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# Derivatizing Reagents for Detection of Organic Compounds By HPLC

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***Authors' contributions***

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

Derivatization is the process of chemically modifying a compound to develope a new compound which has properties that are suitable for analysis using HPLC. Derivatization improves the detectability of a target analyte by reaction with suitable derivatizing agent. Derivatization reactions are simple chemical modification of substance that make it compatible with the selected separation method or transforms substance with a low UV- absorption into highly sensitive product. Derivatization reactions in liquid chromatography modify the solutes adding a chomophore for easy UV detection or a fluorophore for sensitive fluorescent detection. The chemical structure of the compound remains same and just modifies the specific functional group for reacting compounds to derivative of deviating chemical and physical properties in order to make them detectable. Introduction of certain elements or groups through chemical derivatization may enhance the detector's response helpful for the elucidation of structure of analytes. In conclusion, the present review describe various derivatization reagents for pre-column and post column derivatization process in HPLC by UV-visible and fluorescence detection are summarized along with reactions and some practical aspects. The commonly used derivatizing reagents in HPLC are 1-fluoro-2, 4-dinitrobenzene, ninhydrine, 4-N-N-dimethylaminoazobenzene-4'-sulfonyl chloride, benzoyl chloride, phenyl isocyanate for UV-visible detection and o-phthalaldehyde, fluorescamine, 1-dimethylaminonapthalene-5-sulfonyl chloride (DNS-Cl), 9-fluorenylmethyl chloroformate (Fmoc-Cl), benzofurans for Fluorescence detection.

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*Keywords: Derivatization; UV-visible detection; fluorescence detection; pre-column derivatization; post-column derivatization.*

## 1. INTRODUCTION

Derivatization is chemical reaction which converts an analyte in such a way as to allow its detection. This process whereby a molecule is chemically altered to enhance its detection by a particular analytical method is called derivation. [1-3] The chemical structure of the compound remains the same and just modifies the specific functional group of reacting compounds to derivative in order to increase their sensitivity to UV absorption and fluorescence detection. [4,5] Chemical derivatization is used to convert the non-UV absorbing analytes into highly sensitive products that are easily chromatographed or detected. In pharmaceutical analysis, derivatization is commonly employed if the Active Pharmaceutical Ingredient does not have a chromophore for UV detection or a fluorophore for fluorescence detection [6].

In this paper, the derivatization method for HPLC connecting with UV-VIS detection will be described. The term derivatization means basically the reactions caused by chemical reagents or physical methods to convert a poor detector-responding analyte into a highly detectable product which enhanced chromatographic properties. In other words, derivatization aims to increase the detectability of the analyte.

Derivatization in HPLC can be performed either before the chromatographic analysis (Pre-column derivatization) or after the column separation and before detection (Post-column derivatization).

### 1.1 Pre-Column Derivatization

In pre-column derivatization, the reaction is generally performed manually in vials before HPLC injection i.e. the compounds are derivatized before their injection in the column, but can also be automated. [6,7] This technique allows more flexible working conditions (reaction time, solvent reaction, elimination of excess reagent, etc. [3] Separation of the coloured reagent or UV-absorbing material takes place after their modification by derivatization, the excess of derivatizing agent and the by-products can be easily removed; derivative stability is necessary. [6] This technique is often used to promote improved chromatographic response of the analytes under investigation. [3,7] Important characteristics of this technique involve completion of reaction, stability of the resultant derivatives and ease of preparation. [4]

### 1.2 Post-Column Derivatization

Post-column derivatization has been proposed to overcome the drawbacks associated with pre-column mode. Post-column derivatization technique is typically used for compounds with low or no response to the selected detection system. [4,8,9,10].

In post-column derivatization, the derivatizing reagent is added not only to the mobile phase but also to the target analyte/eluted compound. The reaction is performed in a reactor which is placed between the column and the detector. This is accomplished by inserting a mixing "T" or chamber at the end of the column and pumping system (pump). The derivative is in a reaction coil, the length of which is controlled by the time required for completion of the reaction, and is then measured in the detector. [5,6,11].

### 1.3 Uv-Visible Detection

UV detection is the most commonly used technique in HPLC but it sometimes lacks sensitivity or selectivity for trace analysis of compounds. [4,11,12] Chemical derivatization modifies substances with a low UV absorption into highly sensitive products. [4,12] The chromatographic detection can be improved by the derivatization of the compounds which do not possess a chromophore. [4] There have been a variety of chemical reagents that have been used to add a chromophore to a particular type of functional group. [12] The reagents which are used in UV-visible detection are usually highly conjugated aromatic compounds containing a highly reactive group to react with a functional group of the analyte. [4,10,12].

