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# DETAILED REVIEW ABOUT PREGABALIN AND ITS DERIVATIZATION TECHNIQUES

Pharma

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## ABSTRACT

Pregabalin capsules, extended-release (long-acting) tablets and oral solution (liquid) are used to relieve neuropathic pain (pain from damaged nerves) and also used in spinal cord injury and to treat fibromyalgia (a long-lasting condition that may cause muscle stiffness and tenderness, tiredness, pain, and difficulty falling asleep) as well as in epilepsy, so determination of pregabalin in various dosage form is very important, but direct determination of pregabalin in pharmaceutical dosage form is quite difficult as pregabalin is an aliphatic agent (absence of any chromophoric group). Addition of chromophoric group – derivatization of pregabalin is necessary for effective determination of pregabalin in pharmaceutical dosage form. Derivatization of pregabalin is done with various derivatizing agent – benzyl chloride, ninhydrin, , Gibb's and MBTH reagent 1-Fluoro-2,4-dinitrobenzene for spectroscopic method. For HPLC method, pre coloum and post coloum derivatization is done with Na-5- fluoro-2, 4-dinitrophenyl-5-L-alanine amide, o-phtaldialdehyde/2-mercaptoethanol.

## KEYWORDS

Pregabalin, derivatizing agents, benzyl chloride, ninhydrin, 1-Fluoro- 2,4-dinitrobenzene, o-phtaldialdehyde

## INTRODUCTION

Pregabalin chemically known as [S-[+]-3-isobutyl GABA or (S)-3-(amino methyl)-5- methylhexanoic acid which is structurally and pharmacologically related toGABA[1].First drug which received an approval labeling from Food and Drug Association (FDA) for the treatment of diabetic neuropathy and post-herpetic neuralgia is Pregabalin[2,3]. Preclinical and clinical studies have shown the efficacy of pregabalin either as monotherapy or in combined dosage form with analgesics in managing the neuropathic pain and related symptoms [4, 5]. Pregabalin binds potently to the α2-δ subunit, an auxiliary protein associated with voltage-gated calcium channels in the CNS, attenuating depolarization-induced $Ca^{2+}$ influx in nerve terminals ,reduce nor adrenaline, glutamate, and modulating calcium influx in calcium channels. Pregabalin exhibits linear pharmakokinetics. There is no protein binding or hepatic metabolism. It is renally excreted. No drug—drug interactions have been identified during its development, but may potentiate the effects of lorazepam and alcohol. Pregabalin is approved for treatment of partial seizures and for the treatment of diabetic neuropathy and neuropathic pain from postherpetic neuralgia. Additionally it is useful for physiological conditions associated with alcoholism, psychomotor stimulants, insomnia, gastrointestinal damage, psychiatric disorders such as mania and bipolar disorder, inflammation.[2,3]

Pregabalin is an aliphatic chain without presence of any significant chromophore group, which makes difficulty in its quantification by general HPLC-UV methods. Routine analysis of the drug in bulk powder and pharmaceutical preparations in research laboratories and pharmaceutical industry requires a relatively uncomplicated and a more cost effective method like UV/visible spectrophotometry or spectrofluorometry. Indian pharmacopoeia 2010 reported analytical technique, but only for HPLC method. Therefore, modified method with using derivatizing agents such as benzyl chloride, ninhydrin, Gibb's and MBTH reagent 1-Fluoro-2,4- dinitrobenzene were usually used to make better spectroscopic determination. For HPLC method, pre coloum and post coloum derivatization is done with Na-5-fluoro-2,4-dinitrophenyl-5-L-alanine amide, o-phtaldialdehyde. As a successor of pregabalin has been shown to be effective in several models of neuropathic pain, incisional painand inflammatory pain.The present review summarizes the all derivatives of Pregabalin which is used in effective quantification of drug in dosage form.[1,2,3,4,5]

## FOR SPECTROSCOPIC METHOD:

### Structure of Pregabalin and its suitability for derivatization.

Pregabalin chemically (S)-3-(aminomethyl/)-5-methylhexanoic acid. The molecule is highly statured, aliphatic chain with single bonds except for C=O bond. Such bonding chemistry of Pregabalin, makes it poor candidate for UV light absorption and hence making it almost ineligible for analysis by techniques which explore phenomenon UV radiation such as HPLC-UV or UV-Spectrophotometry. Although presence of COOH group makes its estimation pretty easy by titration from bulk API lots but is unsuitable for analysis pregabalin in the dosage forms which contain interfering excipients.

The structure as shown in the Figure-1, has two functional groups, carboxylic acid (COOH) and primary amine (Nh2), both groups amenable for derivitization. The derivatization of drugs for the purpose of analysis, needs to be simple, cost effective, safe and environment friendly. Most importantly, the dervitizing reactions essentially be short, single step and can be carried out at general laboratory conditions. The presence of both primary amine and carboxylic acid groups in the structure makes molecule more explicable for modification into UV into absorbing species, however considering structural constrains, it appears that exploring amine groups appears to less complicated.

