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Aims and Scope

Journal of Applied Chemistry is a peer-reviewed, journal that publishes original research articles as well as review articles related to all aspects of applied chemistry. These includes the fields of analytical, inorganic, organic, physical and applied chemistry area. Review articles discussing specific areas of chemistry of current chemical importance are also published.

The journal welcomes publications of high quality papers on theoretical developments and practical applications in applied chemistry. Original research papers, state-of-the-art reviews, and high quality technical notes are invited for publications.

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Formulation of Paint using Natural Pigment from Lawsonia Inermis Leaves

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ABSTRACT

The increased application of paint in coating industry for interior and exterior decoration and corrosion inhibition, has inspired research in paint formulations. However, due to the toxic effect of preparatory chemicals used in paints, and the restrictive environmental legisla- tion, research efforts have been directed to a more green process. In this work colouring matter from Lawsonia inermis leaves were ex- tracted using water and analyzed by TLC and FTIR spectroscopy. White emulsion paint was formulated using pigment volume concen- tration (PVC) of 0.07%, the colouring matter extract was dispersed providing colour for the paint. The paint produced with the natural colourant was found to have similar properties with the paint produced using commercial pigment. Paint produced with aqueous extract faded under exposure to 5000 watts Tungsten Lamp after 48 hours but showed excellent fastness to rubbing. The extract induced excel- lent brownish colouration in the paint which maintained excellent, opacity, adhesion and good water resistance.

Keywords: Paint; Paint Formulation; Lawsonia Inermis Leaves; PVC; TLC; FTIR.

1. INTRODUCTION

An acceleration of the rate of scientific discovery had a growing impact on the development of paints from the eighteenth century to the present day. The quality of our environment is deteriorating day by day due to the threat impose by harmful chemical substances as a result of industrialization, in which largest cities reaching saturation points are unable to cope with the increasing pressure on their infra- structure (Lakherwal, 2014). Paints can be defined as a fluid, with viscosity, drying time and flowing properties dictated by formulation, normally consisting of a vehicle or binder, a pigment, a solvent or thinner and a drier which may be applied in relatively thin layers and which changes to a thin opaque continuous layer on surfaces (Nwakaudu and Oghome, 2012). Several types of paints have been classi- fied, among which include solvent (water and oil) based paint and substrate (exterior, interior, automotive, marine and industrial) based paint (Adamu et al., 2014). Paints are used for decoration and also play a vital role in protecting major assets from the natural processes (Suma et al., 2009). The constituents generally used for the production of emulsion house paints include pigments, solvents, extenders, binders and additives (Abdulsalam and Yahaya, 2010). Due to perceived safety and physiological advantage of the natural colourants over synthetic ones, interest are being geared into search of new natural colourants and the verification of the safety of existing ones (Duangmal et al., 2004). They are also used for protecting surfaces against environmental effects like UV-radiation, chemical invasion and mechanical stresses (Oladipo et al., 2013). Due to increasing use of synthetic colourants which are mostly toxic as well as for envi- ronmental safety, it has become imperative to develop alternative source with similar properties (Omojola and Alabi, 2013). Poly (vinyl acetate) the widely used binder in paint formulation suffers poor hydrolytic stability especially under alkaline condition, poor weathering stability, poor wet adhesion and high-water sensitivity. It has only limited application in exterior coating because of the high Tg (30°C)

and minimum film forming temperature (MFT) 20 °C making its film too hard and inflexible limiting its use in paint application (Suma et al., 2009).

Henna plant is a tall flowering tree standing about 5 m tall, native to tropical and subtropical regions of Africa, Asia and Northern nations and it's considered as harmless. The well-known compound is Lawsone occurred in the henna plant leaves. Phytochemists gave the com- pound the trivial name Lawsone due to its origin, the henna plant Lawsonia inermis. Lawsone is an intact glycosidase, able to split the glycosidic bond, when brought into contact with hot water. Therefore, Lawsone has been extracted by means of maceration, digestion and infusion (Mahkam et al, 2014).

There were number of works conducted by different researchers using locally available source materials to produce emulsion paint. Therefore, this present work focuses on formulation, characterization and application of an emulsion paint using poly (vinyl pyrrolidone) as binder and natural colouring matter from Lawsonia inermis leaves to provide colour for the paint. 156

2. MATERIALS AND METHODS

Chemicals and Materials

Dried Lawsonia inermis leaves was purchased from Rimi market, Kano State of Nigeria. Poly(vinylpyrrolidone) (Sigma Aldrich), Cal- gon; Sodium polymetaphosphate (PubChem: 24968) obtained from Clear Paint and Chemicals Company Limited, Technology Incuba- tion Center, Kano, was used as water softener, Hydroxylethylcellulose (Ashland Specialty Chemical), was used for thickening purpose. Calcium carbonate (Specialty Minerals), was used as extender and Titanium dioxide (Winchems) was used for pigmenting. Ammonia (BHD), and biocide (Medimark Scientific Ltd) were purchased from Clear paints and used as preservatives. In order to prevent foaming, Anti-foaming agent was used. All chemicals and solvents used were of analytical grade and were used without further purification. The glass wares were washed with chromic acid and detergent, rinsed with distilled water and dried in oven before used.

Extraction of Colouring Matter from Lawsonia Inermis Leaves

Hot water extraction procedure was adopted (Saeed et. al., 2013) with little modification in the solid: solvent ratio. Here 100 g each of the plants was soaked in 1000 ml distilled water and heated at 100 °C for 45 min under stirring. The system was then allowed to cool and filtered using a whatman filter paper to remove insoluble foreign particles. The filtrate was then concentrated using rotary evaporator from which the rotary flask was filled with the extract, and mounted in the rotor vapour, the machine was adjusted to insert the evaporat- ing flask into water bath, which was set at 60 °C. A vacuum pump was connected with the rotor vapour which exert the required pressure 72 atm. A recycling chiller was attached with the condenser which condense the vapour and collected via receiving flask. The samples was further dried using freeze drying machine and kept air tight container for further analysis.

Characterization

Determination of Extract Percentage Yield

The yield of the plant extract were determined as reported elsewhere (Anokwuru et al., 2011) using the formula576i9c

$$\frac{W_2 - W_1}{W_o} \times 100\ \%$$

Where: W_2 is the weight of the extract and the container,
W_1 the weight of the container alone and
W_0 is the weight of the initial dried sample.

FTIR Studies

The chemical functional groups of the extract were revealed using the Fourier transformed infra-red spectroscopic technique. Sample was directly placed on a cuvette and the transmittance spectrum of both the extract and the formulated paint were recorded using Shimadzu FTIR model No. 8400S.

TLC Separation and Analysis

The various components of the extract were separated using Thin Layer Liquid Chromatography. An aliquot of the extract was dissolved in ethanol to produce a very dilute (about 1%) solution. A small amount of the solution was transferred on one end of a TLC plate which quickly evaporates leaving behind small spot of the extracts on the TLC plate. The spotted end was placed into a beaker containing four different solvents (N-Hexane, ethyl acetate, chloroform and methanol), which then travels up the plate from the original spot by capillary action. The spots travelled almost to the top of the plate. Three different colours were observed; green, light and dark brown.

Paint Formulation

The paint formulation was initiated by first dissolving 2.5 g of sodium polymetaphosphate (Calgon) in 750 ml of distilled water. To this solution, 37.5 g of (TiO_2) was added and shaked vigorously in a plastic container. For the purpose of gloss 325 g $CaCO_3$ was added to the mixture and stirred for 20 minutes. A specified amount of Poly (vinylpyrrolidone) was added as binder under stirring condition. For thickening purpose 5 g of Hydroxylethylcellulose was added. Finally, the preserving agents were added in equal proportion.

Paint Property Assessments

Drying Time

The drying time of the formulated sample was determined. The paint sample was applied on a substrate (interlocks) and drying time was determined by exposure to air and recorded.

Weathering Test

Weather ability (i.e. outdoor durability) of emulsion paint coating like other coating systems is expressed as change in gloss, opacity, colour retention, vertical wall sagging, rubbing, resistance to staining and light fastness test. All these processes are influenced by nature, like solar radiation, temperature, moisture, chemical pollutants, oxygen, and microorganisms. The sample of the formulated paint pattern was exposed in an open air during the weathering test for 240 hrs.

Fastness Tests

Determination of Light Fastness

The test was carried out on the painted samples with use of a 500 watts fading lamp. The procedure involved aligning both the eight blue standards and the painted samples, until the last specimen faded. The rating of the painted samples was carried out to assess the degree of fading.

Determination of Rubbing Fastness

Rubbing fastness is the resistance of the dyed material or a paint substrate to rubbing. In the present research the test was conducted on the painted interlock blocks both in dry and wet condition.

3. RESULTS AND DISCUSSIONS

Percentage Yield of the Plants Extract

The initial weight of the container alone $W_1 = 34.1274$ g

Weight of the initial dried sample $W_0 = 100$g

Weight of the extract + weight of the container $W_2 = 27.6742 + 34.1274 = 61.8016$g

Therefore % yield of the *Lawsonia inermis* plant $= \frac{61.8016 - 34.1274}{100g} \times 100$

$= 27.6742\%$

Determination of Pigment Volume Concentration (PVC)

PVC for 37.5g of pigment: The pigment volume concentrations (PVC) for the formulated paint was determined. The PVC for 37.5g of pigment was found as represented from the table below. From table 1, emulsion paint was prepared at the PVC of 0.07 (7%) CPVC. CPVC which is the stage where air occupying the interstitial spaces of the pigment has been displaced by binder. Any increase in pig- ment concentration above CPVC level lead to voids in the paint film with a consequent increase in permeability, and below CPVC leads to the separation of pigment particles from each other. The CPVC increases with increasing amount of dispersing agent indicating that the close packing of pigment particles is intimately associated with degree of dispersion. When the pigment dispersed on the formulated paint is below the critical pigment volume concentration, the paint may become rucked in patches as a result of efflorescence (Udeozo et al., 2013).

Table 1: PVC (Pigment Volume Concentration) for 37.5g of TiO_2 Pigment

Component	Weight	Density	Volume
Pigment	37.5	4.23	8.86
PVP	125	1.2	104.16

$$PVC = \frac{V_P}{V_p + V_b}$$

Where V_P and V_b are the Pigment volume and volume of the binder respectively.

$$PVC = \frac{8.86}{8.86+104.16} = \frac{8.86}{113.02}$$

$= 0.07$

CPVC = 0.07 × 100

CPVC = 7%

Thin Layer Chromatographic Technique of the Plant Extracts

The Thin Layer Chromatographic Technique (TLC) of the plant extracts was conducted. The number of components in the mixture were traced based on the colours appear on the TLC plate by observing their appearance, where the hues obtained due to stationary and mobile phases were clearly seen.

The results presented here were obtained from Thin Layer Chromatography (TLC), which is a method for analyzing mixtures by separat- ing the compounds in a mixture. TLC can be used to help determine the number of components in a mixture, the identity and purity of compounds. The number of components in the mixture were traced based on the colours appear on the TLC plate by observing the ap- pearance of a product or the disappearance of a reactant. It can also be used to monitor the progress of a reaction. Thin Layer Chromatog- raphy (TLC) is a sensitive technique from which microgram (0.000001g) quantities can be analysed and it takes little time for an analysis (about 5-10 minutes). Figure 1 present the TLC of Lawsonia inermis where the colours obtained due to stationary and mobile phases were clearly seen.