Derivatization can also improves the resolution of closely eluted compounds and chromatographic retention of polar compounds include aliphatic amines, amino acids, and compounds that have carbonyl and hydroxyl substituents because the derivatives are usually more hydrophobic than the underivatized analyte. [4,13]

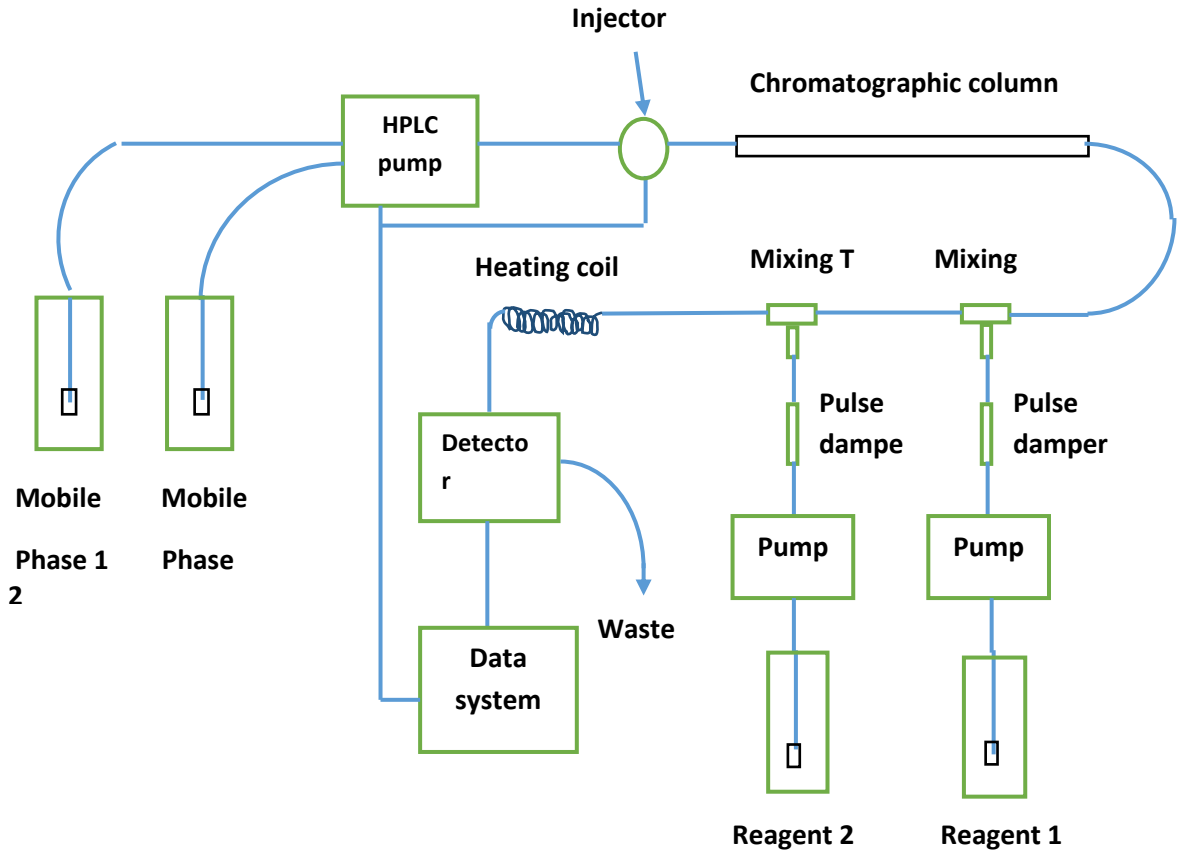



**Fig.1. Post-column derivatization setup in HPLC. [5]**

## 2. NITROBENZENE

### 2.1 Fluoro-2, 4-Dinitrobenzene (FDNB) [1,2,3,6,14,15,16]

FDNB is also called as Sanger's reagent. FDNB specifically reacts with N-terminal amino group. Mostly the reaction of FDNB performed in an alkaline medium (borate buffer). FDNB reacts with amino acid or primary amine or secondary amine to form a derivative 2, 4-dinitrophenyl. (Fig. 1.1).

### 2.2 Fluoro-3-Nitrotrifluoromethylbenzene (FNBT) [1,2,3,17,18]

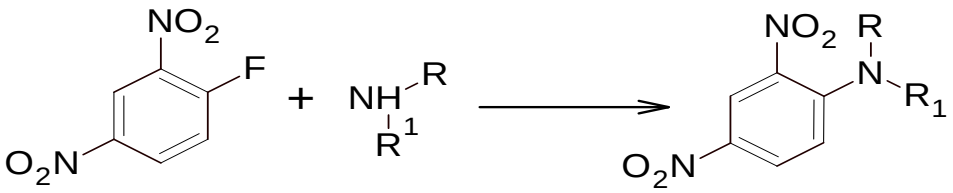
FNBT reacts with polyamine to form N-2'-nitro-4-trifluoromethylphenyl polyamine derivative which absorbs radiation of 242 nm and 410 nm. FNBT does not react with secondary amine or molecules containing polar groups. The reaction of FNBT with primary amine is shown in Fig. 1.2.

### 2.3 2, 4, 6-Trinitrobenzene-1-Sulfonic Acid (TNBS) [1,2,3]

The N-nitrophenyl derivative was formed in an aqueous medium of pH 8 and at room temperature, without any undesirable secondary reaction. The N-nitrophenyl derivatives have a high molar absorptivity at 340 nm.