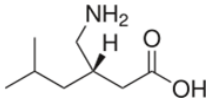



**Figure 1: Structure of Pregabalin**

### Benzoylation of Pregabalin: Derivatization for enabling its analysis.

The benzoylation generally reaction of primary amine with or OH group with benzoyl chloride resulting in the formation of an amide or ester. The benzoylation of phenolic OH requires drastic conditions of high temperatures of 125-175$^0$C with addition of benzoyl chloride. On other hand, benzoylation at primary amine was reported to be relatively less complex [6] [7]

Benzoylation of amines involves, reaction acyl chloride with an amine so that an amide is formed, together with a proton and a chloride ion. Addition of a base is required to neutralize this acidic proton, otherwise the reaction will not proceed. Generally the aqueous solution of a base is slowly added to the reaction mixture to neutralize incoming proton. For zwitterion molecules such as amino acids, base also keeps COOH group ionized and prevents amine group being protonated. General scheme for benzoylation of amines is given below. [8]

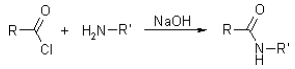



**Figure 2: Scheme for benzoylation of amines.**

The name "Schotten–Baumann reaction conditions" is often used to refer biphasic reaction involving water and an organic solvent. The base within the water phase neutralizes the acid, generated in the reaction, while the starting materials and product remain in the organic phase, often dichloromethane or diethyl ether. The presence of a base prevents the amine reactant from being protonated, which would make it unable to react as a nucleophile. [8]

Pregabalin also has primary amine which makes it fit for benzoylation

reaction. Hence the molecule was explored for this purpose. The pregabalin is allowed to react with benzyl chloride which gives benzoylated derivative of pregabalin. [6] In the study stock solution of pegabalin (1mg/ml) was prepared in methanol. The 1 parts of pregabalin is allowed to react with one part of benzyl chloride in presence of NaOH solution and was kept on stirring for about four hour. Few drops of HCl were added for precipitate out final product. The product was filtered and dried. [6] This benzoylated reaction product is highly UV sensitive and shows maximum absorbance at 223nm. [6] The limit detection was 0.3 µg mL-1 and limit of quantification was 0.87 µg mL-1. [6] In some cases, special attention is dedicated to the optimization of reaction conditions, such as choice of solvent, reagent concentration,[reaction time, molar ratio of the reaction, proposed site of interaction, pH, and temperature. None of such conditions are optimized in this report. Moreover the UV max of 223 is close to some commonly used solvents and specificity of method is not anticipated to be great using such solvents and other excipients which absorb at same wavelength. The lower absorption maxima may due limited very few double bonds that are coming from benzoylation [6]

**Nucleophilic substitution reaction of pregabalin: derivatization for enabling its analysis.**

Ninhydrin (2,2-dihydroxyindane-1,3-dione) which can be considered as the hydrate of indane-1,2,3-trione generally used to detect ammonia or primary and secondary amines. In a ninhydrin reaction system, various parameters are affected toproduct formation and stability and sensitivity of the analysis involving amount of reactant and reaction time, pH of medium and temperature. In that reaction condition require amine group in basic condition with addition of ninhydrin at temperature $70-_{80}0_{C.}$[9] [10]

The carbon atom of a carbonyl group bears a partial positive charge enhanced by neighboring electron withdrawing group. So the central carbon of a 1, 2, 3-tricarbonyl compound will more electrophilic, so indane-1, 2, 3-trione reacts readily with nucleophiles, including water. Ninhydrin will form a stable hydrate of the central carbon because of the destabilizing effect of the adjacent carbonyl groups. [10]

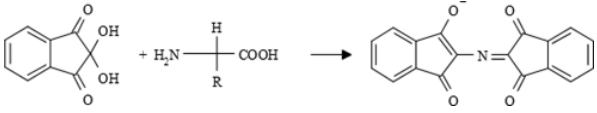


**Figure 3: Scheme for reaction of amines with ninhydrin**

Amine group can condensed with a molecule of ninhydrin and will give Schiff base (2-(1, 3-dioxoindan-2-yl) iminoindane-1, 3-dione). In this reaction, there must be presence of alpha hydrogen to form the Schiff base. Therefore, amines bound to tertiary carbons do not react further and thus cannot be reacted. The reaction of ninhydrin with secondary amines which will also effectively derivatize and form iminium salt, yellow–orange colored product. [10] [12]

Pregabalin also containing primary amine which makes it fit for this reaction. Hence the molecule was explored for this purpose. Formation of purple coloured complex by reaction of pregabalin with ninhydrin by heating at a temperature of 70-75°C for 20 minutes. Reaction involves removal of a water molecule from ninhydrin hydrate will form 1,2,3-indantrione which will form form Schiff's base with the amino group of pregabalin resulting in the ketamine. Removal of the aldehyde RCHO generates an intermediate amine 4 (2-aminol,3-indandione) [13]. Condensation of this intermediateamine with another molecule of ninhydrin follows to form the expected chromophore (Ruhemann's Purple). The rate determining step in ninhydrin reaction is the nucleophilic-type displacement of a hydroxy group of ninhydrin hydrate by a non- protonated amino group. Resulting colored complex which could be effectively measured spectrophotometrically at 402.6 nm[13]. The limit detection was 6.0 µg mL- 1 and limit of quantification was 20.0 µg mL-1. [13]