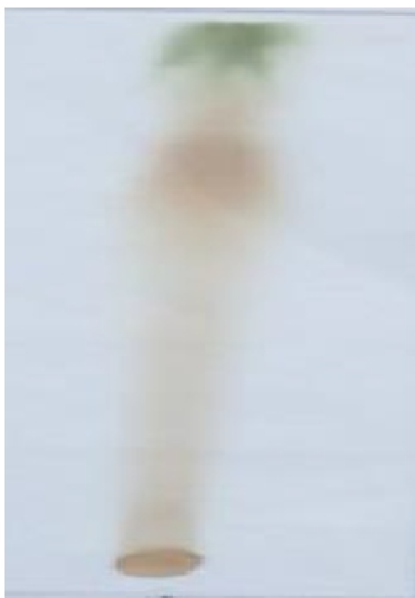

Fig. 1: TLC Image of L. Inermis Leaves Extracts Showing Different Components.

The figure shows three (3) colours from Lawsonia inermis namely; green, light and dark brown. When the extracts were mixed with pol- yvinylpyrrolidone as a binder and other additives in emulsion paint formulation, brown colour was observed from Lawsonia inermis, while the remaining colours were not seen. It was also observed that there is good compatibility between the extracts and the binder dur- ing paint formulation.

FTIR Spectral Characterization of the Plant Extracts and the Formulated Paints

The results presented here were based on the use of Fourier Transform Infra-red Spectroscopy (FT-IR) Machine from which the exami- nation of the spectra reveals the presence of some functional groups found on Lawsonia inermis Extract.

Table 2 also revealed the FTIR spectrum of Lawsonia inermis leaves extract. Peaks observed at 3402 cm^{-1} was due to O-H stretching while that at 2924cm^{-1} was attributed to C-H stretching (sp3). Other peaks at 1635 and 1141cm^{-1} were due to the presence of C=C con- firming their presence from the structure of the active compound of henna leaves. FTIR Spectrum of the white emulsion paint was repre- sented in table 3. The characteristics peaks found at 3448 and 2931cm^{-1} were due to O-H stretching and C-H stretching (sp3). All the peaks were present even after paint formulation confirming that there was no chemical reaction during the formulation.

Lastly, result from Table 3 highlight the FTIR of emulsion paint formulated with Lawsonia inermis leaves extract as colour impacting agent. Characteristic peak at 3410cm^{-1} was indicating O-H stretching, a sharp peak at 2931cm^{-1} was due to the presence of C-H stretching (sp3) and peak at 1419cm^{-1} band was attributed to C-C from the aromatic ring of benzene of Lawsonia inermis leaves extract.

Table 2: Summary of FTIR Results of Extracts from Lawsonia Inermis Leaves

Peaks	*Lawsonia inermis*	Functional Group Assignment
1	3402	O-H
2	2924	C-H (sp^3)
3	1635	C=C
4	1141	C=C

Table 3: Summary of FTIR Results of Emulsion Paint Samples

Peaks	Emulsion paint	Functional group Assignment	Emulsion paint with *Lawsonia inermis*	Functional Group Assignment
1	3448	O-H	3410	O-H
2	2931	C-H (sp^3)	2931	C-H (sp^3)
3			1419	C-C (Ar)

Application of Formulated Paint

The performance characteristics of the paints were determined in terms of drying time, opacity, adhesion, resistance to rubbing, pigment volume concentration (PVC), light fastness and hue observed where recorded in Table 4. The paint formulated coloured with the extract were applied on interlock blocks and dried at room temperature.

Hue Observed

Three different hues on the TLC of Lawsonia inermis namely; green, light and dark brown, respectively were observed.

Drying Time

Drying or curing time of the paints on interlock blocks was observed and recorded. From the result obtained, white emulsion paint dry fast at 8mins, while emulsion paint coloured with Lawsonia inermis extracts dries at 15mins. This indicate that Lawsonia inermis extract delays the drying of the paint to some extent due to oily nature of the extract.

Adhesion

The paints samples were applied on interlock blocks and adhesion of the paint on the substrate was observed visually from which all the paint samples; White emulsion paint and paint coloured with the extracts from Lawsonia inermis shows excellent adhesion.

Opacity (Hiding Power)

From the result obtained opacity (hiding power) of the paint was measured by visual assessment; five different observers rated the degree of opacity of the paint formulations. From the formulations of the paints white emulsion paint shows excellent opacity while emulsion paint coloured with Lawsonia inermis extracts indicate good hiding power when applied on the interlock blocks.

Light Fastness Property

The light fastness test was carried out on the paint samples using cupboard paper, from which the formulated paint coloured with Law- sonia inermis extract having grade 4 comparing with the 8 blue wool standard.

Light Fastness Rating Key: 1= Poor; 2= slightly poor; 3= Moderate; 4= Fair; 5= Good; 6=Very good; 7= Outstanding; 8= Excellent

Rubbing Fastness Property

The obtained results deduced that both at dry and wet conditions the paint samples tested for staining white cotton fabric have good re- sistance to rubbing, from which the formulated paint with Lawsonia inermis had excellent fastness both in dry and wet conditions with rating 5.

Rubbing Fastness Rating Key: 1= Poor; 2= Fair; 3= Good; 4= Very good; 5= Excellent.

Table 4: Paint Property Assessment

Paint Samples	Hue	Adhesion	Opacity	Drying Time (min)	Water Resistance	Light fastness	Rubbing fastness
White Emulsion Paint	White	Excellent	Excellent	8	Excellent		
With Lawsonia inermis	Brown	Excellent	Excellent	15	Good	4	5

4. CONCLUSION

In this research, paint was successfully formulated by the use of Lawsonia inermis leaves extract and has also been explored to provide colour in paint formulations, percentage yield of the plant extract was calculated. Thin layer chromatographic technique (TLC) revealed different colours from the TLC plates under observation. The bands in the FTIR spectra of the plant extract and the formulated paints reveals same functional groups indicating that there was no chemical reaction taking place during paint formulation. From the result ob- tained, it can be concluded that extracts from the plant have the same effect of impacting colour with the toxic inorganic pigments used in paint industry and can also be used to replace them and turn out to be of great contribution in commercial sectors. As such the paints formulated can be considered a satisfactory article of commerce.

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Comparative Viscometric Study of Pure and Acetylated Gum Arabic using Different Plot Methods

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ABSTRACT

Acetylation of gum Arabic was achieved using acetic anhydride as solvent. The ester group formed was confirmed by FTIR spectra having absorption band of 750 cm^{-1} – 700 cm^{-1}. Viscometric study of the pure and acetylated samples was carried out. Relative viscosity of acetylated gum was found to be higher than that of the pure gum. Intrinsic viscosity was determined for the two samples using different plot methods taking Huggin's plot as standard. The intrinsic viscosity was found to be 86.43 cm3/g and 64.59 cm^3/g for acetylated and pure gum arabic respectively. Relative errors of other methods for the two samples was compared to that of Huggins and the plots that are most comparable to Huggins with relative errors less than 5% are; Martin, Lyon-Tobolsky, Staudinger-Heuer, Maron-Reznik and our proposed method. The proposed method which was a modification of the Kreiser method gave relative error less than 2 %, for both pure and modified gum. Whereas the Kreiser method gave relative error greater than 15 % for both methods. The critical concentration for the samples was found to be 0.0116 g/cm^3 and 0.0155 g/cm^3 for acetylated and pure gum respectively. This shows that there was no molecule- molecule entanglements during viscosity measurements.

Keywords: Intrinsic Viscosity; Acetylation; Gum Arabic.

1. INTRODUCTION

Gum Arabic exudate is gummy, dry and edible. It is usually obtained from stems and branches of Acacia senegal and Acacia seyal that have high content of non-viscous soluble fibre (Williams, 2000). It is a salt of complex polysaccharides that is neutral or slightly acidic in nature, containing Ca^{2+}, Mg^{2+} and K^{+}. Its most distinguishing character among other gums is that it is extremely soluble in water. The exudate is found mainly in unhealthy trees that are affected by diseases, drought or poor nutrition. The gum comes out through wounds carved in the bark of the tree in liquid drops, which then becomes hard with time. The tree's taxonomic classification is genus; Acacia, subfamily; Mimosoidene, family; leguminosae (Smolinske, 1992).

The gum itself comprise of different materials but may be separated in to three major parts. 88.4% of the gum is arabinogalactan with 0.35% protein content and has molecular weight of 3.8×10^5 Da. 10.4% is arabinogalactan protein with 11.4% protein and 4.5×10^6 Da molecular weight. The third part (1.2%) is glycoprotein with 47.3% protein content with $2.5{\times}10^5$ Da molecular weight (Randell et al., 1989).

Gums obtained from acacia species have high molecular weight (Baldein et al, 1999), are used as gels and thickeners (Savary et al., 2009), have emulsifying properties (Huang et al, 2001; Islam et al., 1997), stabilization properties (Dickinson, 2001) and can be used for micro- encapsulation (Renard et al, 2002). It can also be used in pharmaceutical industries ((Nasir et al, 2010), has biotechnology applications (Ben-Zion and Nussinovitch, 1997) and as an adhesive (Cochrane, 1996)

Viscometry is an analytical method used to characterize polymer properties in dilute solutions. It allows fast and very simple way of determining structure, polymer concentration, polymer chain dimensions, molecular weight, and other thermodynamic properties of a polymer in solution (Kulicke and Clasen 2004).

Viscosity can be defined as resistance to flow, reflecting the frictional forces of all molecules (of both solute and solvent) present in solution.

Capillary viscometers commonly measure the kinematic viscosity, which is the ratio of viscosity to the density of solution. To measure viscosity using a capillary viscometer, a certain amount of the polymer solution is placed in the capillary that has two marks at different levels. The flow time for the solution to pass between two lines marked in the viscometer is proportional to the kinematic viscosity of the solution.

The viscosity of a solution is expressed as the sum of viscosity of the dissolved polymer and the viscosity of the solvent (Kulicke and Clasen 2004). Relative viscosity therefore, is defined as the ratio of solution viscosity to solvent viscosity (Kulicke and Clasen 2004). In order to describe an incremental change in solvent viscosity upon dissolution of a polymer, the term specific viscosity is often used. The reduced viscosity is defined as the specific viscosity divided by the concentration of the polymer.

In order to obtain the intrinsic viscosity of a polymer, the reduced viscosity data is extrapolated to zero polymer concentration. It should be noted that the units of intrinsic viscosity and reduced viscosity are "volume/mass". The intrinsic viscosity can also be treated as the natural ability of a polymer to increase the viscosity of a solution (Huggins 1942). Therefore, the intrinsic viscosity is dependent on the shape, size and molecular weight of a polymer.

Dilute solution of a polymer can be defined as such a solution in which the polymer macromolecules are sufficiently far apart from one another so that their mutual interactions are eliminated and only polymer-solvent interactions take place.

The most important requirement for reliable intrinsic viscosity measurement is that the tested solution must be sufficiently dilute to eliminate polymer-polymer inter-chain interactions, so only polymer-solvent interactions and perhaps some intra-chain forces govern the size and conformation of individual polymer chains. At this state, the solution is said to be ideal-dilute, and the polymer molecule only interacts with the solvent. Therefore, the intrinsic viscosity of a polymer is only defined by the dimension of this single coil in solution (Kulicke and Clasen 2004). As the concentration of a polymer is gradually increased, spaces between molecules is reduced, and the molecules become more compacted producing a change in flow behavior. In diluted solutions, polymer coils must be spatially separated to prevent the for- mation of mechanical entanglements between the polymer chains. This process takes place at specific concentration called the critical concentration(C). Above this concentration, flow behavior of polymer coils is dominated by intermolecular interactions, and at concentrations below, the flow behavior is mainly due to polymer-solvent interaction. Therefore volume occupied by polymer coils is inversely proportional to the critical concentration.

Truly dilute polymer solutions are Newtonian while the presence of entanglements usually leads to non-Newtonian effects, such as time- dependent flow or visco-elasticity (Robinson et al. 1982). Lovell

(1989) argued that in the most general case the transition concentration is $C \approx \frac{1}{[\eta]}$ at to ensure truly dilute solution conditions.