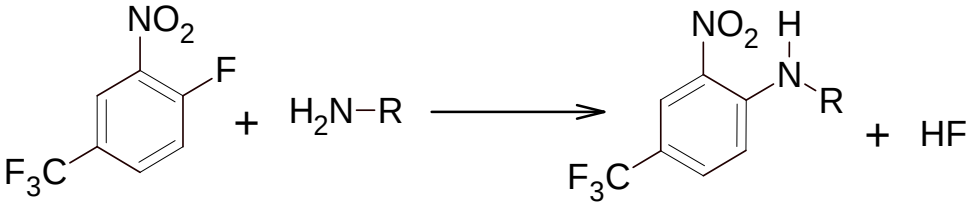
### 2.4 Ninhydrine [1,2,3,6]

Both pre-column and post-column derivatization methods are used for the determination of amino acids in proteins. The former is characterized by an ion-exchange separation and post-column derivatization with ninhydrine. Ninhydrine reacts only with primary amines and giving blue-violet coloured compound (diketohydrindylidene-diketohydrindamine) (Fig. 1.4). The ninhydrine method originated by Moore and Spackman [19,20] and has become a classical method because of its suitability for automation, reproducibility and accuracy despite low sensitivity, high cost instrumentation and time consumption.



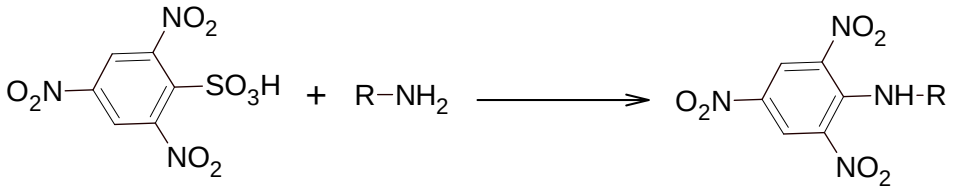

FDNB R = H or R 2, 4-dinitrophenyl

**Fig. 1.1. Reaction of amine with FDNB**



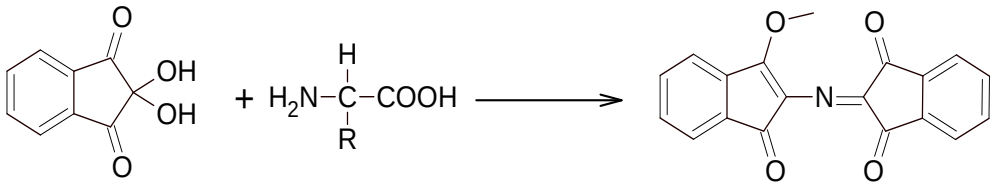

FNBT 1° Amine N-2'-nitro-4-trifluoromethylphenyl

**Fig. 1.2. Reaction of primary amine with FNBT**




TNBS 1° Amine N-nitrophenyl

**Fig. 1.3. Reaction of primary amine with TNBS**




Ninhydrine Amino acid

**Fig. 1.4. Reaction of amino acid with ninhydrine**

## 3. ARYLSULFONYL CHLORIDE

### 3.1 4-N-N-Dimethylaminoazobenzene-4'-Sulfonyl Chloride (DABS-Cl) [1,2,3,6]

DABS-Cl is one of the derivatizing agent for amino acid. DABS-Cl reacts with amine to form sulphonamide derivative (Fig. 1.5). The resulting sulphonamide show an absorbance maximum at 420 to 450 nm. Lammens and Verzele [21] developed a procedure in which derivatization was performed at 50° C and at pH 8.9 for short time, and gave a quantitative yield. Reverse-phase HPLC with isocratic elution and detection at 464 nm was employed.

### 3.2 Acyl Chloride

#### 3.2.1 Benzoyl chloride [3,6]

Benzoyl chloride is the preferred derivatizing agent polyamines, and it reacts with most of the naturally occurring di-amines and polyamines

(tosyl-, dansyl or benzoyl chloride). Benzoyl chloride is particularly advantageous because it implies short derivatization procedure and has a long elution time. A rapid and simple method using benzoyl chloride was originally described by Redmond and Tseng [22]. The derivatization method was improved by dissolving benzoyl chloride in methanol thereby enhancing the reaction with polyamines. The benzoylated polyamines are eluted by RP-HPLC using methanol-water (60:40) as the mobile phase. The sensitivity of this method is 100 p/mol. The benzoylated polyamines can be stored upto 3 weeks at -20°C.

### 3.2.2 Acetic anhydride [2,3]

Tertiary aliphatic amine can be separated on HPLC column packed with Nucleosil 5N $(CH_3)_2$ resin, and detected by post-column derivatization after reaction with a color reagent consisting of an acetic anhydride solution of citric acid. The reaction temperature was 120°C. UV detection was performed at a wavelength of 550 nm.