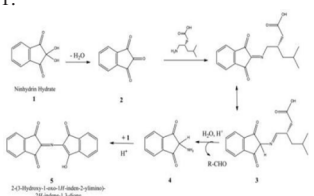


**Figure 3: Scheme of Pregabalin with ninhydrin[7]**

**Michael addition reaction: derivatization for enabling its analysis.**

The Michael addition reaction involve reaction of primary amine or secondary amine with sulfonate group of 1,2-Naphthoquinone-4-sulfonate (NQS) resulting in the formation of highly chromogenic compound.[14] NQS can react in basic medium around pH 10 and moderate temperatures with both primary and secondary amino groups to produce spectrophotometrically detectable derivatives. [14]

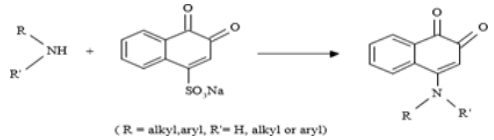


**Figure 4: Scheme for Michael addition of amines.**

Reaction involves, reaction of 1,2-Naphthoquinone-4-sulfonate (NQS) with an amine in basic environment will result in highly coloured product. Basic condition is required to maintain stability of reagent, otherwise reagent instability can be observed. 1,2-Naphthoquinone-4-sulfonate (NQS) which mainly reacts with the guanidino moieties of some aminoglycosides and forms fluorescent derivatives. [14]

Pregabalin also has primary amine which makes it fit for Micheal addition reaction or nucleophilic substitution reaction. Hence the molecule was explored for this purpose. The pregabalin is allowed to react with 1,2-Naphthoquinone-4-sulfonate (NQS) in basic condition which gives derivative of pregabalin. [17][18]

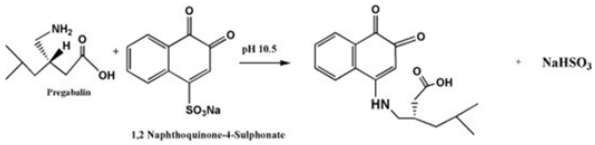


**Figure 5: Scheme of Pregabalin with 1,2-Naphthoquinone-4-sulfonate (NQS) [8]**

In the study working standard solutions of pregabalin were prepared to obtain final concentrations range (2-25µgmL-1) in borate buffer solution having pH 10.5 followed by 1.0 mL of NQS solution (0.5%) were added and shaken and heated in thermostatically controlled water bath at 55 ± 5°C for 10 min. The reaction was stopped by cooling under tap water, and getting final concentration volume with distilled water. Resulting product is highly UV sensitive and shows maximum absorbance at 473 nm against a reagent blank prepared simultaneously. [17][18] The limit detection was 0.14 µg mL/1 and limit of quantification was 0.47 µg mL/1. [17] [18] In that type of reaction, special attention is dedicated to the various experimental parameters involving pH, volume of buffer, concentration of reagent solution, temperature, heating time, diluting solvent, stability of product. [17][18]

**Coupling reaction: derivatization for enabling its analysis.**

The reaction of primary amine with or OH group with Dichloroquinone chloroimide resulting in the formation of highly UV sensitive coloured complex. The reaction requires high temperature with addition of dichloroquinone chloroimide[19][20] Coupling reaction of amines involves, reaction of dichloroquinone chloroimide with amine and will form coloured complex. In this reaction, gibbs reagent couples with amine by elimination of HCl and results in coloured complex measured at specific wavelength.[21][22]

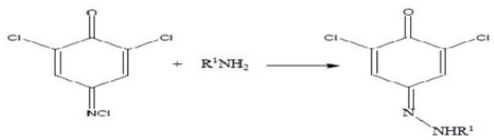


**Figure 6: Scheme for coupling reaction of gibbs reagent with amines**

Additionally, the charge transfer (CT) reactions are also studied in the determination of drugs. This reaction are easy based on complex formation with some electron acceptors. Dichloroquinone chloroimide are strong electron acceptors and can be applied for determination of electron donor drugs.[21][22]

Pregabalin also contain primary amine group, so can be effectively reacted under same condition and can give effective high

chomogenous product showing high UV absorbance. Solution of Gibb's reagent prepared by dissolving specific amount of reagent in methanol (0.5gm in 100ml methanol will give 0.5%). Standard solutions of pregabalin in methanol are prepared having final concentrations were transferred into a series of volumetric flasks. [9]For formation of a coloured complex between 2, 6-dichloroquinone chlorimide (gibb's reagent) and pregabalin, to each flask, add 1.5ml of 0.5% gibbs reagent. Mixture was then kept aside for 5min and heated for 10 min. Contents were diluted up to 10 ml with methanol. Absorbance of each solution was measured at 400 nm against the reagent blank. [22]

