiley and sons, New York.
- [2] Kline, W.F. ; Matuszewski, B.K. and Bayne, W.F. *J. Chromatogr.* 1990, 534, 139.
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2.2.2.3 Ferrocene

Tanaka *et al.* have developed highly selective and sensitive HPLC/ECD derivatization reagents for amine or alcoholic compounds which have an electrophore ferrocenyl group [1]. The ferrocenyl moiety can easily be oxidized and is detected selectively in the presence of other electroactive compounds, such as phenols and aromatic amines. Fig. 38a shows the structure of *N*-succinimidyl 3-ferrocenyl propionate, a labeling reagent for amines.

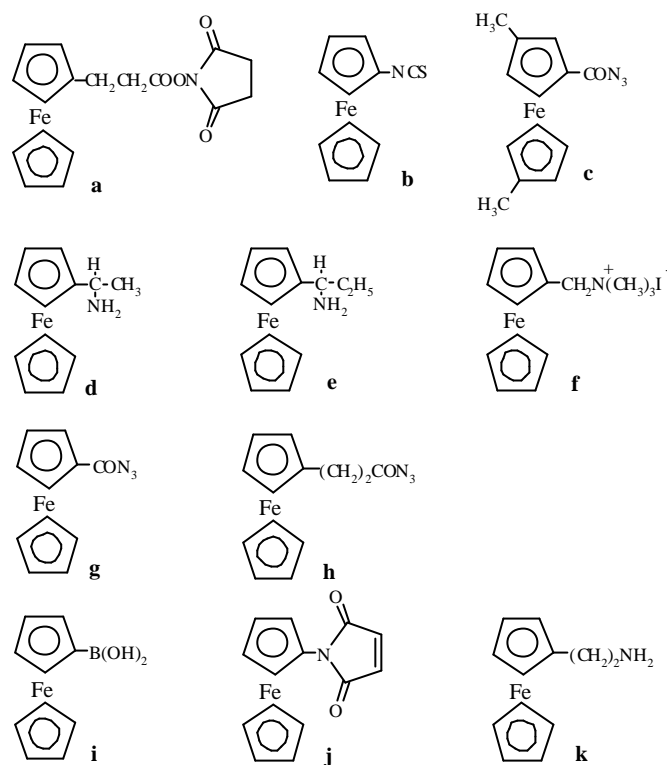


Fig. 38 Structure of ferrocene derivatization reagents [3]

Quantitative condensation of arylalkylamines, such as PEA and tryptamine with *N*-succinimidyl-3-ferrocenylpropionate, is performed at room temperature for 20 min. in an acetonitrile 0.05 borate buffer (pH 8) (1:1). Maximum sensitivity can be obtained with a glassy carbon electrode at + 0.4 V with an Ag/AgCl reference electrode. Limit of detection is 0.2 pmol [1]. *N*-succinimidyl-3-ferrocenylpropionate is the best reagent with ferrocene as electrophore, in terms of reactivity, stability and electrochemical properties [2]. Kubab *et al.* have developed two reagents for amines, ferrocene carboxaldehyde and ferrocene carboxylic acid chloride (FAC), with a maximum sensitivity between + 0.50 V and + 0.75 V vs Ag/AgCl, respectively, and with a limit of detection of 0.5 pmol [4]. FAC reacts both with primary and secondary amines to produce stable amides, which are reversibly oxidized in an aqueous acetonitrile solution at moderate potentials (+ 0.22 V vs Ag/AgCl).

Analytical applications

Ferrocenylisothiocyanate allows to determine GABA in the brain with a potential of + 0.50 V. The limit of detection is 0.05 pmol [5].

References

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2.2.2.4 Salicylic acid chloride

o-Acetylsalicyloyl chloride was developed as a pre-column derivatization reagent for aliphatic amines. Initially, this compound reacts with an amino group to form an *o*-acetylsalicylamide, which is spontaneously hydrolyzed under alkaline conditions to form 2-hydroxybenzamide (Fig. 39).

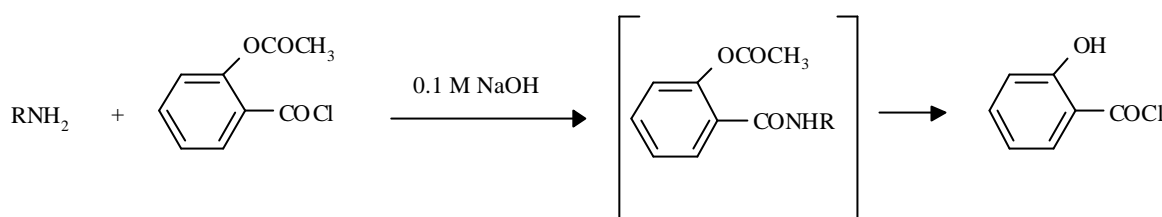


Fig. 39 Reaction of *o*-acetylsalicyloyl chloride with an amine in alkaline solution [3]

The free phenolic group can be detected electrochemically at low concentrations in an aqueous solution after separation by HPLC [1].

Aliphatic amines, such as propylamine, butylamine, 2-pentylamine, dimethylamine, diethylamine and dipropylamine, react with salicylic acid chloride to produce electroactive amide derivatives with good chemical stability. The method is sensitive enough to detect derivatives in ECD in picomole range (+1.2 V vs Ag/AgCl).

Analytical applications

The method allows to determine amines in plasma and urine by using 2-propylamine and dipropylamine as reference substances. After a solid-phase extraction and derivatization, the compounds are detected in solid-phase biological material at nanogram level [2].

- [01461](#) **O-Acetylsalicyloyl chloride** purum; $\geq 98.0\%$ (AT)

References

- [1] Smith, R.M. ; Ghani, A.A. ; Haverti, D.G. ; Bament, G.S., Chamsi, A.Y. and Fogg, A.G. *J. Chromatogr.* **1988**, 455, 349.
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2.2.2.5 2,4-dinitrofluorobenzene

The nitrophenyl group is ideally suited for ECD operating in reduction mode. However, its electrochemistry is not yet fully understood.

In 1987, Chang *et al.* developed pre-column derivatization for improved detection in HPLC-photolysis-electrochemistry [1].

Pre-column derivatization with 2,4-dinitrofluorobenzene (Sanger reagent) improves the chromatographic and detection properties of amino alcohols and amino acids. 2,4-Dinitrophenyl derivatives are separated by reverse-phase HPLC and the column eluate is sent through a photochemical reactor to produce electroactive species and, thence to an electrochemical detector containing a single or dual electrode system. This approach provides limits of detection in the low parts-per-billion range with a linearity of three orders of magnitude.