### 3.2.3 Phenyl isocyanate (PIC) [1,2,3,6]

Phenyl isocyanate (PIC) reacts with primary and secondary amines to form N-N'-disubstituted urea (Fig. 1.7). UV detection measured at 255 nm. Bjorkqvist proposed a method in which derivatization was carried out in N-N'-dimethylformamide, and aliphatic alcohol was added to destroy the excess of reagent. The separation was by RP-HPLC with detection at 240 to 260 nm.

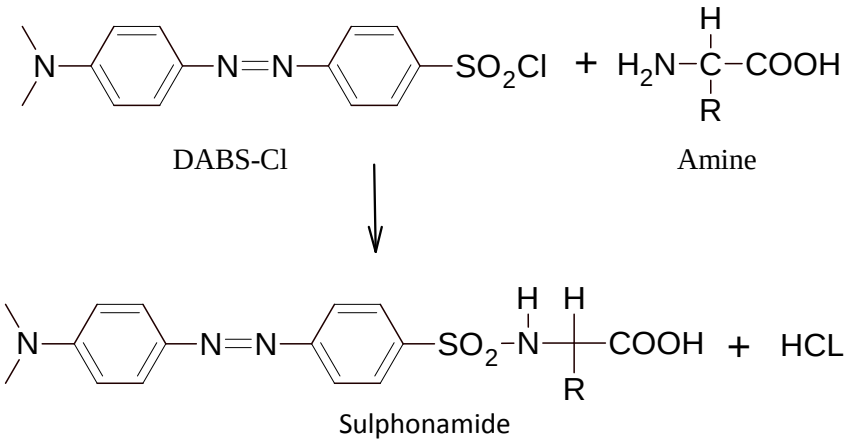



**Fig. 1.5. Reaction of amine with DABS-Cl**

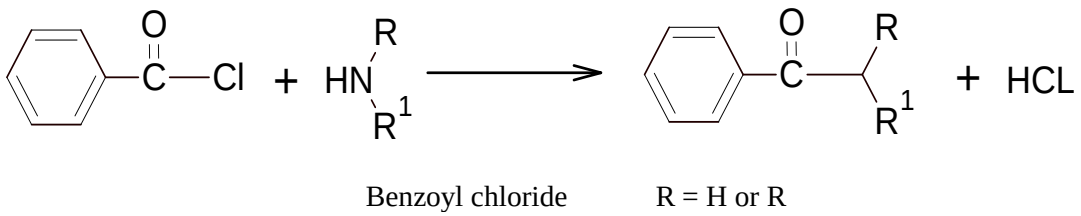



**Fig. 1.6. Reaction of amine with benzoyl chloride**

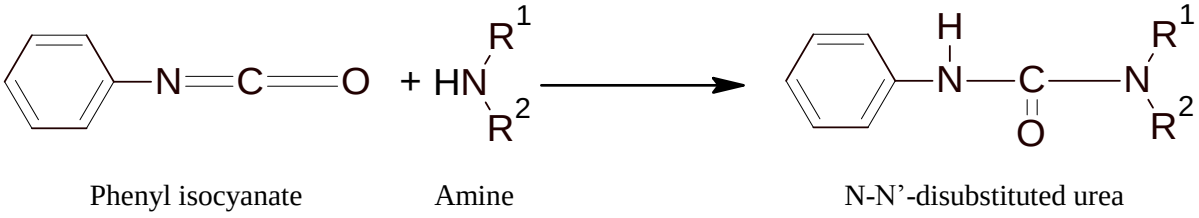



**Fig. 1.7. Reaction of amine with phenyl isocyanate**

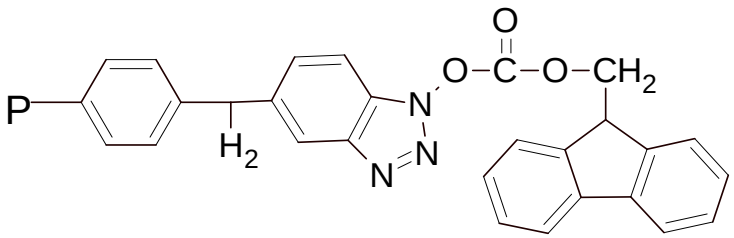



**Fig. 1.8. Polymeric Benztriazole - FMOC**

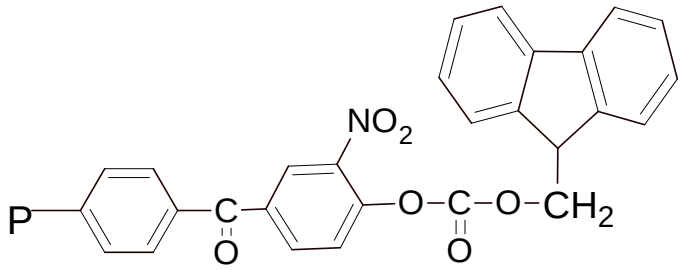



**Fig. 1.9. Polymeric 3-nitro-4-[[9-fluorenylmethoxy) carbonyl] oxy benzophenone**

### 3.3 Solid-phase Reagent with UV or VIS Light Absorbing Moiety

#### 3.3.1 Polymeric benzotriazole activated reagent containing FMOC group [2,3,23]

A polymeric benzotriazole reagent containing the FMOC group **(Fig. 1.8)** for UV and fluorescence detection, was applied in the determination of cadaverine and putrescine, normally occurring polyamines in human urine [24]. Polymeric FMOC reagent was also used for the determination of volatile amines in air by on-line solid phase derivatization and HPLC with UV and fluorescence detection. [25] An improved polymeric reagent, a fluorenyl attached polymeric *o*-nitrobenzophenone reagent (Fig. 1.9) was presented by Gao et al*.* [26].