Industries that use gums as raw materials are in continuous search for better gums with improved physicochemical characteristics, higher quality and lower cost of production. In this work, we seek to determine the feasibility of obtaining intrinsic viscosity of pure and acetylated (modified) gum Arabic using different equations, and also compare their viscosity average molecular weights.

2. MATERIALS AND METHODS

Materials

Arabic gum was obtain from Malam Musa Maiunguwa at kurmi Market. Ethanol, Acetic Anhydride and diethyl ether were obtained from BDH. Some of the glassware used are; beakers, pipettes, majoring cylinder, volumetric flasks. The equipment used are; clinical thermom- eter, Ostwald viscometer, constant temperature magnetic stirrer, and analytical weighing balance.

Sample collection

The raw exudate of Acacia Senegal gums was bought from Kurmi Market in Kano State. Malam Musa Maiunguwa assured us that the gum was from Acacia senegal which was obtained from Acacia tree in one of the bushes in Sokoto State, Nigeria.

Sample identification

The exudate sample of the gum was taken to the Department of forestry, fishery and wild life in the faculty of Agricultural science at Kano University of Science and Technology Wudil for further identification, and was identified and certified as Acacia senegal gum.

METHODS

Sample purification

Impurities such as pieces of tree bark were removed by hand, and the gum was crushed to obtain smaller chunks. The gum then hydrated in distilled water for three days. The mucilage obtained was forced to drain through a calico cloth and 95% ethanol was used for precipitation of the gum. The precipitate was washed with diethyl ether, dried, powdered and stored in a desiccator for further use (Oluyemisi et al, 2010).

Sample modification

Acetic anhydride was used for this modification. 10g of the gum was dissolved in 50ml of distilled water. This makes 20%w/v concentration of the mixture. 5g of acetic anhydride corresponding to 50% by weight of dry gum was added to the mixture. The mixture was heated at 70oC for three hours, allowed to cool, dry and was then powdered (Sowunmi, 1990).

Preparation of gum solutions

Pure and modified gum samples were dissolved in distilled water to obtain 0.1%, 0.2%, 0.3%, 0.4% and 0.5% w/v concentrations. Solutions were gently stirred and slightly heated to obtain a uniform solution. Samples were then allowed to cool overnight.

Density measurement

Measurement of densities was carried out with the use of relative density bottles. The R.D bottle was first washed with chromic acid, rinsed with distilled water and then with alcohol, allowed to dry, and then weighted. It was then filled with distilled water and stoppered. The R.D bottle was then weighted again. Water is then removed, the bottle was washed again with alcohol and dried. It was thereafter filled with experimental liquid as before and weighted again. The density of the experimental solution was determined using the formula below.

$$d_1 = \frac{w_3 - w_1}{w_2 - w_1} \times d_2$$

Where:

d_1 = is the Density we are looking for

d_2 = is the density of water

w_1 = weight of density bottle

w_2 = weight of the density bottle + distill water

w_3 = weight of the density bottle + Sample

Viscosity measurements

Capillary viscometers commonly measure the kinematic viscosity which is the ratio of viscosity to the density of a solution. The Ostwald viscometer was clamped to retort stand and dipped in to a 1000cm3 beaker which was filled with water. The sample solutions and reference solvent were analyzed under a temperature controlled thermostatic bath. Samples were then inserted in to the viscometer by the use of pipette until the down loop was filled. Suction pump was used to absorb the sample from the down loop to high loop where the two marks are provided. The solution was released to flow from the top mark till it reached the lower mark, and the time of flow was recorded using digital stop watch at 30oC, for each sample.

(Leo, Chem. Anal. Ed., 9 (2), 85-90, December 1937).

Intrinsic viscosity calculations

Viscosity of a solution containing even the slightest amount of a solute (polysaccharide), is always greater than that of a pure solvent. This is as a result of the larger size of the polysaccharide molecule as compared to that of the pure solvent. The viscosity determined using a capillary viscometer can be calculated using the equation below.

$$\eta = A\rho t \qquad (1)$$

Where η indicates viscosity, ρ (g/cm^3) is the fluid density, A (cm^2/s^2) is called instrumental constant of the viscometer, and t is the time of flow.

The relative viscosity of a solution is given by:

$$\eta_r = \eta_s / \eta_0 = \rho_s . t_s / \rho_0 . t_0 \qquad (2)$$

ηr is the relative viscosity, ηs is the viscosity of solution and ηo is the viscosity of solvent. ρs and ρo are the viscosities of solution and solvent respectively. Also ts and to represent time of draining of solution and solvent respectively.

Specific viscosity or rather increment in relative viscosity is given by the equation below.

$$\eta_{sp} = \eta_r - 1 \quad (3)$$

Reduced viscosity is given by the first term of the Huggins equation.

$$\eta_{red} = \eta_{sp/c}$$

In Huggins' method (Huggins, M. L. 1942.), intrinsic viscosity [η] is defined as the ratio of the increase in relative viscosity (ηsp) to concentration (c in g/cm³) when the latter tends towards zero.

$$\eta_{sp}/C = [\eta] + K_H [\eta]^2 c \quad (4)$$

Where K_H is Huggins constant.
The Kraemer (Kraemer, E. O. 1938) propose the following equation:|

$$\ln(\eta_r)/c = [\eta] + K_K[\eta]^2 c \quad (5)$$

Where K_K is Huggins constant.
The Martin (Martin, A. F. 1942) proposes the following equation:

$$\ln(\eta/C) = \ln \eta + K_M [\eta] d^2 \quad (6)$$

where K_M is Martin's constant.
Fuoss (Fuoss, R. M. 1948) propose the following equation:

$$C/\eta_{sp} = 1/[\eta] + K_{fs} 1/[\eta] \, c^{1/2} \quad (7)$$

Where K_{Fs} is Fuoss constant.
Fedors (Fedors, R. F. 1979.) propose the following equation:

$$\frac{1}{\eta^{1/2} - 1} = \frac{1}{[\eta]c} - \frac{1}{[\eta]C_{max}} \quad (8)$$

Where C_{max} is polymer parameter concentration.

Heller (Fedors, R. F. 1979.) propose the following equation:

$$\frac{1}{2}c\left(\frac{1}{\eta_{sp}} + \frac{1}{\ln \eta_r}\right) = \frac{1}{[\eta]} - K_{He}c \quad (9)$$

Where K_{He} is Heller's constant.

Lyons & Tobolsky (Lyons, P. F., Tobolsky, A. V. 1970; Quadrat, O. 1977) propose the following equation:

$$\ln\frac{\eta_{sp}}{C} = \ln[\eta] + \left(\frac{K_{L-T}[\eta]c}{1-bc}\right) \quad (10)$$

where K_{L-T} and b are constant.

Baker (Baker, F. 1913 and Lewandowska, K. et al 2001) propose the following equation:

$$\eta_r^{1/n} = 1 + [\eta]\frac{c}{n} \quad (11)$$

$$n = \frac{1}{1 - 2K_H} \quad (12)$$

With $0.25 < K_H < 1$, and $K_H = 0.9204$

Tager (Tager, A. 1978) propose the following equation:

$$\eta_r^{K_B} / \eta_{sp} = 1/[\eta](K_{T1} + K_{T2}c^2) \quad (13)$$

Where K_{T1} and K_{T1} are Tager's constant.
Budtov (Budtov, V. P. 1976) propose the following equatior

$$\eta_r^{K_B} = 1 += 1 + K_B[\eta]c \quad (14)$$

Where K_M is Martin's constant. This method is not applicable to intrinsic viscosity and molecular weights as high and $K_M > 05$. Solomon & Gotesman (Solomon, O. F. et al 1967) propose the following equation:

$$1 + \frac{1}{3}\eta_{sp} = [\eta]^{\frac{\eta_{sp}}{c}} \quad (16)$$

Arrhenius-Rother-Hoffmann (Hoffmann, M. at el 1957) propose the following equation:

$$\frac{In\eta_r}{c} = [\eta] + K_A \ln\eta_r \quad (17)$$

Where K_A is constant.
Kreisa (Kreisa, J. at el 1960) propose the following equation:

$$\frac{\eta_{sp}}{c} = [\eta] + K_{Kr}\frac{\eta_{sp}^2}{c} \quad (18)$$

Where K_{Kr} is Kreisa's constant.
Staudinger & Heuer (Hoffmann, M. 1957) propose the following equation:

$$In(\frac{\eta_{sp}}{c}) = In\,\eta [\,] + K_{S-H}[\eta]c \quad (19)$$

Where $K_{S\text{-}H}$ is a constant.
Schramek [Schramek, W. 1955.] propose the following equation:

$$(\frac{\eta_{sp}}{c})^{1/n} = [\eta]^{1/n} + K_{Sch}c \quad (20)$$

Where K_{Sch} is Schramek's constant.

This work proposes the following equation

$$\left(\frac{\eta_{sp}}{c}\right)^2 = [\eta]^2 + K_{Kr}\ {}^2\left(\frac{\eta^2_{\square\square}}{c}\right) \quad (21)$$

Where K_{Kr} is Kreaser's constant

Critical concentration

The critical concentration C_* is calculated using the formula below

$$C_* \approx \frac{1}{[\eta]} \quad (22)$$

At $C_* > \frac{1}{[\eta]}$, the flow behavior is governed by intermolecular interactions of the polymer coils; while at $C_* < \frac{1}{[\eta]}$, the interaction is dominated mainly by the polymer-solvent interactions.

Molecular weight

Mark-Houwink-Sekurada equation was used to calculate the viscosity average molecular weight of the gum. This equation relates the intrinsic viscosity of the gum [η], and the average molecular weight. The equation is given by

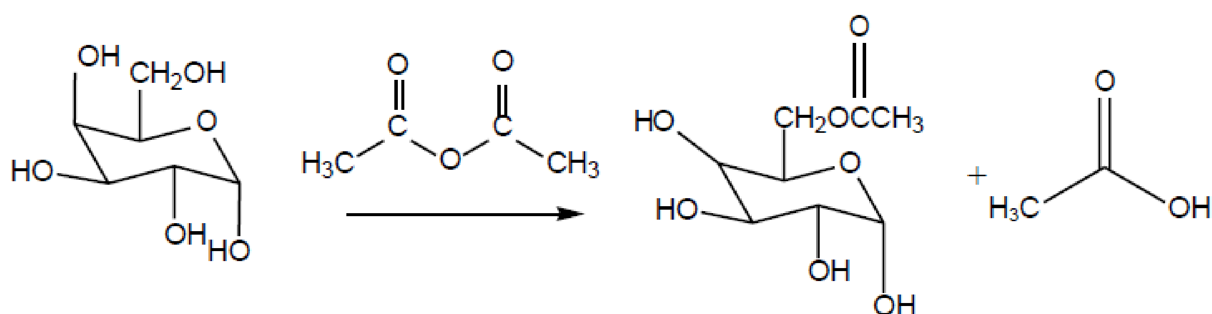
$$[\eta] = K\,M^a$$

'K' and 'a' are constants called the MHS parameters. These parameters are specific for a particular polymer and solvent, and at a particular temperature. The exponent 'a', is a measure of solvent quality. It is also related to structure, orientation and flexibility of polymer bonds. (Kulicke and Clasen 2004; Picout and Ross-Murphy 2007). From the MHS equation, the intrinsic viscosity values can be plotted on a logarithmic scale as a function of the logarithm of the molecular weight to obtain a straight line with slope equal to 'a'.