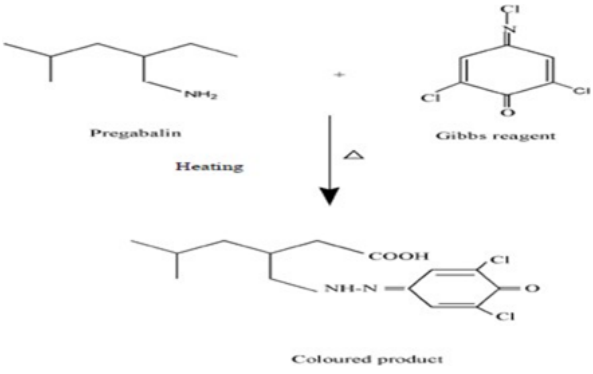



**Figure 7: Scheme for coupling reaction of gibbs reagent with Pregabalin**

This coupling reaction product is highly UV sensitive and shows maximum absorbance at 400nm. [22] Limit of detection was 2.457 µg/ml and limit of quantification was 7.448 µg/ml respectively. [22]

**Oxidative coupling reaction: derivatization for enabling its analysis 3-Methyl-2-Benzothiazoline Hydrazone (MBTH reagent)**

The oxidative coupling reaction of 3-Methyl-2-Benzothiazoline Hydrazone (MBTH reagent) was taken by reaction of 3-Methyl-2-Benzothiazoline Hydrazone in presence of feCl3 as catalyst. Optimum reaction condition required for effective oxidativecoupling reaction involved 1% FeCl3 and 2ml of 0.5% MBTH at ambient temperature and resultant product will remain stable for at least 35 minutes.[22][23]

Reaction involves oxidative coupling of 3-Methyl-2-Benzothiazoline Hydrazone (MBTH reagent) by iron, thus MBTH loses 2 electrons and one proton forming an electrophilic intermediate, which will react as active coupling species. This intermediate undergoes electrophilic substitution with amines and will form coloured product. General scheme for oxidation of 3-Methyl-2-Benzothiazoline Hydrazone is below. [23]

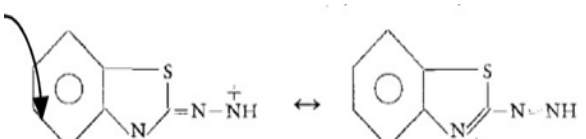


**Figure 8: Scheme for oxidation reaction of MBTH reagent**

Pregabalin has also primary amine which makes it fit for this oxidative coupling reaction. Hence the molecule was explored for this purpose. MBTH solution was prepared by dissolving specific amount of reagent in distilled water reagent (0.5gm in 100ml distilled water will produce 0.5% w/v) .The pregabalin is allowed to react with $^{3}$Methyl-2-Benzothiazoline Hydrazone in presence of FeCl3 which will form electrophilic intermediate, this intermediate undergoes electrophilic substitution with the drug to form the colored product. Standard solutions of pregabalin was prepared in methanol, followed by addition of 2 ml of MBTH, 2 ml of ferric chloride was added and the volume made up to the final mark with distilled water and allowed to stand for 20 minutes. The green colored complex was developed and stable for 2 hrs. [22] Maximum absorption at 668 nm was obtained. Limit of detection was 2.3665 µg/ml and limit of quantification was 7.1714 µg/mL [22]The optimum conditions for the reaction were carefully studied. Reproducible results were obtained in the temperature range of 20–40 °C [22].The reaction between the MBTH reagent and pregabalin was represented in scheme 9.

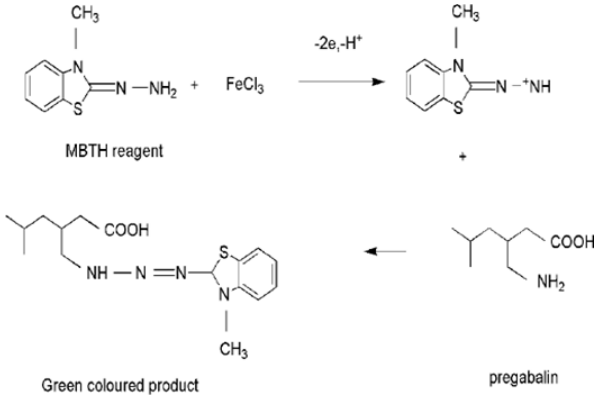



**Figure 9: Scheme for oxidative coupling reaction of MBTH reagent with pregabalin**

**2,3-Dichloro-5,6-dicyano-1,4-benzoquinone and 7,7,8,8 tetra-cyano- quinodimethane**

2,3-Dichloro-5,6-dicyano-1,4-benzoquinone reagent is also undergoes oxidative coupling reaction with primary amine.

The oxidative coupling generally reaction of primary amine with or OH group with **2,3-Dichloro-5,6-dicyano-1,4-benzoquinone or** 7,7,8,8-tetracyanoquinodimethane resulting in the formation of an highly colored complex species.. The oxidation of phenolic OH requires drastic conditions of high temperatures of 60$^{0}$C with addition of specific reagent for 1 hr.