## 4. FLUORESCENCE DETECTION

Fluorescence detection is a very sensitive procedure used in HPLC. Fluorescence takes place when a molecule absorbs a photon, typically in the UV range, which causes the emission of another photon at a higher wavelength in the visible range. [4,5] Generally many compounds have natural fluorescence, but in case if any compound or analyte is not fluorescent then it can be induced by creating a derivative. Derivatization is used for producing fluorescent compound. In addition, the unique property of fluorescent can allow for the selective identification of a molecule in a complex mixture. [6,27] Fluorescence usually has a wider linear range of response vs. concentration compared to optical (UV-VIS) absorbance and is quantifiable at lower concentrations.

### 4.1 Primary Amines

#### 4.1.1. O-pthalaldehyde (OPA) [1,2,3,6,28]

OPA is non-fluorescent itself but it is widely used for derivatization of primary amines and amino acids. OPA reacts with primary amino group in an aqueous basic medium in the presence of alkylthiol (such as 2-mercaptoethanol) to form a highly fluorescent isoindolederivative (Fig. 2.1.).

The derivatization reaction was performed in a mixture of borate buffer (pH 6-8 for amines, pH 9.7-10.0 for amino acids) and methanol at room temperature in 2 minutes. Excitation of fluorescence can be achieved at 340 nm. Fluorescence emission is measured at 455 nm. The OPA reaction can be applied to post column derivatization because of its short reaction time and fluorogenic property (non-fluorescent).

#### 4.1.2. Fluorescamine [1,2,3,4,6,28]

Fluorescamine reacts with primary amines and amino acids in borate buffer (pH 9.5-10) at room temperature. The reaction is complete in few minutes and forms a fluorescent Pyrrolidone

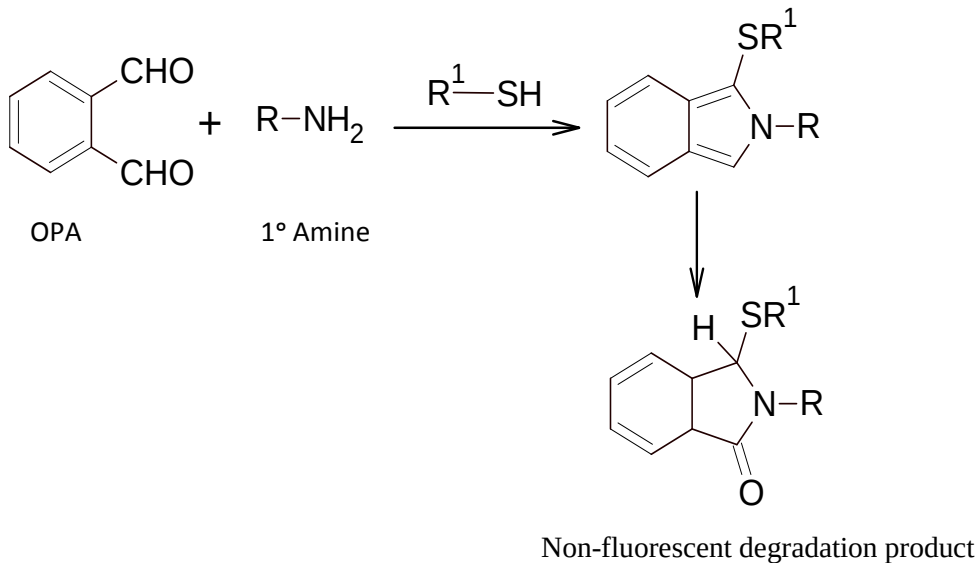



**Fig. 2.1. Reaction of primary amine with OPA**

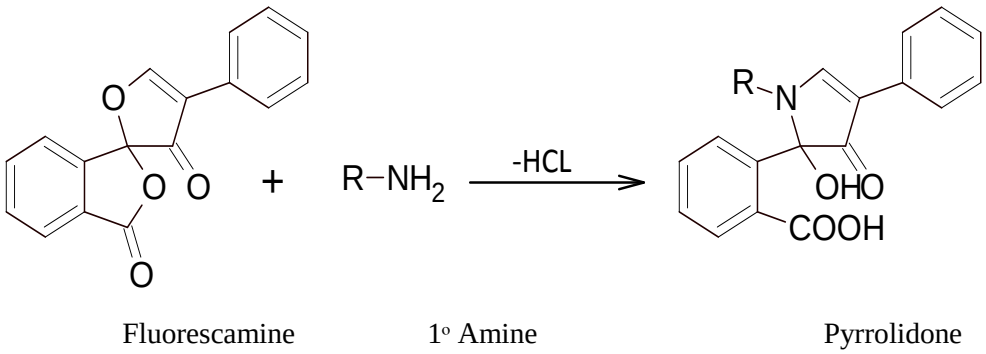



**Fig. 2.2. Reaction of primary amine with fluorescamine**

(Fig. 2.2). Fluorescamine is a non-fluorescent compound, but it offer enhanced detectability of primary amino compounds with fluorescence detection. Fluorescamine reacts with compounds with nucleophilic functional groups such as primary amines, secondary amines, alcohol, water, etc. but only primary amines form a fluorescent derivative. Therefore, fluorescamine is specific reagent for compounds of primary amino group.