In 1995, Leube and Fischer reported pre-column derivatization and post-column irradiation for the ECD method [2].

Analytical applications

Leube and Fischer describe a method for the quantification of renin inhibitor I [2].

- [42080](#) **2,4-Dinitro-1-fluorobenzene** BioChemika; ≥99.0% (GC) , DNPF; FDNB; 1-Fluoro-2,4-dinitrobenzene; Sanger Reagent

References

- [1] Chang, M.Y. ; Chen, L.R. ; Ding, X.D. ; Selavka, C.M. ; Krull, I.S. and Bratin, K. *J. Chromatogr. Sci.***1987**, 25, 460.
- [2] Leube, J. and Fischer, G. *J. Chromatogr. B***1995**, 665, 373.

2.2.2.6 1,2-diphenylethylenediamine

As shown in § 2.1.2.3.1, catecholamines are converted into trihydroxyindoles with $[\text{Fe}(\text{CN})_6]^{3-}$, and are detected by fluorescence. However, dopamine cannot be detected this way. Therefore, the reaction between 1,2-diphenylethylenediamine and catecholamines, or other catechol compounds giving fluorescent diphenylquinoxaline derivatives [1], is replaced by an electrochemical method which allows to determine dopamine and other catecholamines after a pre-column derivatization with 1,2-diphenylethylenediamine. The limit of detection is 2 fmol [2].

Analytical applications

Electrochemical detection, at + 0.6 V with an Ag/AgCl reference electrode, allows to determine norepinephrine and epinephrine, as well as dopamine, with limits of detection at 0.3 pg and 0.5 pg, respectively [3].

- [42747](#) N,N'-Diphenylethylenediamine purum; ≥97.0% (NT) , 1,2-Dianilinoethane; N,N'-Ethylenedianiline

References

- [1] Nohta, H. ; Mitsui, A. and Ohkura, Y. *Anal. Chim. Acta* **1984**, 165, 171.
 [2] Mori, K. *Life Sci.* **1987**, 41, 901.
 [3] van der Hoorn, F.A.J.; Boomsma, F.; Man in't Veld, A.J. and Schalekamp, M.A.D.H. *J. chromatogr.***1989**, 487, 17.

2.2.2.7 Bolton and Hunter reagents

In 1979, Shimada *et al.* developed highly sensitive electroactive derivatization reagents for primary and secondary amines: *N*-hydroxysuccinimide esters (Bolton and Hunter type reagent) formed by the condensation of vanillic acid and homovanillic with *N*-hydroxysuccinimide [1].

Analytical applications

Sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate (sulfo Bolton and Hunter type reagent) was chosen for the chemical derivatization of β-phenethylamine, PEA. PEA reacts with excess reagent at room temperature and at pH 10; the reaction is complete after 10 min. (Fig. 40).

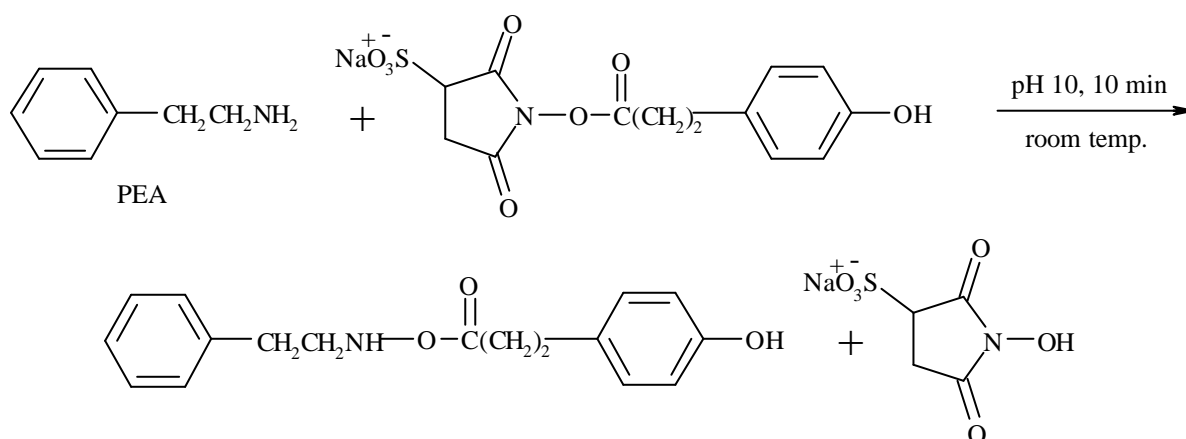


Fig. 40 Derivatization of PEA with the sulfo Bolton and Hunter type reagent [4]

In a HPLC system, the Bolton and Hunter type PEA derivatives give a symmetrical peak. The detector response for a single concentration of PEA increases with the electrode voltage and produces an optimal signal to noise for + 0.55 V [2].

Cyclohexylamine and benzylamine are derivatized with 3-(4-hydroxyphenyl)propionic acid-*N*-hydroxysuccinimide ester to produce the corresponding electroactive amines. For cyclohexylamine, the limit of detection is 0.1 μM [3].

References

- [1] Shimada, K. ; Tanaka, M. and Nambara, T. *Chem. Pharm. Bull.* **1979**, 27, 2259.
- [2] Gusovsky, F. ; Jacobson, K.A. ; Kirk, K.L. ; Marshall, T. and Linnoila, M. *J. Chromatogr.* **1987**, 415, 124.
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- [4] Toshimasa Toyo'oka (édité) *Modern Derivatization Methods for Separation Sciences*, **1999**, John Wiley and sons, New York.

2.2.2.8 Other reagents

PEA and piperidine, as well as primary and secondary amines, react with *N*-(4-anilinophenyl)isomaleimide (APIM) which is prepared with a pre-column derivatization reagent in an acetonitrile borate buffer solution at 0.05 M (pH) (1:1). Derivatization of amines is complete after 20 min at room temperature. The derivatives formed with APIM are sensitive to electrochemical detection. The limit of detection for a PEA-APIM derivative is 0.1 pmol. The electrochemical response is linear in the range of 0.1 -1.0 ng of PEA [1].

A polymeric anhydride containing an *O*-acetylsalicyl group is utilized as labeling moiety for primary and secondary amines. Derivatives are detected by an electrochemical detector in oxidative mode and oxidative ECD, after post-column photolysis (HPLC-*hv*-ECD). Derivatization improves the limits of detection by 3-4 orders of magnitude [2].