3. RESULTS AND DISCUSSION

Acetylation of gum Arabic

Acetic anhydride, acetic acid or even vinyl acetate are used in the modification of starches to produce starch acetates. During the reaction process, hydroxyl groups on the glucose units are replaced with acetyl groups to form esters. Certain factors like reactant concentration, time of reaction, catalyst and pH determine the number of acetyl groups formed in the starch matrix. (Durdica et al., 2015). In this project acetic anhydride is used instead of acetic acid. This is because acetic acid is a weak acid and its reaction with starch hydroxyl groups is reversible and the equilibrium constant does not favor the product side. The reaction is represented below



FTIR

Fig. 1 and 2 shows the FTIR of pure and acetylated gum arabic. The spectra of acetylated gum show the processing of new peak around 750 cm^{-1} to 700 cm^{-1} attributed to *COOR* stretching indicating the presence of an ester. The peak is seen to be absent in the native gum

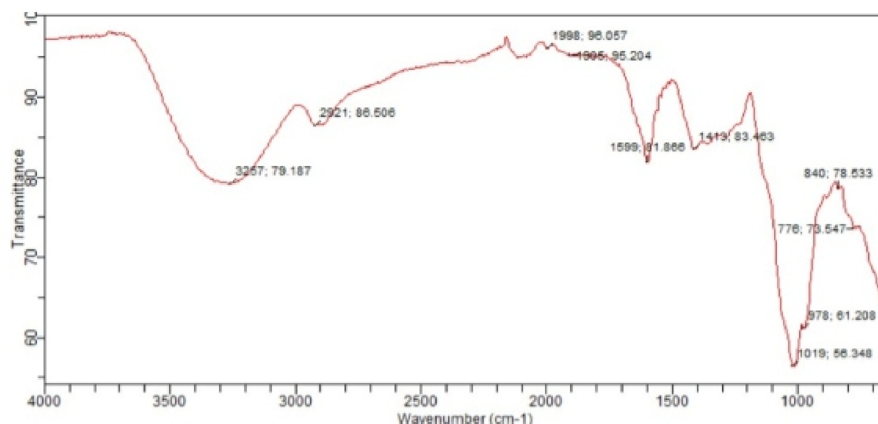


Fig. 1: Ftir Spectra of Pure Gum Arabic.

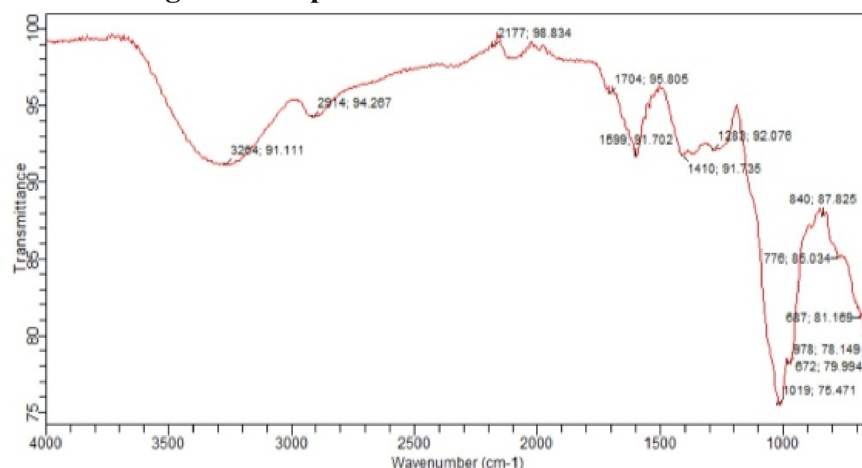


Fig. 2: Fitr Spectra of Acetylated Gum Arabic.

Densities

Fig. 3 compares densities of pure and acetylated gum samples at different concentrations. The trend shows at all concentrations, modified gum sample has a higher density compared to the pure sample.

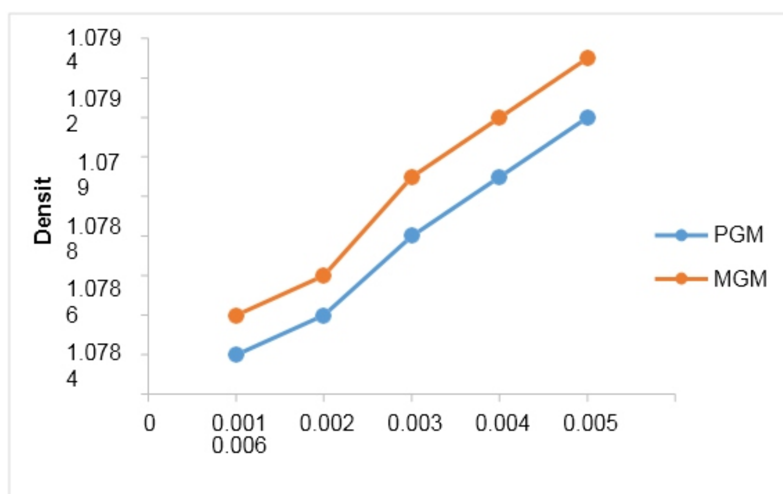


Fig. 3: Densities of Pure and Modified Gum Arabic.

Relative viscosity

In relative viscosity, the viscosity of the solute solution is determined relative to that of the solvent. Then from their mathematical relationship a lot of polymer parameters can be calculated. The relative viscosity of pure and acetylated gum Arabic is presented in fig 4. From the figure, it is seen that relative viscosity of acetylated gum Arabic is greater than that of pure gum Arabic. This however, does not agree with the findings of Muhamedbegović et al. (2012), who worked on acetylation of potato starch. In their work, they found a decrease in viscosity after acetylation of the starch. Berski et al. (2011) made similar observation when they acetylated Oat starch. Saartrat et al. (2005) also observed same trend after acetylation of Canna starch. The relative viscosity of acetylated starch is dependent on the uniformity of acety- lation. That is whether the acetylation reached the inner lamellae of granules or just restricted to its outer part (Huang et al. 2007). According to Saartrat et al. (2005), the relative viscosity of starches could either decrease or increase after acetylation. This is due to the disruption of either inter or intra molecular bonds during the acetylation process.

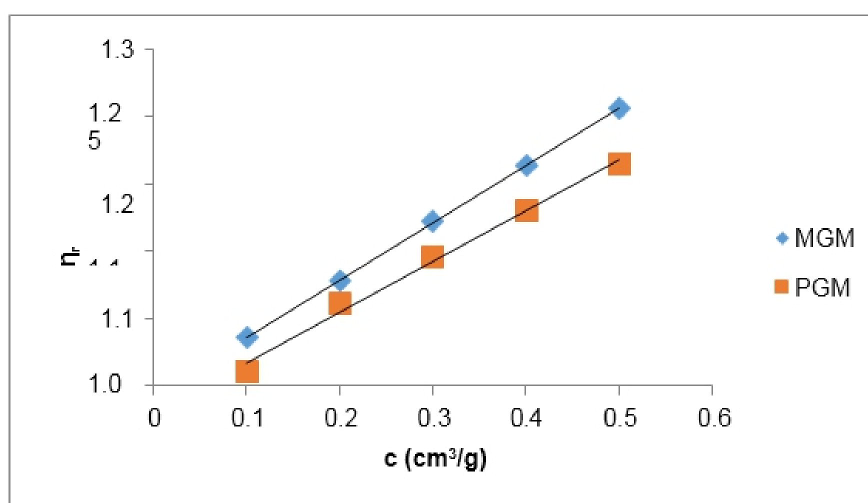


Fig. 4: Relative Viscosities of Pure and Acetylated Gum Arabic.

Intrinsic viscosity

Intrinsic viscosity is the contribution of individual molecules to the viscosity of a solution. It gives insight into polymer shape, structure, molecular weight and other hydrodynamic parameters. In this work, multi-concentration viscosity measurement approach was used, and therefore, several solutions were prepared with different gum concentrations (Nicholson, 2017). The intrinsic viscosity of pure and acety- lated gum Arabic is determined using different plot methods. Table 1 shows the intrinsic viscosity of pure gum Arabic. The value differs considerably with that reported by Anderson and Rahma (1967).). This might be attributed to difference in geographic location of the parent tree, or due to difference in purification methods. However, the intrinsic viscosity falls within the range reported by Renard et al., (2006) and Yebeyen et al., (2009). Taking the Huggins plot as standard, the intrinsic viscosity was determined from the plot of reduced viscosity as a function of concentration. Those plots that have relative errors (RE) less than 5% are considered feasible and comparable to Huggin's method. Those methods with RE greater than 5% are considered poor and not comparable to Huggin's method. The results in table 1 shows Kreamer (RE 2.4%), Martin (RE 1.3%), Lyon-Toblosky (RE 2.12%), Staudinger-Heuer (RE 2.12%), Maron-Reznik (RE 3.46%) and our proposed method (RE 1.85%), are good and comparable to Huggins method.

Table 1: Intrinsic Viscosities of Modified Gum Arabic Using Different Plot Methods

Method	Huggins	Kraemer	Martin	Fuoss	Fedors	Arrhenius-Rother-Hoffmann
[η] (cm^3/g)	86.43	83.59	88.28	80	106.38	95.033
R2	0.812	0.8354	0.8619	0.7943	0.9807	0.8479
RE%	-	3.29	2.14	7.44	23.08	9.95
Method	Heller	Lyon-Tobolsky	Tager	Budtov	Maron-Reznik	Kreisa
[η] (cm^3/g)	92.59	87.9	63.29	66.07	83.25	112.95
R2	0.817	0.8606	0.9015	0.997	0.7745	0.7058
RE%	7.13	1.7	26.77	23.56	3.68	30.7
Method	Staudinger-Heuer	Square	Square Root	Mean	Proposed Method	
[η] (cm^3/g)	87.89	75.92	114.06	45.43	85.45	
R2	0.8606	0.5945	0.9075	0.8477	0.5898	
RE%	1.7	12.16	31.97	47.43	1.13	

Table 2: Intrinsic Viscosities of Pure Gum Arabic Using Different Plot Methods

Method	Huggins	Kraemer	Martin	Fuoss	Fedors	Arrhenius-Rother-Hoffmann
[η] (cm^3/g)	64.59	63.04	65.43	100	68.96	68.23
R2	0.9615	0.9635	0.9717	0.9767	0.9807	0.9815
RE%	-	2.4	1.3	54.8	6.76	5.64
Method	Heller	Lyon-tobolsky	Tager	Budtov	Maron-Reznik	Kreisa
[η] (cm^3/g)	64.9	65.96	59.17	53.95	62.35	74.536
R2	0.9878	0.9706	0.8506	0.9984	0.9219	0.9537
RE%	0.48	2.12	8.39	16.47	3.46	15.4
Method	Staudinger-Heuer	Square	Square Root	Mean	Proposed Method	
[η] (cm^3/g)	65.96	58.46	79	32.57	63.4	
R2	0.8606	0.8302	0.9848	0.9775	0.9374	
RE%	2.12	9.49	21.8	49.57	1.84	

In table 2, the acetylated gum Arabic shows a higher intrinsic viscosity than the native gum. The viscosity of acetylated gum is 86.43 which is higher than that of pure gum (63.40). This could be due to increase in swelling power of the acetylated gum. Acetylation also increases emulsifying ability of gums, thereby increasing the viscosity, (Samia et al., 2009). Introduction of acetyl groups also reduces bond strength between gum molecules and thereby increasing swelling and solubility of the molecules. This enhances access of water to amorphous areas, increasing water holding capacity of the gum matrix and developing a more organised structure leading to a higher resistance to deformation and achieving a higher peak viscosity (Hovers and Susulski, 1985). Other methods were also used to find the intrinsic viscosity. The ones that were most comparable to Huggins and have lower RE values are; Kreamer (RE 3.29%), Martin (RE 2.14%), Lyon-Tobolski (RE 1.7%), Staudinger-Heuer (RE 1.7%), Maron and Reznik (3.68%) and our proposed method (RE 4.10%). The rest have RE values greater than 5% and are considered not comparable to Huggins.