## 5. PRIMARY AND SECONDARY AMINES

## 5.1 Sulfonyl Chlorides

### 5.1.1 1-dimethylaminonapthalene-5-sulfonyl chloride (DNS-Cl) [1,2,3,6]

DNS-Cl reacts with primary and secondary amino compound under slightly alkaline condition (alkaline pH) and forms a fluorescent derivative. The reaction time (30-120 minutes) depends on the type of amino acid compound. The reagent is hydrolyzed in the derivatization procedure to produce a highly fluorescent 1-dimethylaminonaphthalene-5-sulfonic acid (Fig. 2.3). Hence this reaction is mainly used for pre-column derivatization. The derivative of amino acids are separated on reverse-phase column with UV-visible or fluorimetric detection.

### 5.2. Carbonyl chlorides

### 5.2.19-fluorenylmethyl chloroformate (Fmoc-Cl) [1,2,3,6]

9-Fluorenylmethyl chloroformate (Fmoc-Cl) is used in the pre-column derivatization of primary and secondary amino compounds. The reaction proceed in buffered solution (borate buffer pH 8) within 2 minutes to form a stable and fluorescent derivative (Fig. 2.4). However, Fmoc-Cl and its hydrolyzed product are highly fluorescent, hence

they have to be removed by extraction with an organic solvent, pentane.

## 5.3. Isocyanate and Isothiocyanate

### 5.3.1 Phenyl isocyanate [2,3,28]

Phenyl isocyanate reacts with primary and secondary amine to form urea (Fig. 2.5). It also reacts with alcohols, phenols, water, carboxylic acids. The reaction completes within few minutes at room temperature and for this reaction requires excess reagent.

Isocyanates react quite rapidly with water and alcohols to give urethanes. For this reason they are usually replaced by the less reactive isothiocyanates.

### 5.3.2 Phenyl Isothiocyanate [2,3,28]

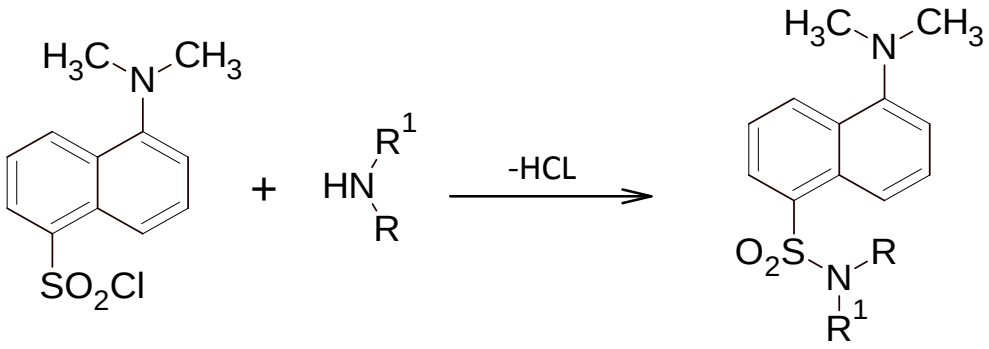
It is also known as Edman's reagent. It is not only used in the pre-column determination of amino acids, but also in the microanalysis of peptide sequence. The reaction is performed in an acidic environment and the derivative was determined by fluorimetric detection (Fig. 2.6).

### 5.3.3. Benzofurans [1,2,3,6,16]

Halogenobenzofurazan reagents such as NBD-Cl (4-chloro-7-nitro-2, 1, 3-benzoxadiazole) and NBD-F (4-fluoro-7-nitro-2, 1, 3-benzoxadiazole) react with both primary and secondary amino compounds (Fig. 2.7). The reaction proceeds under alkaline condition (pH 8-9) at 50°-60° C. The reaction with NBD-F is ten times faster than with NBD-Cl, and is complete in 1 minute. Both the reagents NBD-Cl and NBD-F are used for post-column derivatization. NBD-Cl also reacts with aliphatic amine, amino acids, peptides and proteins.