Chiavari *et al.* report using ECD in HPLC with dansyl derivatives of biogenic amines [3]. Putrescine, cadaverine, 1,6-diaminohexane, tryptamine, histamine, tyramine, spermine and spermidine are derivatized with DNS-Cl. Limits of detection with ECD (+ 0.55 V) range between 0.18 to 1.0; with UV absorption, between 4 and 8 pmol; and with fluorescence, between 0.52 and 1.25.

References

- [1] Shimada, K. ; Tanaka, M. and Nambara, T. *J. Chromatogr.* **1983**, 280, 271.
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3 Derivatization for the resolution of chiral amines

Many pharmacological compounds have asymmetric carbons; those with a therapeutic interest are used in their pure enantiomeric form. Usually, drugs are prescribed in a racemic form, even if individual enantiomers possess different properties (activity, toxicity and pharmacokinetics). HPLC is the most adequate technique for enantiomer separation and works in two ways: direct separation with a stationary chiral phase, and indirect separation, after previous formation of a diastereoisomer with the adequate chiral reagent and separation on a conventional stationary phase.

The choice of the reagent for the separation of chiral molecules is of the greatest importance in indirect methods involving a derivatization step. A pair of mirror image enantiomers is labeled with a chiral derivatization reagent in order to generate two diastereoisomers. Separation depends on the various physicochemical properties of the diastereoisomers (stereochemistry and stability) with the achiral stationary phase. In fact, the choice of the reagent determines the precision and repeatability of the quantitative analysis. Further, the presence of two or more reactive functional groups within the analyte makes the labeling process more difficult.

Ideal chiral derivatization reagents present the following characteristics:

- 1) High chemical and optical purity.
- 2) Stability and no racemization during storage.
- 3) Negligible racemization in derivatization conditions.
- 4) Reaction rates and yields essentially the same for each enantiomer. For many reagents, this is not the case.
- 5) Resulting diastereoisomers exhibit adequate detector response during sample analysis.

The main reactions for chiral amines (amino acids and aminoalcohols) are based on the formation of amides, carbamates, urea and isourea (Fig. 41). Diastereoisomeric amide reactions are widely used for the resolution of various primary amines.

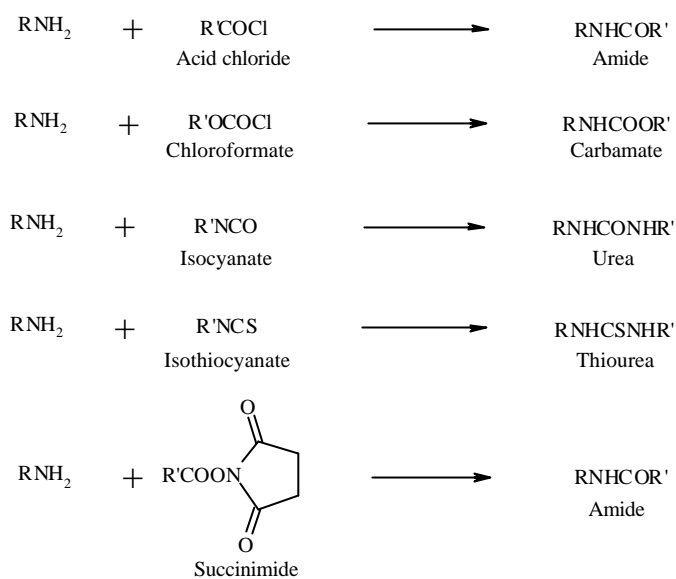


Fig. 41 Derivatization reactions forming optically active amines [1].

References

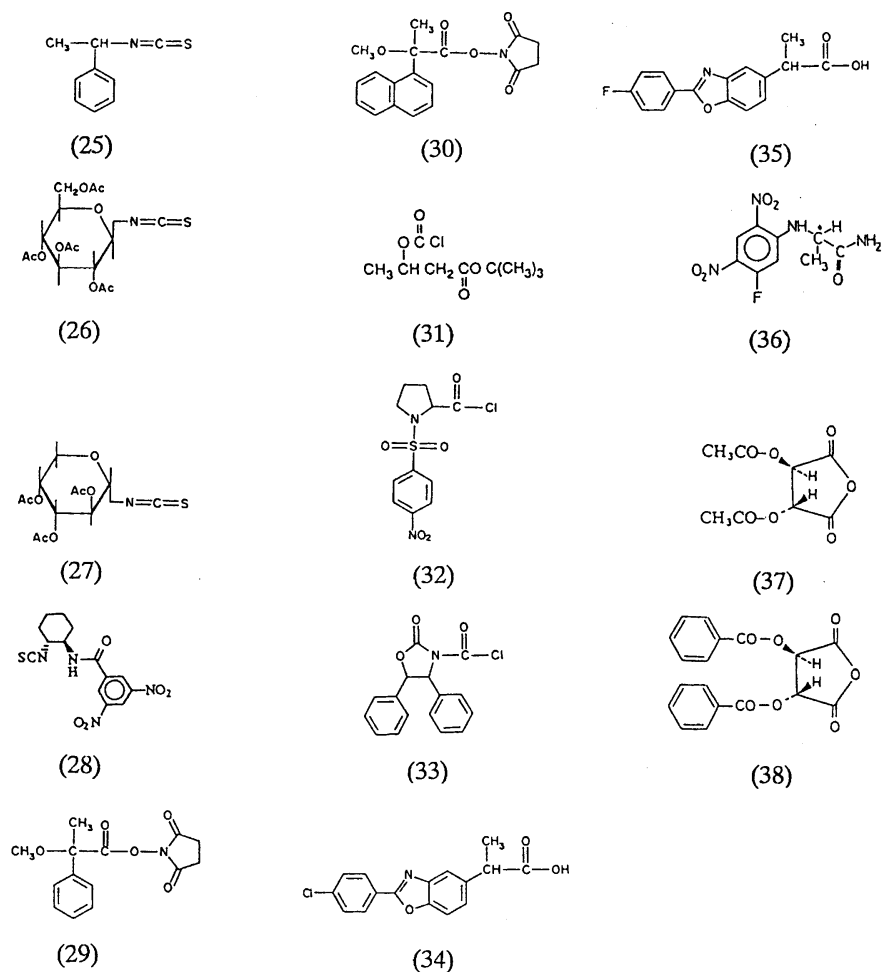
- [1] Toshimasa Toyo'oka (édité) *Modern Derivatization Methods for Separation Sciences*, 1999, John Wiley and sons, New York.