Reaction involves primary amine group as n-electron donors with 2,3-dichloro-5,6- dicyano-1,4-benzoquinone (DDQ) and 7,7,8,8-tetracyanoquinodimethane (TCNQ) as π-acceptors which gives highly colored complex species, as π-acceptors such as TCNQ and DDQ are known to yield charge transfer complexes and also radical anions with a strong electron donors.[23][24]

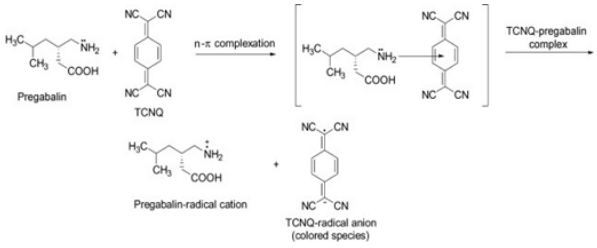



Figure 10: Scheme for oxidative coupling reaction of TCNQ reagent with pregabalin Pregabalin also contain primary amine which makes it fit for this oxidative coupling reaction. In this reaction, pregabalin standard stock solution was prepared in acetonitrile and 1.5 mL of DDQ or 2 mL of TCNQ solution were added. The volume was brought to 1.5 mL (for DDQ) and 0.5 mL (for TCNQ) with acetonitrile and 1.5 mL of DDQ or 2 mL of TCNQ solution were added. For DDQ method, reaction mixture was heated for 15 min at 60 ℃ and allowed to stand for 5 min at room temperature, whereas for the TCNQ method, specific colour development was obtained after heating on a water bath at 60 ℃for 15 min. After cooling and diluting up to 5 mL with acetonitrile final absorbance was measured at 494 and 841 nm for DDQ and TCNQ, respectively. For DDQ, limit of detection was 0.343 µg/ml and limit of quantification was 1.145 µg/mL, whereas for TCNQ limit of detection was 0.016 µg/ml and limit of quantification was 0.055 µg/Ml.[23]The colour remained stable for 8 h for these reagents. TCNQ was superior as compared with DDQ reagent according to higher molar absorptivity and lower detection limits. The described methods are suitable for the assay of pregabalin in pharmaceutical formulations without interference from excipients. It can be easily applied to variety of quality control laboratories for the routine analysis of pregabalin in raw materials as well as in pharmaceutical formulations. [23][24]

**Substitution reaction**

**Tetracynoethylene**

The substitution reaction of Tetracynoethylene was taken by reaction of tetracynoethylene in acetonitrile at room temperature. Optimum reaction condition required for effective substitution reaction involved reaction of cyno group with amine groyp at 20$^{0}$C with addition of MeCN. [24]

Substitution of tetracynoethylene involves, intramolecular charge transfer from the amino nitrogen atom of the amidine moiety to the cyano group. So, the resulting complex molecule will be an asymmetric chromophore exhibiting second order nonlinear optical properties. Addition of amines to a cyano group is facilitated by the presence of electron- acceptor in the α-position with respect to that group. General scheme for reaction with amines is given below. [24]

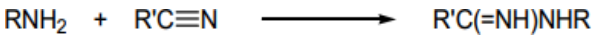



Figure 11: Scheme for reaction of cyno group with primary amine Pregabalin also has primary amine which makes it fit for this reaction. Hence the molecule was explored for this purpose. The pregabalin is allowed to react with tetracynoethylene which gives substituted derivative of pregabalin. [6] Specific procedure for derivatization of pregabalin with this method still not reported. But resulting complex could give maximum absorbance in range of 300 – 700 nm.[24]

**Dehydrogenation Reaction/ Nucleophilic Substitution Reaction:**

Dehydrogenation reaction involves reaction of 2,3-Dichloro-5,6-dicyano-1,4- benzoquinone/ Chloranil with primary amine and will form 2, 5-bis-amino derivatives with specific temperature condition. Optimum reaction condition require $20^0$ C temperature for 24hr with 78% humidity. [25]

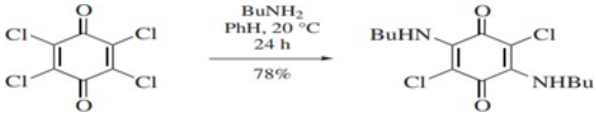



**Figure 12: Scheme for substitution reaction of chloranil with primary amine**

Pregabalin also containing primary amine which makes it fit for this reaction. Hence the molecule was explored for this purpose. Formation of yellow coloured complex by reaction of pregabalin with chloranil by incubation at a temperature of 20°C for 60 minutes. Reaction involves chloro atom substitution by amino group present in pregabalin, resulting in formation of yellow coloured chromogen. The formation of charge transfer complexes (n→π) is favored in the presence of organic polar solvents and in absence of basic buffers. Resulting highly UV sensitive colored complex which could be effectively measured spectrophotometrically at 352 nm [25] The limit detection was 0.18 µg mL-1 and limit of quantification was 0.6 µg mL-1. [25] This methods can be recommended for routine analysis of pregabalin where sophisticated equipments are unavailable. This proposed method is simple, accurate and less tedious than chromatographic procedures. Considering the limits of detection and/or concentrations ranges, the proposed method is more sensitive than other previously published methods including spectrop hotometric and spectroflurimetric methods. There is no specific interference from added excipients, additives, co administrated drugs, which can consider as advantage of proposed method. So, this advantage encourage the application of developed method in routine analysis of drug. [25]

**Nucleophilic Substitution reaction**

2,4-Dinitrophenylhydrazine (2,4-DNP or 2,4-DNPH) reacts readily with aldehydes and ketones through condensation reaction to produce the corresponding hydrazone. The hydrazine is a brightly colored yellow, orange or red compound, which will act as derivatized molecule. Optimum condition required for the reaction 2 minutes at room temperature and resulting complex will stable for 40 mins after complex is formed.