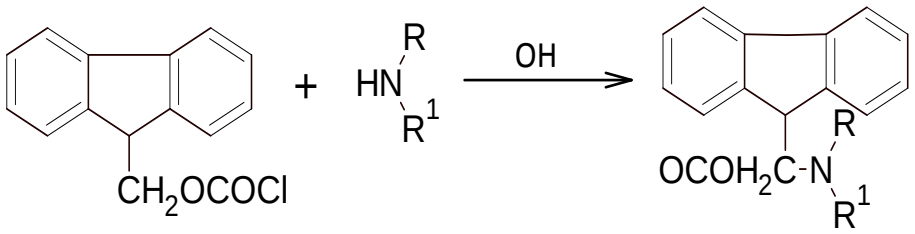
### 5.3.4 Succinimidyls [1,2,3,28]

N-succinimidyl-1-napthylcarbamate (SINC) (Fig. 2.8) reacts with amino acids in an alkaline environment (borate buffer pH 9.5) within 1 minute at room temperature to form naphthylcarbamoyl derivative. Fast reaction and the removal of the excessive reagent by hydrolysis make N-succinimidyl-1-napthylcarbamate suitable for automated pre-column derivatization. 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate (AQC) (Fig. 2.9) have also been developed.



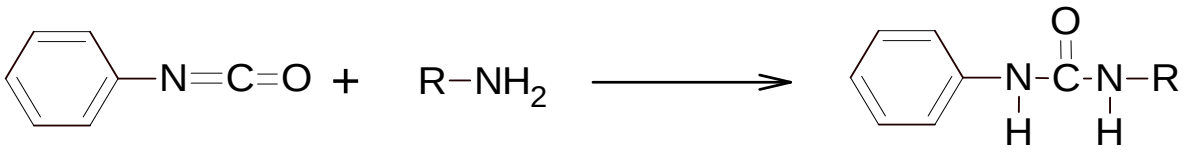

DNS-Cl R = H or R 1-dimethylaminonaphthalene-5-sulfonic acid

**Fig.2.3. Reaction of amine with DNS-Cl**



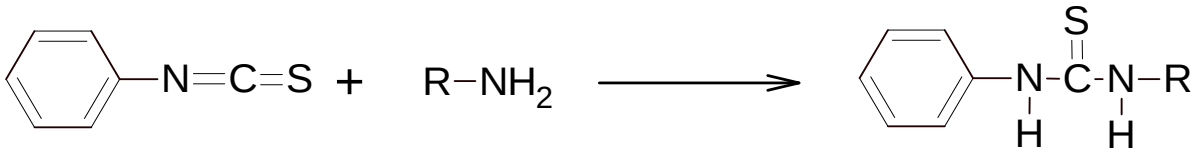

Fmoc-Cl Amine

**Fig. 2.4. Reaction of amine with Fmoc-Cl**



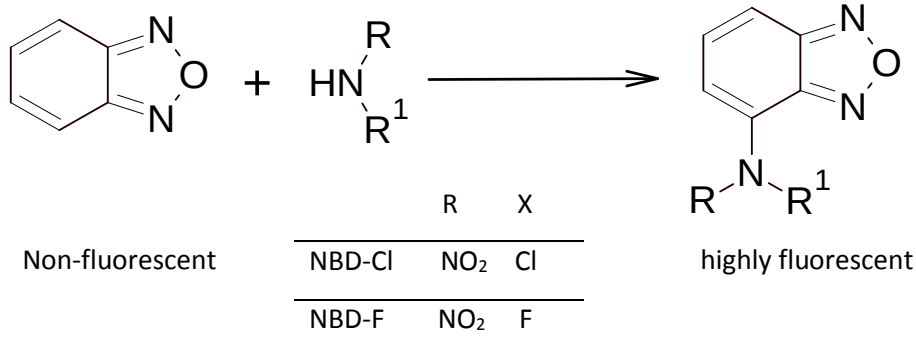

Phenyl isocyanate Amine Urea

**Fig. 2.5. Reaction of amine with phenyl isocyanate**




Phenyl Isothiocyanate Amine Thiourea

**Fig. 2.6. Reaction of amine with Edman's reagent**




Non-fluorescent highly fluorescent

**Fig. 2.7. Reaction of amine with halogenobenzofurazan**

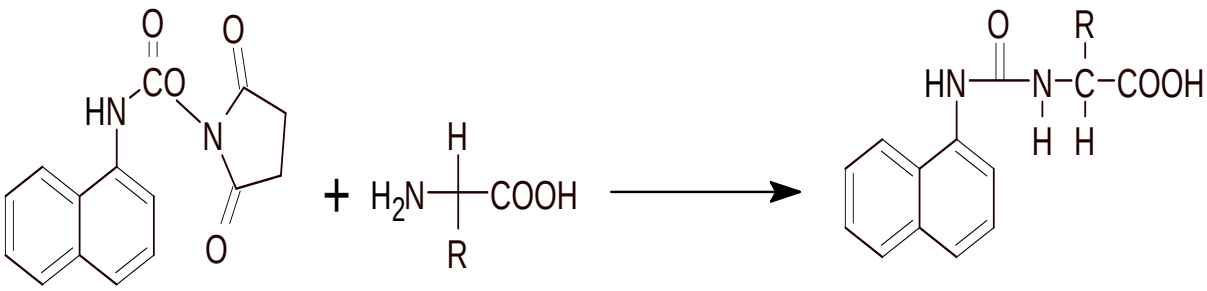



**Fig. 2.8. Reaction of amino acid with N-succinimidyl-1-napthylcarbamate (SINC)**

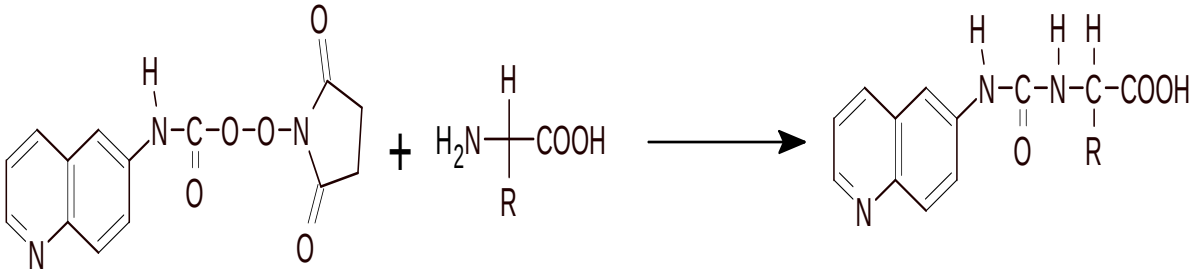



**Fig 2.9. Reaction of amino acid with 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate (AQC)**

## 6. APPLICATION OF VARIOUS DERIVATIZATION REAGENTS USED IN HPLC

**Table 1. Reagents for UV-visible detection, targeted functional group and its application**

| Derivatization reagent | Target | Application | Reference |
|---|---|---|---|
| *1-fluoro-2,4-dinitrobenzene (FDNB)* | 1° & 2° amines, aliphatic hydroxyl group | Pre-column derivatization of aminoglycosides (amikacine, tobramycin) | [29,30] |
| *4-fluoro-3-nitrotrifluoromethylbenzene (FNBT)* | 1° amines & polyamines | Polyamine such as putrescine, spermidine & spermine | [31,32] |
| *2, 4, 6-trinitrobenzene-1-sulfonic acid (TNBS)* | 1° amines, amino acids & peptides | Reacts with amikacine | [33] |
| *Ninhydrine* | 1° amines | Post-column derivatization of aminoglycosides (streptomycin); determination of peptides, amino acids & amines | [34] |
| *4-N-N-dimethylaminoazobenzene-4'-sulfonyl chloride (DABS-Cl)* | 1° & 2° amines, thiols, imidazoles, phenols & aliphatic hydroxyl group | Determination of biogenic amines in complex matrices | [35] |
| *Benzoyl chloride* | Di & Polyamines | Determination of histamine in fish | [36] |