3.1 Detection methods

3.1.1 UV-VIS absorption detection

An ideal derivatization reagent reacts quantitatively with the analyte in order to produce a sole diastereoisomer, without racemization or secondary products. In order to avoid undesirable compounds in samples which absorb in the UV-VIS region, reagents with a visible band are preferable in terms of selectivity.

Although a number of reagents for amino functional groups have been developed and applied to real sample analysis, only few chiral reagents allow enantioseparation. Chiral UV labels for primary and secondary amines are presented below (Fig. 42).



Chiral UV-VIS labels for amines by HPLC: (25) $R(+)$ -1-phenylethyl isothiocyanate (PEIT); (26) 2,3,4,6-tetra-*O*-acetyl- β -D-glucopyranosyl isothiocyanate (GITC); (27) 2,3,4-tri-*O*-acetyl- α -D-arabinopyranosyl isothiocyanate (AITC); (28) (R,R) -*N*-[(2-isothiocyanatocyclohexyl)-(3,5)-dinitrobenzoylamide] (DDITC); (29) *S*-2-methoxy-2-phenylacetic acid *N*-succinimidyl ester; (30) 1- α -methoxy- α -methyl-naphthaleneacetic acid *N*-succinimidyl ester; (31) *S*-tert.-butyl 3-(chloroformoxy)butyrate; (32) 1-[(4-nitrophenyl)sulfonyl]propyl chloride; (33) *cis*-4,5-Di-phenyl-2-oxazolidone-3-carbamyl chloride (benzoxapropen); (34) *S*(+)-2-(*p*-Chlorophenyl)- α -methylbenzoxazole-5-acetic acid chloride (flunoxapropen); (35) *S*(+)-(*p*-Fluorophenyl)- α -methylbenzoxazole-5-acetic acid chloride (flunoxapropen); (36) 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent); (37) (R,R) -*O,O*-diacetyl tartaric acid anhydride (DATAAN); (38) (R,R) -*O,O*-dibenzoyl tartaric acid anhydride (DBTAAN).

Fig. 42 Derivatization reagents for amines by UV-VIS detection [1].

References

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3.1.1.1 Isocyanates and isothiocyanates

Although isocyanate and isothiocyanate reagents afford slower labeling than halogeno reagents, they are appropriate for the resolution of a racemate, after introduction of polar functional groups of urea and isourea. The method has been improved and simplified. Research studies report derivatization times inferior to 15 min. [1-9] and HPLC chromatographic separation in less than 50 min [6].

The drawbacks of these reagents are a marked polar character, a limited UV detection,

as well as solution instability and degradation.

2,3,4,6-Tetra-*O*-acetyl- β -D-glucopyranosyl isothiocyanate (GITC) (26) [11-16] and 2,3,4-tri-*O*-acetyl- α -D-arabinopyranosyl isothiocyanate (AITC) (27) [17-20] are the most important isothiocyanates for amino enantioseparation. They produce (thio-) urea derivatives in presence of a base catalyst. In this family, *R*(+)-1-phenylethyl isothiocyanate (PEIT) (25) [21] is also used.

GITC is a highly selective reagent with a rigid and large cyclic structure. It derives from glucose and is optically very pure. It has 5 asymmetric carbon atoms. Reaction of GITC with amines is presented in Fig. 43.

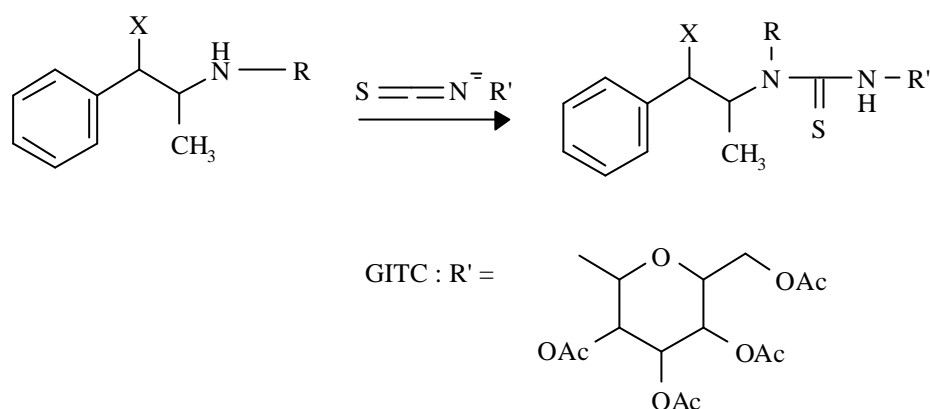


Fig. 43 Reaction of GITC with amines [6]

Reaction time is generally short (between 15 and 30 min.) and resolution of both diastereoisomers good.

R(+)-1-phenylethyl isocyanate (PEIC) (13) [22-27] and (*R*)-1-naphtylethyl isocyanate (NEIC) (14) [28-30] are the main isocyanates used for chiral derivatization. Reaction time of PEIC is very long (>15 hours).

Other isocyanates, such as *S*(-)- α -methylbenzyl isocyanate (MBIC), are mainly used to analyze β -blockers [10] which also contain amino functions. The alcohol function improves the quality of the separation of the two diastereoisomers thus formed.

Derivatization reactions with isocyanate reagents require drastic working conditions (high temperature and long reaction time).

Analytical applications

GITC and AITC are used to derivatize different amines, such as catecholamines, which present a biomedical and pharmaceutical interest [4, 7].

NEIC is used to analyze prenyalmanine in urine and blood [8].

- [70725](#) ***R*(—)-1-(1-Naphthyl)ethyl isocyanate** ChiraSelect; $\geq 99.0\%$ (GC, sum of enantiomers); stab. , (*R*)-NEI , NEIC
- [77968](#) ***R*(+)-1-Phenylethyl isocyanate** ChiraSelect; $\geq 99.0\%$ (GC, sum of enantiomers); stab. ,
- [77970](#) ***S*(—)-1-Phenylethyl isocyanate** ChiraSelect; $\geq 99.0\%$ (GC, sum of

- enantiomers); stab. , MBIC
- **86550** **2,3,4,6-Tetra-O-acetyl- β -D-glucopyranosyl isothiocyanate** ChiraSelect; $\geq 98.0\%$ (HPLC) , GITC
- **90245** **2,3,4-Tri-O-acetyl- α -D-arabinopyranosyl isothiocyanate** ChiraSelect; $\geq 98.0\%$ (HPLC, sum of enantiomers) , AITC

References

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3.1.1.2 Succinimidyls

Amides are formed by reaction with *N*-succinimidyl esters such as *N*-succinimidyl-1- α -methoxy- α -methyl-naphtaleneacetic acid ester (30) [1], and *N*-succinimidyl-(S)-2-methoxy-2-phenylacetic acid ester (29) [2]. These reagents are stable and can be utilized in aqueous medium. However, they are mainly used as a coloring reagent for biological specimens such as cells and organs.