Nucleophilic substitution reaction involves, reaction of 2,4-Dinitrophenylhydrazine with carbonyl group, so that an hydrazone is formed(the lone pair of electrons on the terminal amino group in 2,4-DNPH makes it a strong nucleophile and the condensation starts by the nucleophilic 2,4-DNPH attacking the electrophilic carbonyl carbon), General scheme for nucleophilic substitution of carbonyl is given below. [8]

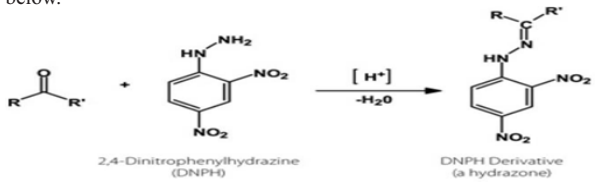



Figure 13: Scheme for reaction of 2,4 dinitrophenylhydrazine with carbonyl group Pregabalin also containing carbonyl group which makes it fit for this reaction. Hence the molecule was explored for this purpose. Standard solution of pregabalin 100 µg/ml was prepared. Addition of 2,4 DNP with simultaneously addition of potassium iodate and sodium hydroxide solution. So, nucleophilic addition of the -NH2 group to the C=O, followed by the elimination of a H2O molecule was take place. Reaction between pregabalin and 2, 4-DNP was completed within 2 minutes at room temperature and the absorbance will remain constant for up to 40 minutes and can be detected at 461 nm. [18] The limit detection was 1.906 µg mL-1 and limit of quantification was 5.77 µg mL-1. [18] It was found that the absorbance of the resulting complex chromogen remains stable for 1 hour. This will increased the convenience of the methods and made it applicable for large number of sample treatment.[18]

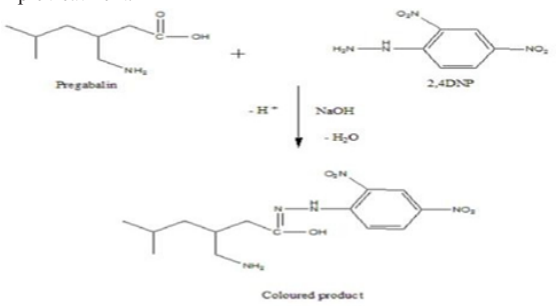



**Figure 14: Scheme for reaction of 2,4 dinitrophenylhydrazine with pregabalin**

**Hydrolysis reaction/ carbonyl substitution reaction**

Hydrolysis reaction/ carbonyl substitution reaction is a reaction of primary amine with or OH group with fluorescamine (4-phenylspiro[furan-2(3H),1''-(3'H)-isobenzo furan]-3,3′-dione) resulting in formation of fluorophors. Carbonyl substitution of fluorescamine requires basic environment with pH range 8 to 8.5 at room temperature to yield highly fluorescent yellow product .[25][26][27]

This reaction involves, reaction of fluorescamine with amine in presence of basic pH so that imines will formed. An imine is a nitrogen analog of ketone in which the C=O group is replaced by a C=NR group, where R = alkyl, aryl, or H. The mechanism for imine formation begins as a nucleophilic addition to the carbonyl group present in fluorescamine resulting in formation of fluorophors.

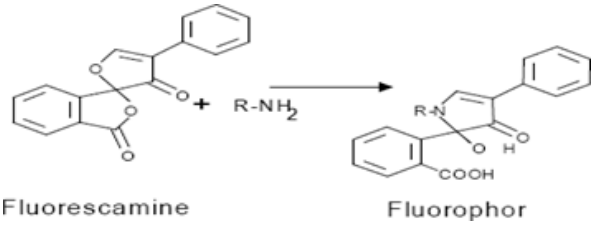



Figure 14: Scheme for reaction of fluorescamine with primary amine Pregabalin also containing amine group which makes it fit for this reaction. Hence the molecule was explored for this purpose. In the study stock solution of pregabalin (1mg/ml) was prepared in borate buffer solution of pH8.5. The 1 parts of pregabalin is allowed to react with one part of fluroscamine in basic medium and were left for 25 min. at room temperature .[25] This reaction product is highly UV sensitive and shows maximum absorbance at fluorescence was measured at 487 nm. [25] The limit detection was 3.2 × 10-3 µg mL-1 and limit of quantification was 9.6 × 10-4 µg mL-1. [25] In some cases, special attention is dedicated to the optimization of reaction conditions, such as reagent concentration, reaction time (30 min), pH of medium. The resulting fluorescence increases almost linearly by time till reaching a maximum at 30 min and will remaine stable for at least 2 hours at room temperature.