| *Acetic anhydride* | 3° aliphatic amines, trialkylamine | Post-column derivatization of aliphatic amines | [37] |
| *Phenyl Isocyanate (PIC)* | 1° & 2° aromatic amines | Excellent derivatization agent for compound with active hydrogen atoms | [38] |

**Table 2. Reagents for fluorimetric detection, targeted functional group and its application**

| Derivatization reagent | Target | Application | Reference |
|---|---|---|---|
| *O-pthalaldehyde (OPA)* | Amino acid, 1° amines | Pre-column derivatization of different biogenic amines in wine (histamine, methylamine, tyramine, putrescine) | [39,40] |
| *Fluorescamine* | 1° amines, amino acids | Derivatize – sulphonamides in foods, ampicilline in biological fluids, determination of amoxicilline | [41,42] |
| *1-dimethylaminonapthalene-5-sulfonyl chloride (DNS-Cl)* | 1° & 2° amines, 1° & 2° alcohols | Determination of biogenic amines such as putrescine, cadaverine, spermidine & spermine | [43] |
| *9-fluorenylmethyl chloroformate (Fmoc-Cl)* | 1° & 2° amines | Determination or analysis of kanamycin in swine tissue | [44] |
| *Isocynates* | 1° & 2° amines, alcohol, water, phenol, carboxylic acid | Analysis of amino acids | [45] |
| *Phenyl isothiocynates* | 1° & 2° amines | Determination of amino acids & peptide sequencing, analysis of | [46] |

| Derivatization reagent | Target | Application | Reference |
|---|---|---|---|
| | | amphetamine | |
| *Benzofurans* | 1° & 2° amines, alcohol, thiols& phenols | Derivatization of fumonisines | [47] |
| *Succinimidyl* | Amino acids | AQC used for derivatization of fumonisines | [48] |

## 7. 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 amines, amino acids or amino compounds. Chemical derivatization with HPLC permit UV-visible and fluorescence detection for routine use to solve selectivity and detectability problems for drug samples not amenable to GC.

A wide variety of chemical reactions are employed to transform target compounds into labelled compounds that are easier to separate by liquid chromatography or to detect with common detectors.

Both pre-column and post-column derivatization employed in HPLC analysis. Chemical derivatization has been accepted as an effective modification technique prior to HPLC analysis. This technique can provide highly sensitive and selective detection of amines by reacting with a chromophore or fluorophore that results in products with strong UV absorption and/or fluorescence emission and which can be detected by HPLC.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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