References

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3.1.1.3 Acid chlorides

The following acid chlorides are derivatization reagents for amines: *N*-trifluoroacetyl-1-prolylchloride [1], (-)- α -methoxy- α -(trifluoromethyl)phenylacetyl chloride [7], (-)-camphanic acid chloride [2], 1-[(4-nitrophenyl)sulphonyl]prolyl chloride (32) [3, 4] and S(+)-benzoxaprofen chloride (34) [5-7].

- [248509](#) (S)-(-)-*N*-(Trifluoroacetyl)prolyl chloride, 0.1 M solution in dichloromethane
- [21287](#) (—)-Camphanic acid chloride ChiraSelect; $\geq 98.0\%$ (AT)

References

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3.1.1.4 Tartric acids

Various (*R,R*)-*O,O*-disubstituted tartric acids [1], such as (*R,R*)-*O,O*-diacetyl tartric acid anhydride (DATAAN) (37) [2], and (*R,R*)-*O,O*-dibenzoyl tartric acid anhydride (DBTAAN) (38), which were synthesized by Lindner *et al.*, react with alkanoamines (propranolol) in aprotic medium containing trichloroacetic acid and produce tartric acid monoesters.

- [31587](#) (+)-Di-O-acetyl-L-tartaric anhydride purum; ≥97.0% (NT)

References

- [1] Lindner, W. ; Leitner, C. and Uray, G. Weber, H. ; Spahn, H. ; Mutschler, E. and Mohrke, W. *J. Chromatogr.***1984**, 307, 145 **1984**, 316, 605.
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3.1.2 Fluorescence detection

Fluorogenic reactions can be classified into two categories: 1) fluorescence generation and 2) derivatization with a fluorescent reagent. For chiral separation by HPLC, pre-column derivatization is more frequently used than post-column derivatization. The reagents used for fluorimetric detection are described below (Fig. 44).

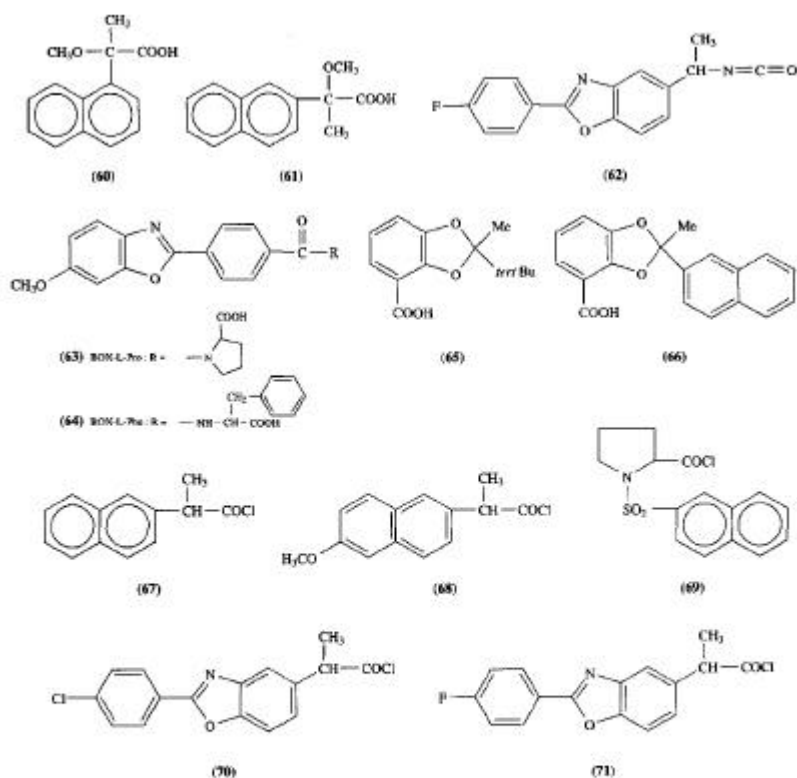


Fig. 6.35. Chiral FL Labels for amines by HPLC: (60) (-)- α -methoxy- α -methyl-1-naphthaleneacetic acids (MM(1)NA); (61) (-)- α -methoxy- α -methyl-2-naphthaleneacetic acids (MM(2)NA); (62) S(-)-flunoxaprofen isocyanate [S(-)-FLOPIC]; (63) N-[4-(6-methoxy-2-benzoxazolyl)]benzoyl-L-proline (BOX-L-Pro); (64) N-[4-(6-methoxy-2-benzoxazolyl)]benzoyl-L-phenylalanine (BOX-L-Phe); (65) 2-tert-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid [R(-)- and S(+)-TBMB-COOH]; (66) 2-Methyl-2- β -naphthyl-1,3-benzodiazole-4-carboxylic acid [S(+)-MNB-COOH]; (67) D-2-(2-Naphthyl)propionyl chloride; (68) S(+)-naproxen chloride [S(+)-NAP-Cl]; (69) S(-)-N-(1-Naphthylsulfonyl)-2-pyrrolideneacetyl chloride; (70) S(+)-benzoxaprofen chloride [S(+)-BZOP-Cl]; (71) S(+)-flunoxaprofen chloride [S(+)-FLOP-Cl].

Fig. 44 Reagents for fluorescence detection [1]

References

- [1] Toshimasa Toyo'oka (édité) *Modern Derivatization Methods for Separation Sciences*, 1999, John Wiley and sons, New York.

3.1.2.1 OPA

Derivatization reactions with OPA (45) (Fig. 44) are important for primary chiral amines [14, 16-18], and especially amino acid enantiomers. The reaction is complete in 5 min at room temperature. However, the resulting isoindole derivatives are usually found to be unstable. In order to avoid this, primary amines are determined by post-column derivatization followed by separation on a RP-HPLC analytical column.

The chiral mercaptans used for the resolution of primary amine enantiomers are *N*-acetyl-L-cysteine (NAC) (47) [1-12], *N*-tert.-butyloxycarbonyl-L-cysteine (BOC-C) (46) [5, 6, 13], *N*-acetyl-D-penicillamine (NAP) (48) [3-6], 1-thio- β -D-glucose (TG) (49) [4, 14], 2,3,4,6-tetra-*O*-acetyl-1-thio- β -D-glucopyranoside (TATG) (50) [4, 14] and *N*-isobutyl-L(or D)-cysteine [15] (Fig. 45).