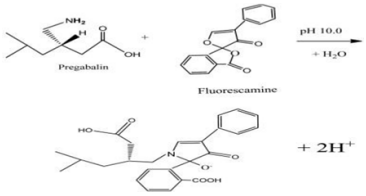



**Figure 14: Scheme for reaction of fluorescamine with Pregabalin**

The proposed method has the advantage of being rapid, simple, and sensitive, with low cost and with no need for prior extraction procedure. So it is well suitable for routine analysis of pregabalin in pregabalin in pharmaceutical preparations in laborateries. [25][26]

**Hplc Column Derivatization**

HPLC is the most popular method for detection of most of the components, which have currently gained attention due to the boom in health foods.UV detection most cases requires using the absorption of the carboxyl group (-COOH) in the 200 to 210 nm range. Some compounds with benzene rings can be detected in the 250 to 300 nm range, it require special reaction procedure and attention. Consequently, pre column or post column derivatization procedure widely used. Since many compounds contain amino groups (-NH2 or -NHR) in their structures, a derivatizing agent that can be selectively react with amino present in compound structure. Mainly two types of derivatization methods- Pre-column derivatization and post column derivatization are used according to compound characteristics.
[ 28][29][30]

**Pre-column derivatization**

Compounds or drugs which are derivertized before injection, and then the reaction products are separated and detected. This concept known as pre column derivatization. Advantage of this method involve less amount of reagent used, increasing sensitivity of drug thus increasing reaction efficiency, unreacted derivatizing agent can be separated in column.

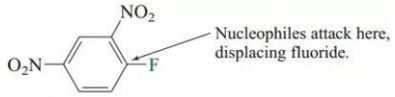
Some disadvantage are also there because the derivatizing reagent is mixed directly with the sample.So, Pre-column derivatization can be considered appropriate for many drugs.Generally, RP-HPLC technique is widely used to separate reaction component. But in some cases, RP -HPLC method is not well suited for separating highly-hydrophilic component such as amino acids. [32]

**Precolumn derivatization involve some reaction for derivatization which include, Nuceophilic substitution reaction in which reagents are used as below,**

**1-fluoro-2, 4-dinitrobenzene**

1-fluoro-2, 4-dinitrobenzene effectively reacts with the amine group in mild basic condition and hydrolysis of the reagent is also occur in alkaline condition. In this type of derivatization reaction most reactive site is carbon which is strongly electropositive because fluorine is attached with carbon atom so, fluoro group of 1-fluoro-2, 4-dinitrobenzene react with N terminal group of drug. Sanger's reagent has also been used for the rather difficult analysis of distinguishing between the reduced and oxidized forms of substance in biological systems in conjunction with HPLC. [33][34]

1-fluoro-2,4-dinitrobenzene(Sanger's reagent) which is chemically trisubstituted, highly activated benzene ring towards nucleophilic aromatic substitution, because all three groups are electron-withdrawing - fluoride is a mildly activating group and an *ortho/para*-director, and the nitro groups in the *ortho*- and *para*- positions to the fluoride are very strongly activating.[34]




1-Fluoro-2,4-dinitrobenzene

**Figure 10: structure of 1-fluoro-2, 4,-dinitrobenzene**

Pregabalin also has primary amine which makes it fit for Nuceophilic substitution reaction. Hence the molecule was explored for this purpose. The pregabalin is allowed to react with 1-fluoro-2, 4-dinitrobenzene which gives substituted derivative of pregabalin. FDNB with primary amines is usually carried out in mild basic medium leads to hydrolysis also. [35] FDNB (0.06M) was prepared by dissolving 1.14 g of the reagent in 100mL of acetonitrile. Appropriate amounts of H3BO3 and KCl were dissolved in water and the pH was adjusted to basic (pH 8.2) by adding 2M NaOH for preparation of 0.25M borate buffer. All solutions were stored at $4^0$C until analysis.

[35] This substituted reaction product is highly UV sensitive and shows maximum absorbance at 360nm. [35] Limit of quantification was1 ng/ml. [35] The time for reaction was exceeded to 20 min for achieving better results. [35] Careful performance for experiment is highly required because 1-fluoro-2, 4-dinitrobenzene is highly skin irritant. Additionally amount of reagent should also be carefully decided as increasing amount of reagent peak area of drug substance will simultaneously increase .Reaction time and temperature also carefully maintained throughout the experiment because it may affected to reaction($65^0$ C for 10 min and $90^0$ C for 5 min).So this reaction require extra attention to its procedure.[35]

**Na-5-fluoro-2, 4 dinitrophenyl-5-l-alanine amide (FDNPAA)**

Nucleophilic substitution reaction generally involves reaction of primary amine with Na-5-fluoro-2,4 dinitrophenyl-5-l-alanine amide (FDNPAA) resulting in formation of diastereomers. Nucleophilic substitution of Na-5-fluoro-2, 4 dinitrophenyl-5-l-alanineamide (FDNPAA) require specific condition of temperature $40^0$C for 60 min with addition of primary amine.[36-39]

Substitution reaction of amines involves, reaction of Na-5-fluoro-2, 4 dinitrophenyl- 5-l-alanine amide with an amine so that diastereomers are formed, due to the stereogenic center that is present in structure of reagent. This makes an indirect enantio separation by means of RP-HPLC. Additionally, elution of enantiomers depends on the stronger intramolecular H-bonding present in both structure.(Generally, D-derivatives shows stronger intramolecular H-bonding which leads to more hydrophobic molecules and thus showing a stronger affinity for the stationary phase on reversed phase columns.) .[36-39]General scheme for reaction of amine is given below