It was observed that not all methods that are comparable to Huggins fitted the two samples well. Heller's method stands out here. It was comparable to Huggins method in the pure gum sample with a relative error of 0.48%, but in the acetylated sample, it became a poor method with a RE of 7.13%. It is still not clear why this happened, but it could be due to the correlation of data between the two samples. In almost all plots used, the native gum has higher correlation than the acetylated gum. This could make some plots agreeable to Huggin's in the native while not agreeable to Huggin's in the acetylated sample.ηsp /cThe Kreiser method which this work modifies gave a very high relative error of 15.4% and 30.7% in pure and acetylated samples respec- tively. The method proposed herein is modification of the Kreiser method and has fitted well and is comparable to Huggins method in both samples with RE values of 1.84 and 1.13% for pure and acetylated gum respectively.

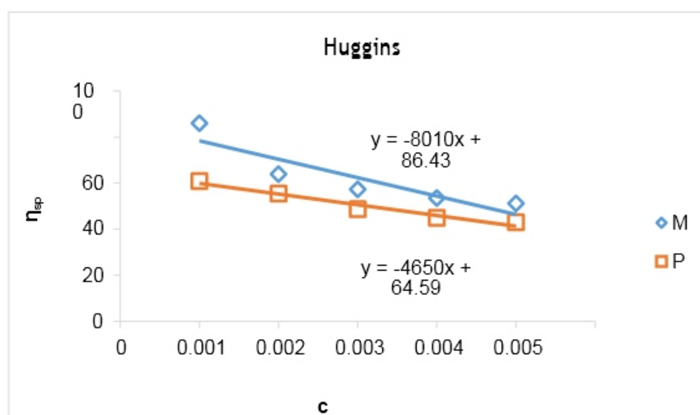


Fig. 5: Huggin's Method.

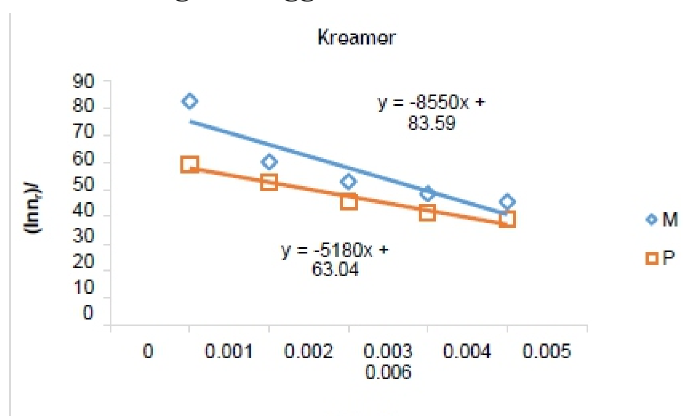


Fig. 6: Kreamer Method.

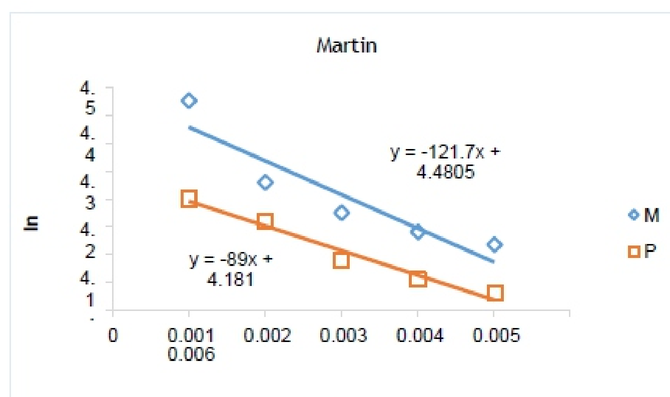


Fig. 7: Martin Method.

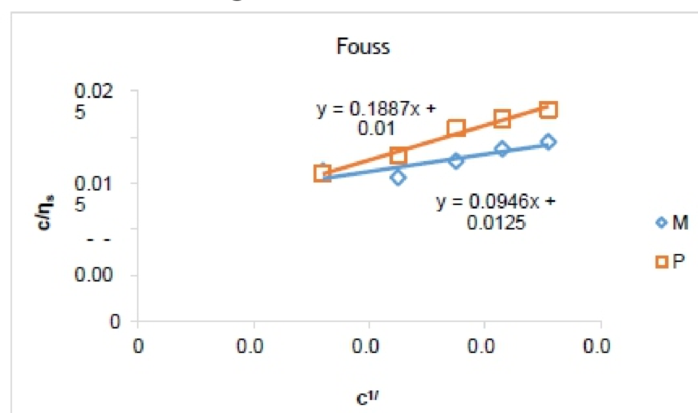


Fig. 8: Fouss Method.

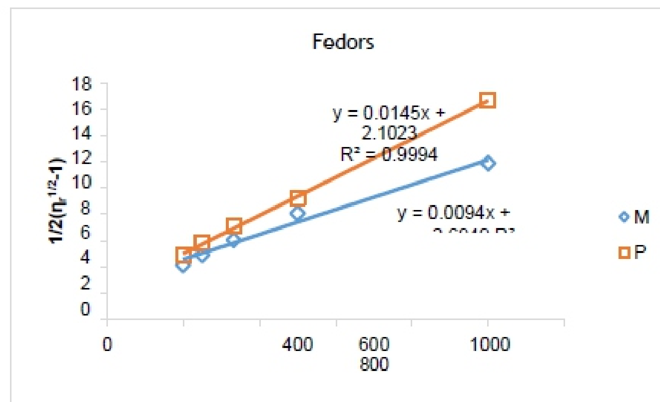


Fig. 9: Fedors Method.

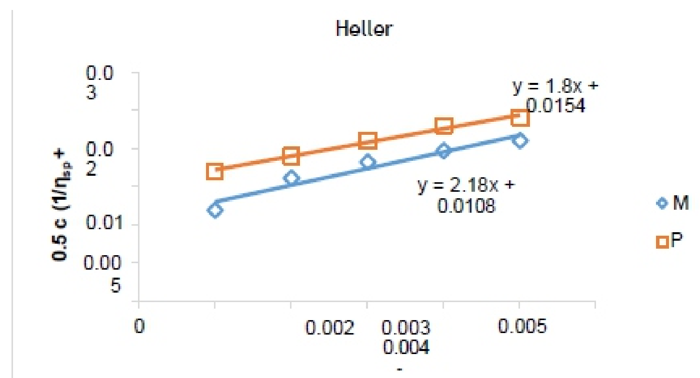


Fig. 10: Heller Method.

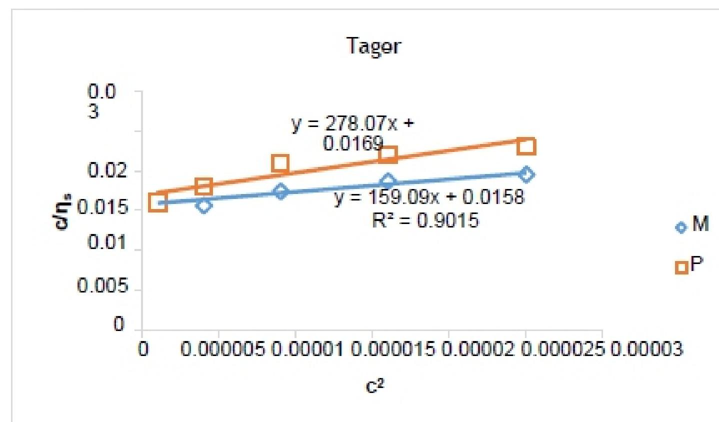


Fig. 11: Tager Method.

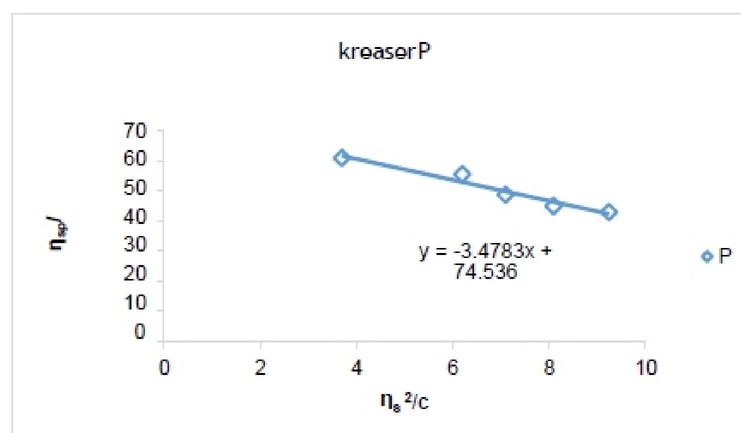


Fig. 12: Kreaser Method for Pure Gum Arabic (P)

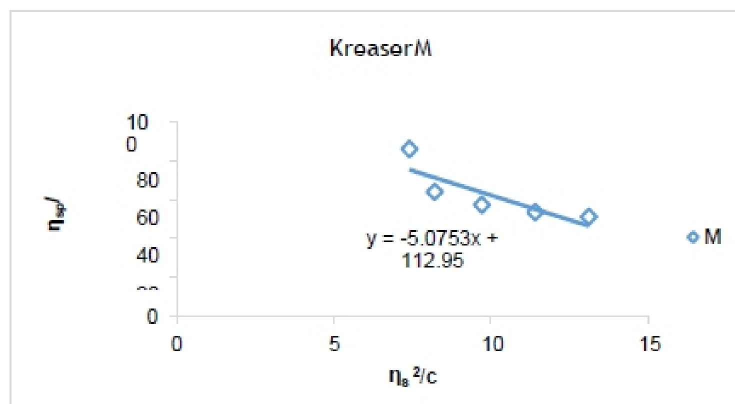


Fig. 13: Kreaser Method for Modified Gum Arabic (M)

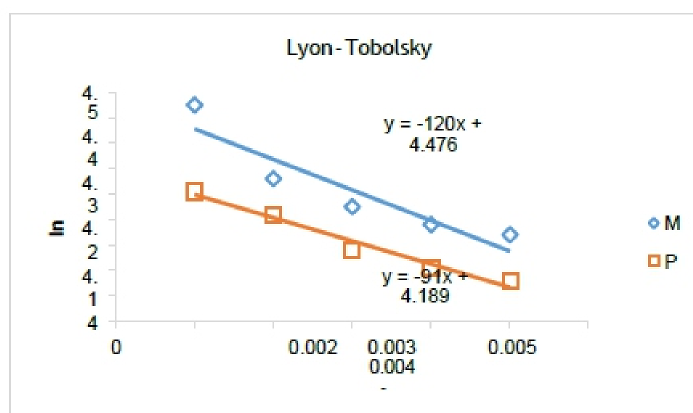


Fig. 14: Lyon – Tobolsky Method.

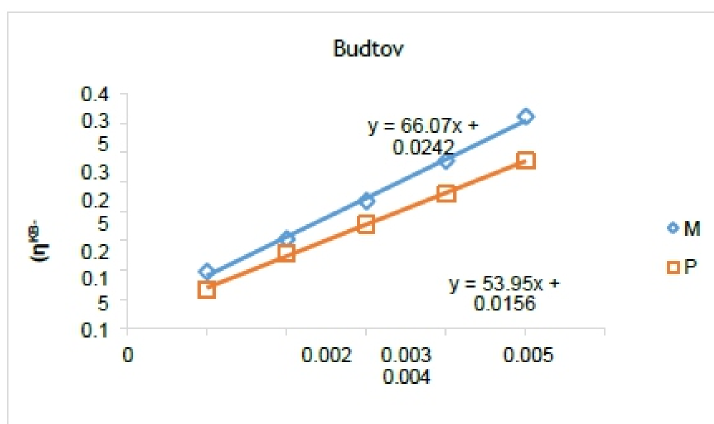


Fig. 15: Budtov Method.