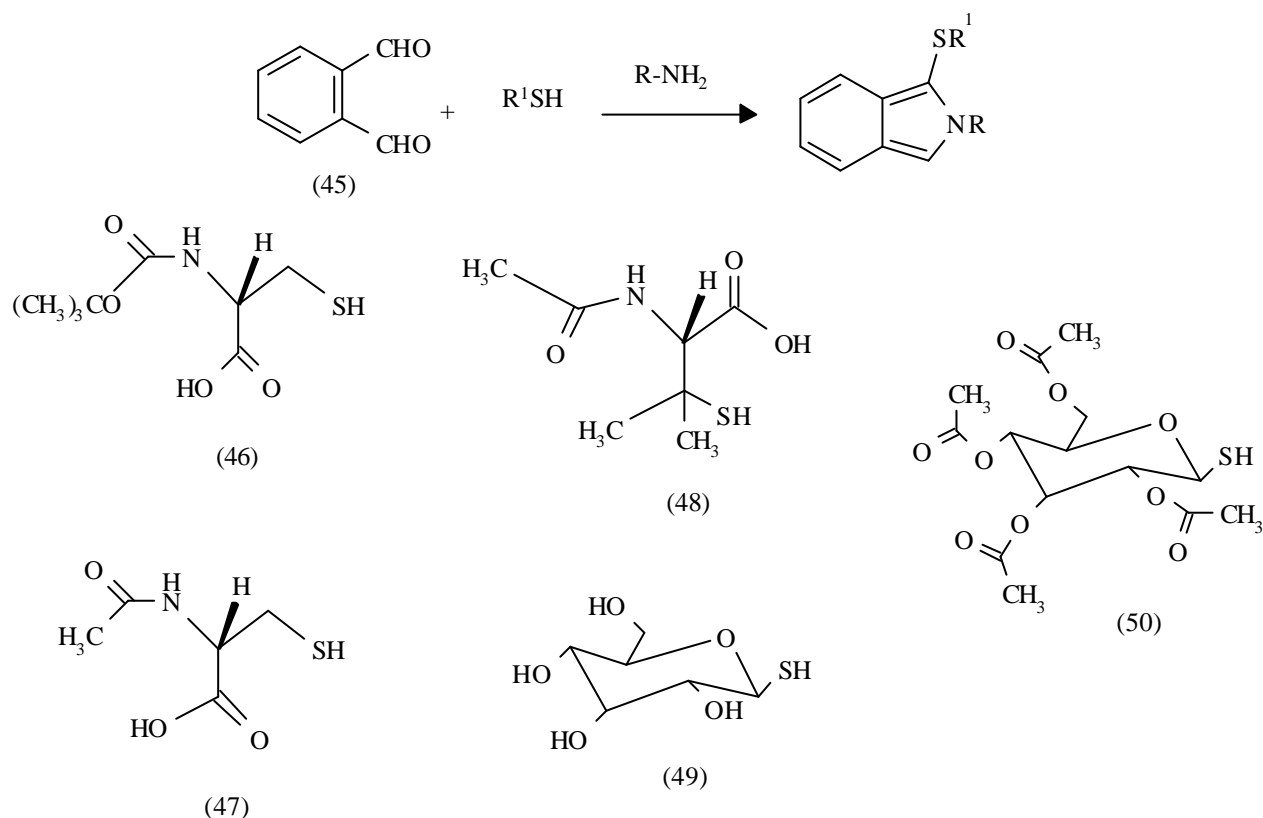


Fig. 45 Structure and reaction of OPA with chiral thiols [19].

The chemical structure of thiols has a great influence in OPA-mercaptan reactions with enantiomeric amines. Using thiols with different chemical structures allows to better comprehend the isoindole structure, and thus improve the stereoselectivity of derivatives.

Analytical applications

Compounds such as L-cysteine derivatives are ideal chiral mercaptans because they

are available in an optically pure form. A number of amines, such as biogenic amines (norepinephrine and norephedrine), amino acids, amino alcohols and medicinal substances containing a primary amine are resolved by combination of OPA with a chiral mercaptan.

ω -*N*-oxalyl diamino acids [6], lombricine [5], tranylcypromine [8] and baclofen [9] are analyzed after pre-column derivatization with chiral OPA/thiols. Desai and Gal [4] describe the enantioseparation of a variety of primary amines and medicinal substances, such as (\pm)-*p*-chloroamphetamine (PCA), (-)-amphetamine (AMP), 3-amino-1-(4-hydroxyphenyl)butane (AHB), (\pm)-1-methyl-3-phenylpropylamine (APB), (\pm)-*p*-hydroxyamphetamine (HAM), rimantadine (RIM), tocainide (TOC) and mexiletine (MEX). Resolution values (R_s) depend on the type of chiral thiols and primary amines chosen. Good resolutions are obtained with TG or TATG.

- [01039](#) **N-Acetyl-L-cysteine** puriss.; $\geq 99.0\%$ (T)
- [01423](#) **N-Acetyl-D-penicillamine** BioChemika; $\geq 99.0\%$ (T)
- [15411](#) **Boc-Cys-OH** ChiraSelect, BOC-L-cysteine
- [79760](#) **Phthaldialdehyde** BioChemika for fluorescence; $\geq 99.0\%$ (HPLC), OPA
- [86550](#) **2,3,4,6-Tetra-O-acetyl- β -D-glucopyranosyl isothiocyanate** ChiraSelect; $\geq 98.0\%$ (HPLC)
- [471275](#) **1-Thio- β -D-glucose, sodium salt hydrate**

References

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3.1.2.2 Chloroformates

Contrary to isocyanates and isothiocyanates, chiral chloroformates have only received a limited attention in amine analysis. Chiral chloroformates are prepared with a chiral alcohol and a phosgene, or with a less toxic and less volatile (triphosgene) substitute. In general chloroformates serve as derivatization reagents for primary and secondary amines, amino acids and alcohol amines.

3.1.2.2.1 (+)-1-ETHYL (9-FLUORENYL) CHLOROFORMATE CHLORIDE (FLEC-CL)

Reaction with chloroformate is fast and forms carbamates. Chloroformate derivatives react quantitatively with primary and secondary amines to produce the corresponding carbamates at room temperature. The most important tag is (+)-1-ethyl (9-fluorenyl) chloroformate chloride (FLEC-Cl). This reagent was synthesized on the basis of Fmoc-Cl [1]. It can also be used for amino acids and medicinal substances containing an amine function.