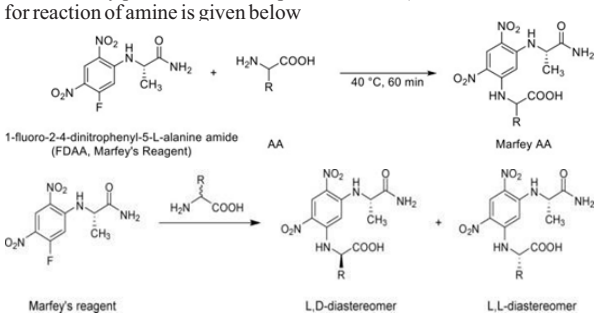



**Figure 11:** Scheme for substitution reaction of FDAA and formation of disteriomers Pregabalin also contain primary amine which makes it fit for this reaction. Hence the molecule was explored for this purpose. The pregabalin is allowed to react with marfey´s which gives derivative of pregabalin. .[36-39] In this study solution of pregabalin was prepared by weighing 80 mg of Pregabalin and dissolved in 2 mL of 1mol/L HCl (pH was set to 7.0 with NaOH solution, up to 10 mL by distilled water.

Solution of FDNPAA was prepared by dissolving 100.0 mg FDNPAA in acetone and stored in the refrigerator, and 20 µL NaHCO3 were added. The solution was closed and heated for 1 hr at 40°C, then HCl was added, and the solution was dried in vacuum desiccation oven with P2O5/KOH. The residue was dissolved in 4 mL DMSO (equivalent to100.0 µg/mL Pregabalin sample). [39] Resulting derivative was determined at 340 nm. [39]

Limit of detection was $1.1 \times 10^{-8}$ g/ml and limit of quantification was $3.3 \times 10^{-8}$ g/ml [39]

**Postcolumn Derivatization**

Post-column derivatization method involves concept for separating the drugs in the column, then delivering and mixing the derivatizing reagent to react with the drugs, before reach to detector. Advantage of this method include excellent quantitative performance, reproducibility, sample component can be separated before reaction, reaction efficiency is less.

Difficulty in increasing sensitivity and high consumption of reaction reagent, which is kept constantly flowing to the detector so, this method is limited by not allowing detection of unreacted reagent in this method can be considered as its disadvantage. The method most commonly used for separation is cation exchange chromatography.[28][29][31]

Post column derivatization method includes some reaction, reduction, hydrolysis, nuclephlic substitution which is done mainly by using one commonreagent**o-phtaldiadehyde**

**Reduction reaction/ hydrolysis reaction/ Nucleophillic substitution**

In this type of hydrolysis reaction, o-phtaldiadehyde reacts specifically with primary amines above their isoelectric point in presence of thiols.

Reaction involves reaction of primary amine or OH group with o-phtaldiadehyde in presence of reducing agent(mercaptoethanol) resulting in formation of fluorescent compound. Resulting complex reported to be more stable.

This reaction involves, reaction of o-phtaldiadehyde with amine in presence of mercaptoethanol so that imines will formed. An imine is a nitrogen analog of an aldehyde or ketone in which the C=O group is replaced by a C=NR group, where R = alkyl, aryl, or H. [41-44]

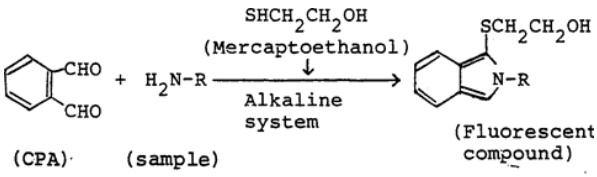



**Figure 12: Scheme for hydrolysis reaction with amines**

The mechanism of imine formation begins with nucleophilic addition to the carbonyl group. In this reaction, the nucleophile is the amine, which reacts with the aldehyde and give an unstable product called a carbinolamine. A carbinolamine is a compound with an amine group and a hydroxy group on the same carbon. Carbinolamines are not isolated, but undergo acid-catalyzed dehydration and will form imines. (Imines are sometimes called Schiff bases or Schiff's bases) Pregabalin is also act as primary amine which makes it fit for this reaction. Hence the molecule was explored for this purpose. The pregabalin is allowed to react with o phthaldihyde which gives fluorescent derivative of pregabalin. [41-44] In this study thiol derivative of pregabalin is very simple and fast. Rection proceed to complete in 1 min at room temperature with using marcaptoethanol as reducing agent. Capillary coil reactor show best performance. Residence time in post column reactor should be at least 60 sec to achieve substantial enhancement of detector response. The increase in residence time was achieved by linear decrease of derivatizing agent. Derivatizingreaction highly depend on pH of reaction medium (It should be above pH 9) UV absorption of the pregabalin derivative was determined at 345 nm[41-44] Limit of detection was 4.8 µg/ml and limit of quantification was 16 µg/ml [41-44] Due to simplicity of reaction and short time period, high stability of complex mainly used this derivatizing agent.

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