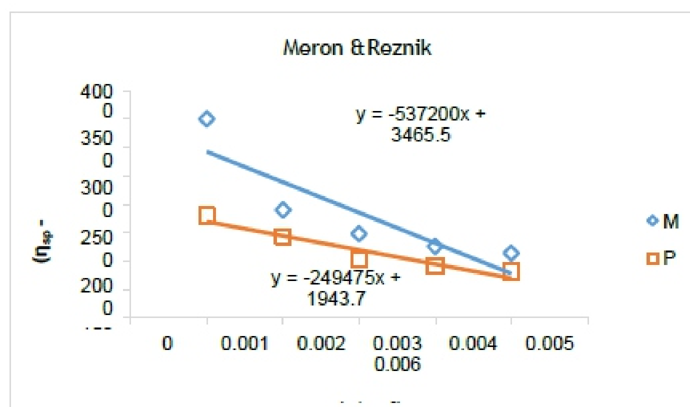


Fig. 16: Meron and Reznik Method.

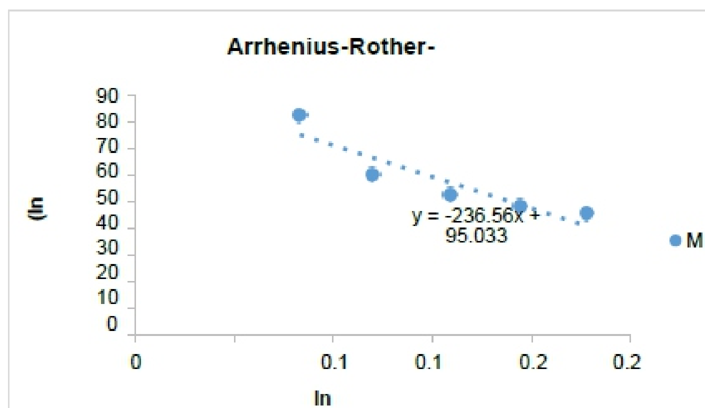


Fig. 17: ARH Method for Modified Gum Arabic (M).

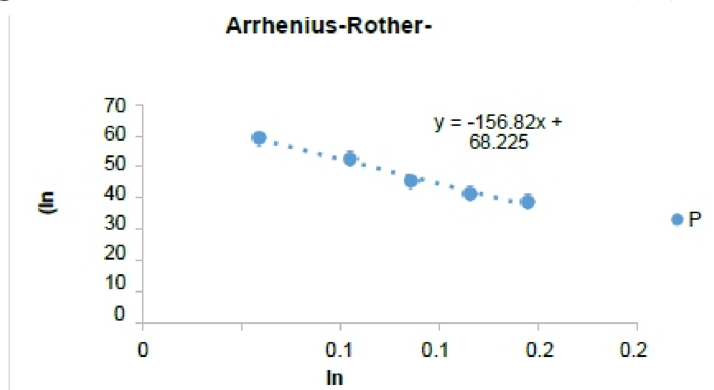


Fig. 18: ARH Method for Pure Gum Arabic (P)

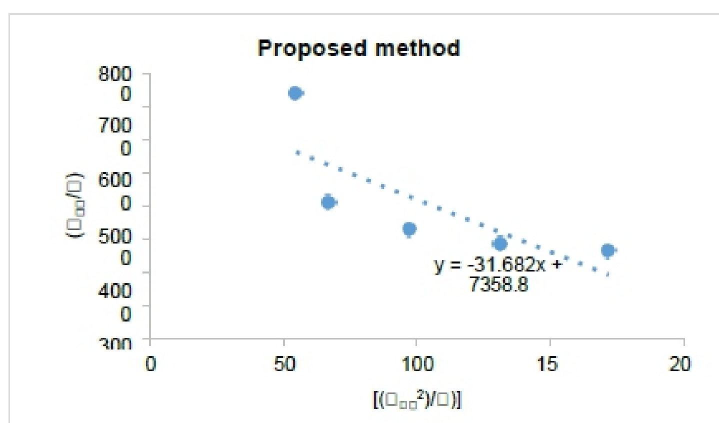


Fig. 19: Proposed Method for Modified Gum (M).

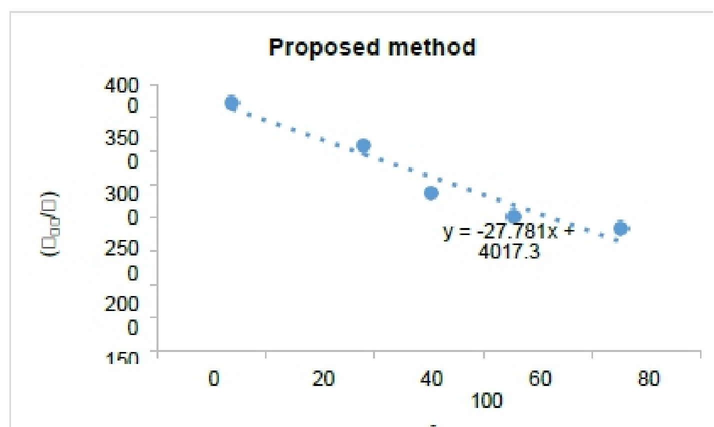


Fig. 20: Proposed Method for Pure Gum (P).

Molecular weight determination

The molecular weight is important in determining functional characteristics of polymers such as strength and processing ability. The vis- cosity average molecular weight of native gum arabic found from this study is 8.64×10^6 $gmol^{-1}$ which is greater than most gums such as xanthan gum (4.05×10^6 $gmol^{-1}$), guar gum (1.45×10^6 $gmol^{-1}$), gellan gum (1.64×10^6 $gmol^{-1}$) and locust been gum (1.6×10^6 $gmol^{-1}$) (Fatemeh et al, 2018). The molecular weight of acetylated gum Arabic was found to be 14.9×10^6 gmol-1 which is greater than that of native gum. This could be due to the substitution of hydroxyl groups with a larger ester group in the modified gum which in turn increases its solubility (Samia et al, 2009). Similar observation was made by Adeyanju et al., (2016). They found that chemical modification of polysaccharides produces products with improved physicochemical and functional properties that are not available from commercial pol- ysaccharides. They acetylated Sweitenia mycropylla gum with acetic anhydride in the presence of sodium hydroxide. The result they found showed that acetylated gum had higher values of solubility, viscosity and swelling index.

Critical concentration

The critical concentration (C) is calculated from equation (21). The value for pure gum arabic was found to be 0.0155 g/cm^3 and that of acetylated gum Arabic was found to be 0.01157 g/cm^3. All these values are above the maximum concentration of gum samples (0.005g/cm^3). This indicates, the solutions are Newtonian and there are no entanglements between the molecules. Also it shows the vis- cosity determined was purely based on molecule–solvent interaction.

4. CONCLUSION

This work showed that the modified gum has a higher density than the pure gum, and as a result, relative viscosities and intrinsic viscosities were found to be greater in the modified gum. Also, from the different plots made, not all fitted well in calculating an accurate intrinsic viscosity for both samples. Some fitted well in the modified sample, and not that well into the pure sample. This work's modification of the Kreiser method gave an intrinsic viscosity that is comparable to the Huggin's method in both samples. The relative error calculated for our modified Kreiser method in far below that of the proper Kreiser method. This makes the proposed method viable. Also on comparing the molecular weights for the two samples, it was observed that the modified sample has a higher molecular weight which could be due the presence of bulkier ester groups.

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Determination and Validation of Pregabalin in Bulk and Pharmaceutical Formulations by Reversed Phase-High Performance Liquid Chromatography

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ABSTRACT

A simple, specific, quick, isocratic Reversed Phase High Performance Liquid Chromatographic method was developed and validated for the analysis of Pregabalin in bulk and 5-different pharmaceutical formulations, the separation was accomplished on a C18, 5µm Reverse Phase column (250 mm × 4.6 mm) using a methanol : water (95:5, v/v) mobile phase. The compound was eluted isocratically at a flow rate of 0.8 ml /min. The UV detector was set at 288 nm for the detection of Pregabalin (PRG). The method was linear over the range of 5-45 µg/ml and validated with respect to accuracy, precision, linearity, and specificity, limit of detection and limit of quantization. Robustness testing was also conducted to evaluate the effect of minor changes to the chromatographic system and to establish appropriate system suitability parameters. This method was used successfully for the quality assessment of 5-different pharmaceutical formulations with good precision and accuracy.

Keywords: Pregabalin; Isocratic System; Validation; RP-HPLC; Different Pharmaceutical Formulations.

1. INTRODUCTION

Pregabalin (PRG) is chemically (S)-3-(amino methyl)-5-methyl hexanoic acid and is not official in any pharmacopoeia. Structural ana- logues of γ-amino butyric acid (GABA) as shown in (Fig. 1). Nature of PRG is a new anticonvulsant and analgesic medication that was recently approved for adjunctive treatment of partial seizures in adults, and for the treatment of neuropathic pain from postherpetic neuralgia and diabetic neuropathy. The site of action of drug is the alpha 2-delta (α2-δ) protein, an auxiliary subunit of voltage gated calcium channels. PRG subtly reduces the synaptic release of several neurotransmitters, apparently by binding to α2-δ subunit and possibly accounting for its action in vivo to reduce neuronal excitability and seizures.

There is no any official, sensitive, accurate, precise and chief analytical method developed for the analysis of pregabalin till now and therapeutic importance of the drug has engendered development of assays for the quantification of PRG. A literature survey regarding PRG revealed that attempts were made to develop analytical methods for PRG using extractive spectrophotometric and spectroflurimetric (Ar- mağan ö.,2009, Önal et al.,2009). LC method with precolumn derivatisation with marfeys reagent (A.S. Jadhav et al .,2007). HPLC analysis of PRG in human serum (Vermeij T A et al.,2004). Liquid chromatography - mass spectrophotometric (LC-MS-MS) (Walash M I et al., 2011, Vaidya VV et al., 2007, Zhang Y et al., 2008). Heteroaromatic analogs of pregabalin and its activity on mouse model (Schelkun RM et al., 2006). method had been reported. All of these methods are very expensive because these methods require long and tedious pretreat- ment of the samples and derivatisation for the analysis of PRG. There is no HPLC method without derivatisation for the analysis of PRG. So, there is need for the development of a HPLC method for the analysis of PRG. Hence, an attempt has been made to

develop a simple, quick, specific, accurate efficient and selective method for the analysis of PRG in bulk and pharmaceutical formulations. This paper de- scribes the Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for estimation of PRG in bulk and 5-different pharmaceutical dosage forms.

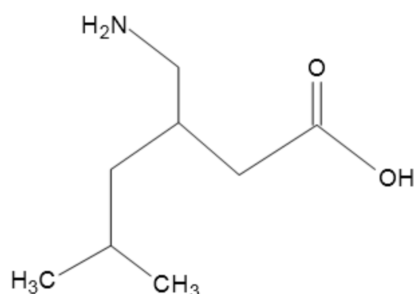

Fig. 1: Structure of Pregabalin.

2. EXPERIMENTAL

Material and methods

Instruments

Analysis by HPLC was performed using an isocratic system. i) The HPLC instrument was used model Younglin Acme-9000 with SP 930 D Pump, variable programmable UV detector, ii) Rheodyne injector with 20µl fixed loop was used. iii) UV/VIS Spectrophotometer, Shi- madzu, Model No UV 1800, iv) Digital pH - meter, Toshniwal instrument manufacture Pvt. Ltd (Mumbai, India). v) Chromatographic analysis was performed by using Autochro-3000 software. vi) The HPLC column used was a Reverse Phase varian C18, 5 µm columns (250 mm × 4.6 mm). vii) The mobile phase filtration unit was Ultipor N66, Nylon 6, 6 membranes (Pall Life Sciences, Mumbai, India).