During derivatization (Fig. 46), a secondary reaction influences the quantity of reagent available for the main reaction. Excess reagent (10 to 100 times) is therefore indispensable but can interfere during fluorimetric detection (λ_{ex} . 265 nm, λ_{em} . 345 nm).

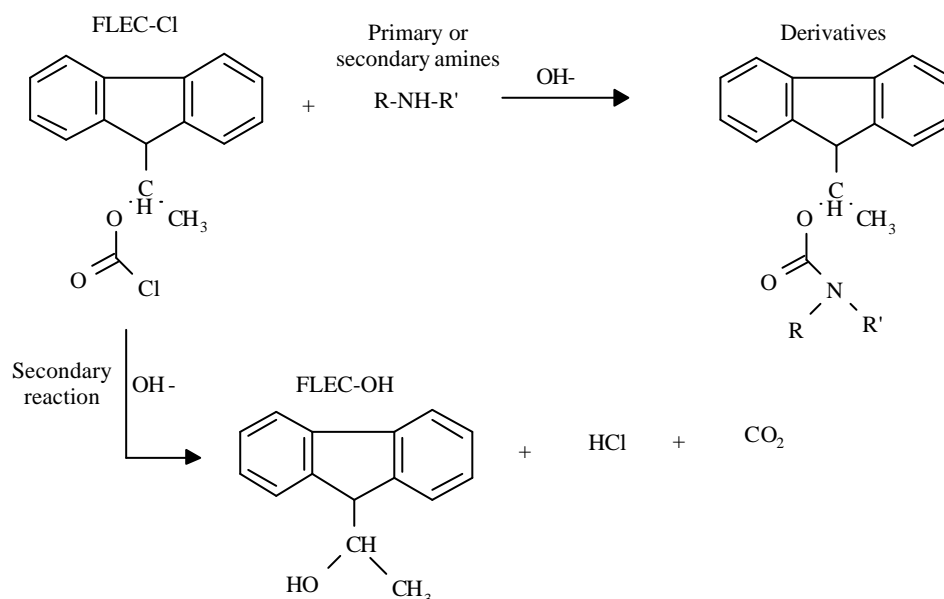


Fig. 46 Formation of the derivative with primary or secondary amines.

The main advantages of this reagent are mild reaction conditions, highly fluorescent derivatives, short derivatization time (2 to 4 min.) and application to primary and secondary amines.

Analytical applications

Rosseele *et al.* describe a derivatization method for atenolol [2].

[23182](#) (+)-1-(9-Fluorenyl)ethyl chloroformate Solution ChiraSelect; ≥18 mM in acetone, (+)-FLEC

[23183](#) (—)-1-(9-Fluorenyl)ethyl chloroformate Solution ChiraSelect; ≥18 mM in acetone, (—)-FLEC

References

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3.1.2.2.2 4-(6-METHOXY-2-NAPHTYL)-2-BUTYL CHLOROFORMATE (NAB-C)

(+)- and (-)-4-(6-methoxy-2-naphtyl)-2-butyl chloroformates (NAB-C) are prepared using nabumetone, an anti-inflammatory non-steroid prochiral agent. The derivatization reaction of amino compounds takes place at alkaline pH, at room temperature, in 2 min. Detection wavelengths are 230 nm (UV detection) or 270 nm (excitation), and 365 nm (emission) for fluorimetric detection [1].

Analytical applications

This reagent is used to analyze metoprolol enantiomers in plasma.

References

- [1] Büschges, R.; Devant, R.; Mutschler, E. and Spahn-Langguth, H. *J. Pharm. Biomed. Anal.* **1996**, 15, 201.

3.1.2.2.3 OTHER CHLOROFORMATES

Other chloroformates are (S)-tert.-butyl 3-(chloroformoxy)butyrate (31) [1] and (-)-menthylchloroformate [2-7]. Prakash *et al.* [2] have shown that the latter can also react with tertiary amines to produce a carbamate.

- [23200](#) (—)-(1R)-Menthyl chloroformate purum; ~97% (GC, sum of enantiomers)

References

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3.1.2.3 Acid chlorides and α -alkyl- α -arylacetic acids

Reactions with acid chlorides are quick and produce amides. Halogeno acids, such as *N*-propyl substituted chloride, are good derivatization agents. Amides are formed by acylation under mild conditions (room temperature or ice bath).

A number of α -alkyl- α -arylacetic acid compounds were initially anti-inflammatory non-steroid drugs (AINS) with a good fluorescence. This is why, since 1984, compounds of this group have been used as derivatization reagents. The first was an enantiomeric pure benzoxaprofen derivative (BOP) which also has a chloride, (S(+)-BZOP-Cl) (70) [1]. However, this method was abandoned because of the excessive time necessary for reagent preparation and derivatization.

Later, naproxene (NAP-Cl) chloride (68) was used [2]; it presents the advantage of being available in a S pure enantiomeric form. Forming an acyl chloride activates it.

Flunoxaprofen (Fig. 47), which has a S(+)-FLOP-Cl chloride (71), is a substance similar to BOP. This compound is as active as NAP but its reaction time is quite long (60 min.); it affords good diastereoisomer resolution [3].

Martin *et al.* have synthesized a new reagent by combining the properties of α -alkyl- α -arylacetic acids with those of isocyanates [4]. Preparation of S-FLOP (FLOPIC) (62) and S-NAP (NAPIC) isocyanates is simple and cost effective. These reagents offer faster derivatization (less than an hour) and are used for primary and secondary amines.

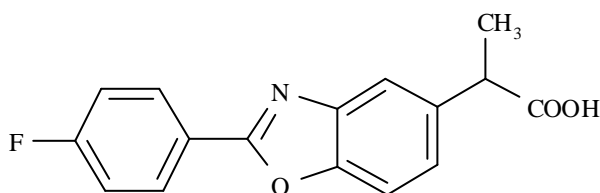


Fig. 47 Chemical structure of flunoxaprofen

References

- [1] Weber, H. ; Spahn, H. ; Mutschler, E. and Möhrke, W. *J. Chromatogr.* **1984**, 307, 145-153.
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3.1.2.4 L-Proline derivatives

3.1.2.4.1 DANSYL-L-PROLINE

Dansyl-L-proline (DLP) (Fig. 48) is used for the enantiomeric separation of a number of amines [1]. It reacts in presence of diethylphosphorocyanidate and triethylamine in 3 min. Average resolution is obtained.