Chemicals and materials

Pregabalin (Sun Pharmaceutical Industries, Jammu, India) used as a standard. Pharmaceutical formulations of Pregabalin such as Neugaba- 75 (Sun Pharmaceutical Industries, Mumbai, India), Pregastar-75 (Lupin ltd, Mumbai, India), Gabanext-75 (Nicholas Piramal India Ltd., Mumbai, India), Gabafit-75 (Glenmark Pharmaceutical Ltd., Mumbai, India), Maxgalin-75 (Sun Pharmaceutical Industries, Jammu, India) were containing labeled amount 75 mg PRG were procured from a local pharmacy store. Methanol, Water HPLC grades were purchased from E-Merck, Mumbai, India. All other chemicals were of analytical grade and used without any further purification.

Chromatographic system and conditions

The proposed method was performed using a following chromatographic condition and are mentioned in Table 1.

Table 1: Chromatographic System and Conditions

Chromatographic mode	Chromatographic conditions
HPLC system	Younglin (acme 9000) with software Autochro 3000
Pump	SP 930 D
Detector	UV- detector
Column	C18, 5 ì m Reverse Phase varian (250 x 4.6)
Mobile phase	Methanol: Water (95:5)
Detection wavelength	288 nm
Flow rate	0.80 ml/min
Sample size	20µl
Column temperature	Room temperature
Run time	10 min
Filtration	By passing through a 0.45 ì m Ultipor filter and ultrasonication for 10 min.
Diluents	The mobile phase used as diluents.

Optimization of chromatographic parameters

Optimizations in HPLC is the process of finding a set of conditions that adequately enable the quantification of the analyze with acceptable accuracy, precision, sensitivity, specificity, cost, ease, and speed.

Optimization of column and mobile phase strength

It was achieved by monitoring varying columns and mobile systems. Silica columns such as a µ Bondapak column with different mobile phases did not give a suitable peak shape for analysis. On the other hand, C18, 5 µm Reverse Phase varians (250 x 4.6) showed better results. For selection of mobile phase, various mobile phase compositions containing acetonitrile: methanol: water in different ratios was tried but the resolution was not found to be satisfactory. It was found that methanol: water gave satisfactory results with sharp well defined and acceptable peak parameters as compared to other mobile phases. Finally, the optimum composition of the mobile phase was selected as methanol: water (95: 5, V/V). Excellent chromatographic specificity with no interference from dosage form excipients was observed. Moreover, a suitable retention time for PRG was achieved. Typical chromatograms obtained from the standard solution of PRG, assay preparation of capsules. Under the chromatographic conditions described, PRG was well resolved and eluted at about 4.4167 min (Fig. 2), the total run time was within 10 min. Good baseline resolution and peak shape can be observed.

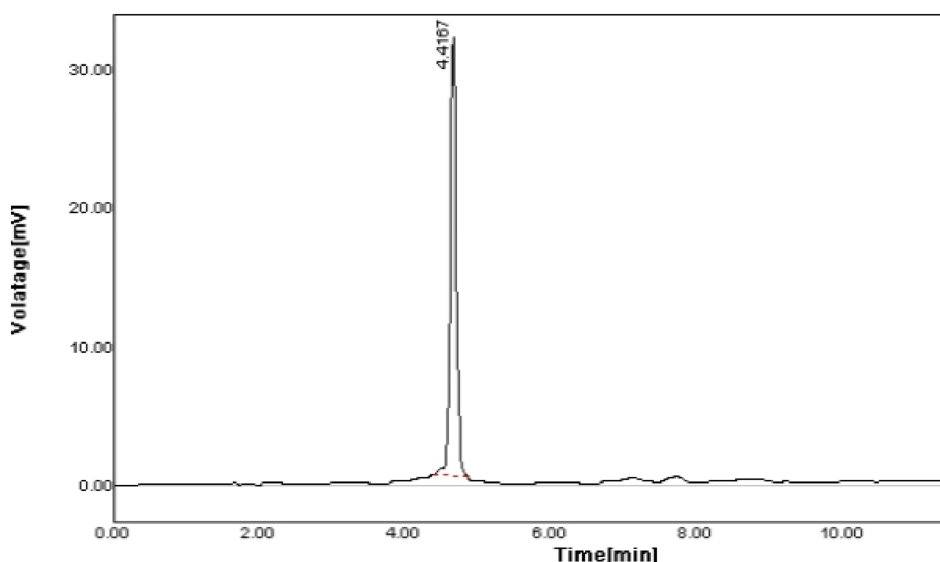

Fig. 2: Chromatogram of Pregabalin (PRG) Standard – R.T:4.4167.

Optimization of detection of UV wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is one that gives good response for the drugs that are to be detected. In the present study, by appropriate dilution of each standard stock solution with mobile phase, various concentrations of PRG were prepared separately. Each solution was scanned in the spectrum mode between the range of 200 to 400 nm and their spectra were seen. The wavelength selected for the analysis was 288 nm at which PRG showed significant absorbance.

System suitability

The system suitability of the proposed method was evaluated after spiking PRG. Following parameters mentioned in Table.2 was suitable for proposed method.

Table 2: System Suitability Parameters

Parameters	PRG (15 ì g/ml)
Retention time (min.)	4.4167
% area	100
Plates	8128.1
Tailing factor	0.9324
Asymmetry	0.993

***Average of nine determinations.**

Preparation of standard stock solutions

Stock solution of PRG in the concentration of 1.0 mg/ml concentration was prepared in mobile phase. All solutions were shown to be stable during the period of study. Standard solutions of PRG (5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0 µg/ml) were prepared by subsequent dilution.

Sample preparation for injection (capsule)

20 capsules were weighed with or without caps and average weight was determined and finely powdered using mortar and pestle. Then ground material, which was equivalent to 25 mg of PRG, was extracted using 10 ml of mobile phase by ultrasonication and the volume was made up to 25 ml by mobile phase. Then 10 ml from the solution was taken and diluted with mobile phase to make up to 100 ml. The final solution (100 µg/ml of PRG) was filtered by 0.45 µm nylon membrane filter by using injection filter.

Preparation of mobile phase

Methanol: water (95:5 v/v), Degassing ensures pump performance and improves detector response. Hence the mobile phase prepared was degassed by ultrasonication, so as to avoid the disturbances caused by dissolved gases. The degassed mobile phase was filtered through
0.45 µm filter to avoid the column clogging due to smaller particles.

Conditioning of the column

Before a new run on HPLC, conditioning of the column was done by passing HPLC grade methanol at 1 ml/min flow rate for 30 min so as to remove the remains of the previous run. The double distilled water was run at flow rate of 1ml/min for 1 h, so as to remove water soluble impurities from on the column.

Loading of mobile phase

Filtered and degassed mobile phase was thus filled in the reservoir. Priming was done for each freshly prepared mobile phase.

Baseline stabilization

The detector was turned on for an hour before the actual run so as to obtain the stable UV light. The mobile phase run was started at required flow rate and the run was continued so as to obtain the stable baseline.

3. METHOD VALIDATION

Calibration curve of pregabalin

50 mg of PRG weighed and dissolved in 50 ml of mobile phase. Then sonicated for 15 min. Suitable dilutions of different concentrations using mobile phase were made from the standard stock solutions. The final solutions were filtered by 0.45 μm nylon membrane filter by using injection filter. The linearity of the method was constructed for PRG reference standard solutions by plotting the concentrations of the compound versus peak area response. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. PRG was found to be linear within range 5.0-45.0 μg/ml.

Solution stability

The stability of the reference PRG sample solutions at room temperature was evaluated with the help of HPLC systems.

Specificity and selectivity

The specificity and selectivity of the proposed method was evaluated by estimating the amount of pregabalin in the presence of common excipients such as magnesium stearate, starch, lactose, glucose, fructose and talc. The ability to separate all the compounds (excipients and substance) from PRG in the sample was demonstrated by assessing the resolution between the peaks corresponding to various substances.

Recovery studies

To study the accuracy, reproducibility and precision of the above method, recovery studies were carried out by addition of standard drug solution to pre-analyzed sample at three different levels. The method was evaluated within the linear range based on the analysis of PRG reference standard samples and pharmaceutical products at 20, 30 and 35 μg/ml. 5 independent analysis were performed at each concentration level within 1 day (intraday precision) as well as for three consecutive days (interday precision). Recovery experiments were carried out by standard addition method.

Robustness Studies

The robustness of a method is its ability to remain unaffected by small deliberate variations in the method parameters. The following changes in the optimum parameter values were examined, the flow rate of the mobile-phase and the proportion of the mobile phase.

Limit of detection (LOD) and limit of quantification (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) of PRG was determined by calculating the signal-tonoise (S/N) ratio of 3:1 and 10:1, respectively according to International Conference on Harmonization guidelines.

4. RESULTS AND DISCUSSION

Method development and optimization

The applied chromatographic conditions permitted to a good resolution of PRG (30 µg ml-1) in standard solution (fig. 2), and in sample solution (fig. 3). No drug deposition was observed during the analysis. The described method has been validated for the assay of PRG using parameters reported below (Mishra AK et al.,2010, United States of Pharmacopoeia 2000, P.D.Sethi 2001, Lloyd R et al., 2012).

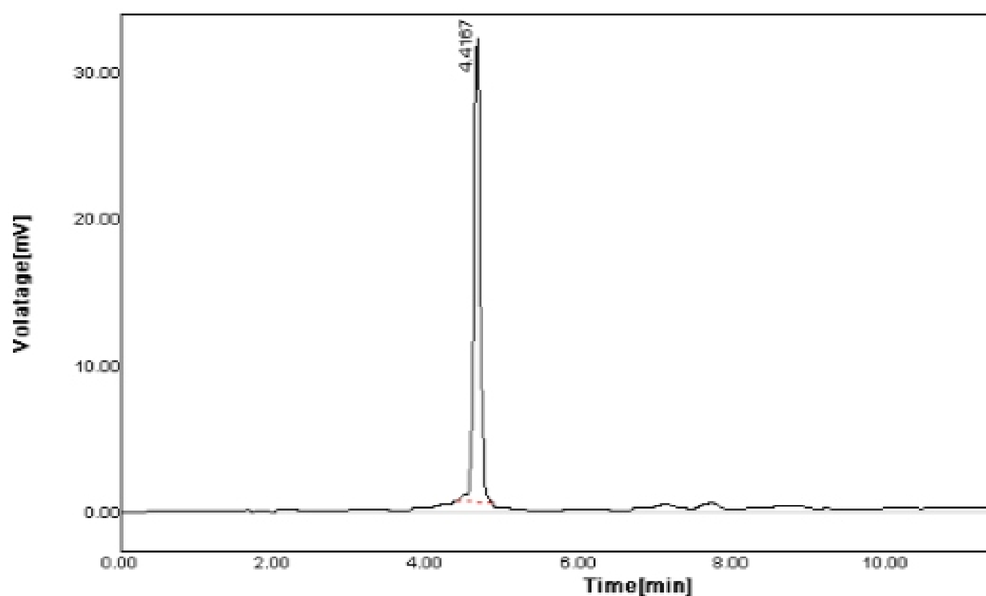

Fig. 2: Chromatogram of Pregabalin (PRG) Standard – R.T:4.4167.