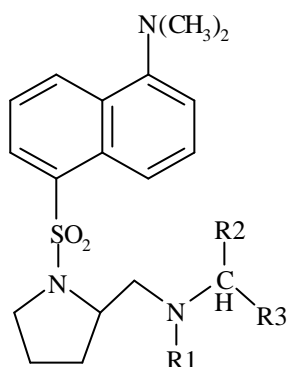


Fig. 48 Dansyl-L-proline derivatives with amines.

3.1.2.4.2 L-PROLINE FLUOROALKYL DERIVATIVES

Fluoroalkyl-L-prolyl acyl chlorides also contain L-proline (Fig. 49).

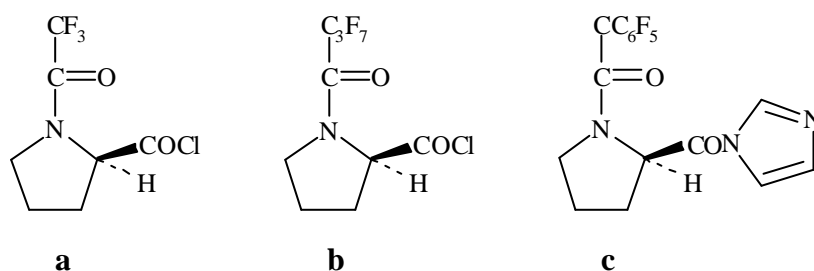


Fig. 49 a) trifluoroacetyl-L-prolyl-Cl; b) heptafluorobutyryl-L-prolyl-Cl ; c) pentafluorobenzoyl-L-prolyl- L-imidazolide.

References

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3.1.2.5 (-)- α -Methoxy- α -(trifluoromethyl)phenylacetic (MTPA) and analogues

(-)- α Methoxy- α -(trifluoromethyl)phenylacetic acid (MTPA) and naphthaleneacetic

analogues, (MM(1)NA) (60) and (MM(2)NA) (61), are other reagents for amine derivatization (Fig. 50). MTPA was first used for GC amine derivatization [1], whereas naphthaleneacetic analogues were applied to the enantioseparation of amino acids by HPLC [2].

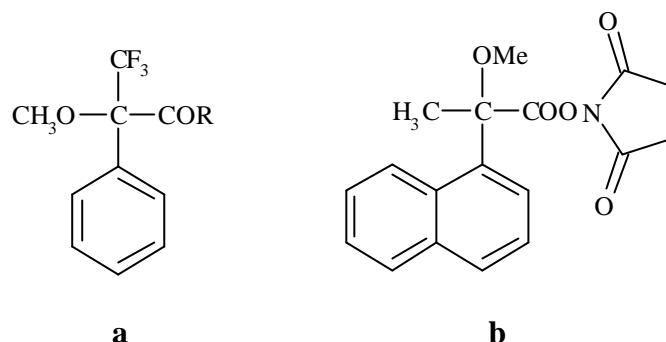


Fig. 50 Chemical structure of (a) MTPA and (b) MMNA.

Derivatization is relatively simple and takes about 30 min., which is longer than with GITC and AITC in the same conditions.

- [65361](#) **R(+)- α -Methoxy- α -trifluoromethylphenylacetic acid** ChiraSelect; $\geq 99.0\%$ (T), (+)-MTPA; Mosher's Acid

References

- [1] Gal, J. *J. Pharm. Sci.* **1977**, 66, 169-172.
 [2] Goto, J.; Hasegawa, M.; Nakamura, S.; Shimada, K. and Nambara, T. *J. Chromatogr.* **1978**, 152, 413-419.

3.1.2.6 Other reagents

Other reagents are:

- *N*-[4-(6-methoxy-2-benzoxazolyl)]benzoyl-L-phenylalanine (BOX-L-Phe) (64);
- 2-tert.-butyl-2-methyl-1,3-benzodioxole-4-carboxylic acid [*R*(-)- and *S*(+)-TBMB-COOH](65);
- 2-methyl-2- β -naphthyl-1,3-benzodiazole-4-carboxylic acid [*S*(+)-MNB-COOH] (66);
- *D*-2-(2-naphthyl)propionyl chloride (67);
- *S*(-)-*N*-(1-naphthyl sulfonyl)-2-pyrrolidene carbonyl chloride (69);

3.1.3 Electrochemical detection

There are many chiral derivatization reagents for UV-VIS and fluorescence, but only few for electrochemical determination. Among these reagents, chiral ferrocene compounds are the most known.

Analytical applications

Shimada *et al.*[1] developed chiral ferrocene compounds, such as *R*(-)-1-ferrocenylethylamine (85) and *S*(+)-1-ferrocenylpropylamine (Fig. 51), for the derivatization of anti-inflammatory non-steroid substances such as naproxen and ibuprofen.

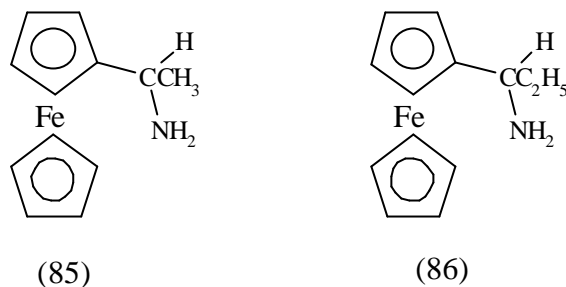


Fig. 51 Chiral derivatization reagents [2].

Aminotetraline stereoisomers, tagged with *R*(+)-1-phenylethyl isocyanate (PEIC) (13), are determined at nanogram level with an electrochemical detector, in oxidation mode.

References

- [1] Shimada, K. ; Haniuda, e. ; Oe, T. and Nambara, T. *J. Liq. Chromatogr.***1987**, 10, 3161.
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4 Conclusion

HPLC derivatization has been the subject of many studies and has led to using a number of reagents which have been successfully applied to substances, such as biogenic amines, toxins, antibiotics and other pharmaceuticals.

However, the difficulty in developing a derivatization method resides in choosing the adequate reagent. This choice depends on the following criteria:

- The detection mode.
- The selectivity: some reagents are very specific and only react with primary amines or with guanidino groups.
- The possibility of working in pre- or post-column mode.
- The compatibility with reaction conditions (temperature, solvent, etc.).
- The enantiomer discrimination, if analysis of chiral amines is required.

Many years of experience in the production and supply of derivatizing reagents have made Fluka a leading supplier of these chemicals. Fluka was first to analyze, specify and guarantee enantiomeric purity used to assay the enantiomeric purity of compounds. Due to our commitment to provide the best analytical solutions to our customers, new applications are continuously developed in our quality control department.

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