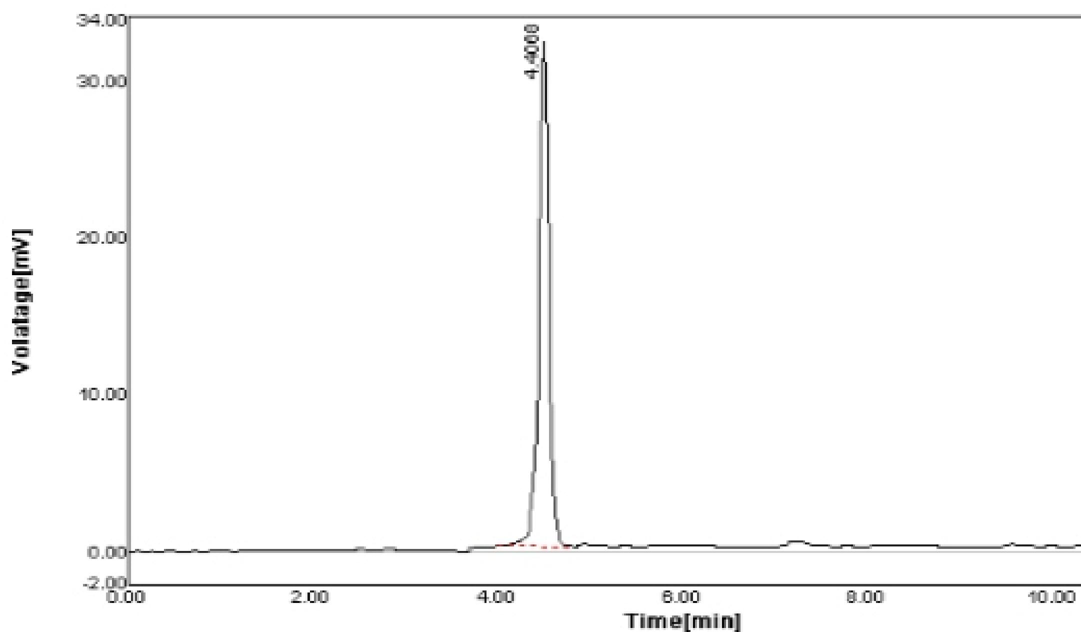

Fig. 3: Chromatogram of Pregabalin (PRG) Sample – R.T:4.4068.

Linearity

AreaUnder the optimum experimental conditions, the concentration vs peak area plot for the proposed method was found to be linear over the range of 5.0-45.0 µg/ml. The parameters for the regression analysis are given in Table.3. Linearity graph of different concentration of PRG shown in fig. 4

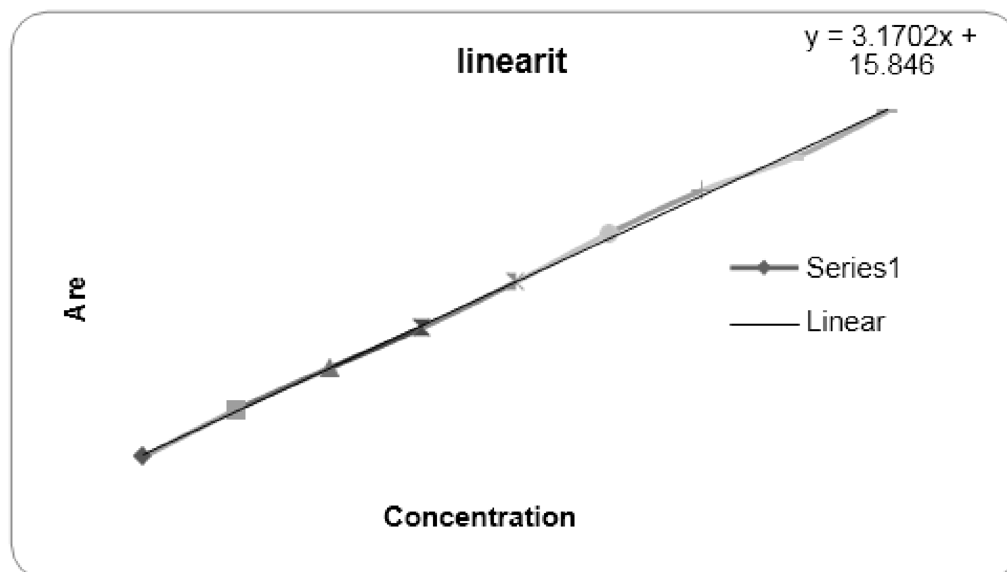

Fig. 4: Linearity of Pregabalin.

Table 3: Summary of Optical and Regression Analysis of the Calibration Curve for PRG

Statistical Parameters	Pregabalin
Linearity range (µg/ml)	May-45
Regression equation	y = mx + c =3.170x + 15.84
Slope (m)	3.17
Standard deviation of slope	0.025
Intercept(c)	15.84
Standard deviation of Intercept	0.353
Correlation coefficient (r)	0.999
Limit of Detection (LOD), µg/ml	0.37
Limit of Quantization (LOQ), µg/ml	1.124

[a]with respect to y = mx + c, where x is the concentration in µg/ml, Y is the peak area.

Precision

The days of precision assays were carried out through replicate analysis (n = 5) of PRG corresponding to 20.0, 25.0, 30.0 µg/ml for the proposed method in pure form (Table 4) and pharmaceutical formulations (Table-5 and 6). The interday precision was also evaluated through replicate analysis of the pure drug and pharmaceutical formulations samples for 5 consecutive days at the same concentration levels as used in the within day precision (Table 5 and 6). As can be seen from the Table 4 that the recovery and relative standard deviation (RSD, %) by intraday and interday precision were in the ranges 98.88 - 100.062%, 0.114-0.245% and 98.92-100.008%, 0.065-0.34%, respectively. As can be seen from Table 5 and Table 6 that that the recovery and RSD by intraday and interday precision were in the ranges 98.41-100.21%; 0.028- 0.502% and 98.07-100.2%, 0.01-1.49%, respectively. The precision results are satisfactory.

Table 4: Summary of Accuracy and Precision Results of the Proposed Method for PRG in Pure Form

Proposed methods	Pregabalin concentration, (µg/ml)	Concentration Found ± SD[a] (µg/ml)	RSD (%)	(%) Recovery
	20	20.008±0.049	0.245	100.062
Intraday assay	25	24.71±0.035	0.141	98.88
	30	29.73±0.033	0.114	99.07
	20	19.99±0.068	0.34	100.008
Interday assay	25	24.72±0.0486	0.196	98.92
	30	29.74±0.0194	0.065	99.15

[a]Mean for 5 independent analyses.

Table 5: Intra-Day Variability of PRG in Different Pharmaceutical Formulations

Pharmaceutical formulations	Pregabalin concentration, (µg/ml)	Concentration Found ± SD[a] (µg/ml)	RSD (%)	(%) Recovery
	20	19.95±0.013	0.067	99.79
Neugaba-75	25	24.68±0.007	0.028	98.74
	30	29.75±0.027	0.09	99.18
	20	20.03±0.039	0.195	100.21
Pregastar-75	25	24.76±0.030	0.123	99.06
	30	29.75±0.020	0.069	99.19
	20	19.92±0.030	0.15	99.62
Gabanext-75	25	24.59±0.120	0.49	98.41
	30	29.76±0.025	0.086	99.2
	20	19.96±0.047	0.235	99.82
Gabafit-75	25	24.74±0.095	0.384	98.98
	30	29.77±0.019	0.065	99.22
	20	19.99±0.030	0.154	99.97
Maxgalin-75	25	24.68±0.124	0.502	98.73
	30	29.76±0.019	0.065	99.22

[a]Mean for 5 independent analyses.

Table 6: Intra-Day Variability of PRG in Different Pharmaceutical Formulations

Pharmaceutical formulations	Pregabalin concentration (ì g/ml)	Concentration found ± SD[a] (ì g mL^{-1})	RSD (%)	(%) Recovery
	20	19.96±0.030	0.15	99.85
Neugaba-75	25	24.77±0.033	0.133	99.1
	30	29.77±0.039	0.131	99.24
	20	19.97±0.029	0.145	99.88
Pregastar-75	25	24.89±0.373	1.49	99.57
	30	29.78±0.049	0.165	99.27
	20	19.97±0.050	0.25	99.84
Gabanext-75	25	24.61±0.061	0.251	98.46

	30	29.82±0.040	0.135	99.43
	20	20.016±0.069	0.344	100.2
Gabafit-75	25	24.77±0.035	0.141	99.1
	30	29.79±0.048	0.161	99.31
	20	19.96±0.033	0.169	99.8
Maxgalin-75	25	24.76±0.110	0.447	98.07
	30	29.76±0.030	0.103	99.2

[a]Mean for 5 independent analyses.

Accuracy

The accuracy of the HPLC assay method was assessed by standard addition method. The results are reported in Table 7 (pure drug) and Table 8(capsule dosage forms) and the recoveries ranged from 99.03±0.116 to 100.34±0.881% for pure drug and 99.29± 0.45 to 99.88±1.32 for dosage forms, respectively. The proposed method results are satisfactorily accurate and precise.

Table 7: Recovery of PRG Using Proposed RP-HPLC Method

Amount of drug added, Ì g/ml	Amount found, µg/ml, (n=3) Mean ± S.D.	(%) Recovery, (n=3) Mean ± S.D.
20	20.06±0.179	100.34±0.881
25	24.758±0.030	99.033±0.116
30	29.73±0.149	99.14±0.508

Assay determination of pregabalin from its capsule formulations

The assay results of samples at three different concentrations were also evaluated by the proposed method and the % recoveries mentioned as below in Table 8.

Table 8: Mean ±S.D. Amount of PRG in Capsule Dosage Forms by Using Proposed RP-HPLC Method

Capsule formulation	Labeled amount of drug, (mg).	Mean ± S.D., amount found, µg, (n=3)*	% Recovery
Neugaba-75	75	74.823±0.99	99.76±1.32
Pregastar-75	75	74.819±0.75	99.76±1.01
Gabanext-75	75	74.947±0.69	99.88±0.85
Gabafit-75	75	74.465±0.38	99.29±0.51
Maxgalin-75	75	74.472±0.33	99.29±0.45

***Average of three different concentration levels.**

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ was found to be 0.37 and 1.124 µg/ml, respectively.

Specificity and selectivity

The specificity of the RP-HPLC method was determined by complete separation of PRG with parameters like retention time (Rt), resolution (RS), and tailing factor (T). The peaks obtained for PRG were sharp and have clear baseline separation. Specificity parameters mentioned in Table 9.

Table 9: Specificity Parameters

Parameters	PRG*(Average±SD)
Tailing factor (T)	0.8761±0.08
Resolution (RS)	0
Retention time (tR)	4.4189±0.109

***(Average±SD) taken for five replicates.**

Robustness

The robustness of the method relative to each operational parameter was checked and investigated. The influences of small changes in the mobile phase composition were studied to determine the robustness of the method, such as the changes in peak area and retention time. The results are summarized in Table 10. The robustness of the method was also assessed by analyzing the active PRG in pharmaceutical formulations. The reference standard sample solution containing 30.0 μg/ml of the drug was assayed and indicating that the proposed method is robust.

Table 10: Robustness Studies

Mobile Phase Composition	Retention Time (tR)	Peak area
Methanol: water (85: 15), 0.7 ì g/ml	4.5167	102.56
Methanol: water (90: 10), 0.8 ì g/ml	4.4	119.43
Methanol: water (75: 25), 1 ì g/ml	3.7833	89.763
Methanol: water (95: 5), 1.2 ì g/ml	3.4	106.54
Methanol: water: Acetonitrile (90: 5: 5), 1.0 ì g/ml	3.2333	100.43
Methanol: water: Acetonitrile (85: 10: 5),0.7 ì g/ml	4.5	131.354

Solution stability

The solution stability was ascertained from HPLC peak area of reference standard samples. The peak area was obtained at 4.4167 min retention time with a UV detector of wavelength of 288 nm (shimadzu, UV-1800). The standard sample solutions were kept at room temperature for 5 days, it was observed that there was no change in peak area of these solutions.

5. CONCLUSION

The proposed RP-HPLC method has been evaluated over the linearity, precision, accuracy, stability, specificity and proved to be convenient and effective for the quality control of PRG in bulk and pharmaceutical formulations. The major advantages of this method include short retention time, without derivatisation with other reagent, stability of the solution, no need for prior separation or purification before analysis, and the applicability of a common HPLC system (Isocratic system, Uvdetector). The short chromatographic time makes this method suitable for the processing of multiple samples in a limited period of time. The method shows no interference from common excipients. Thus, proposed method is rapid, selective, simple, cost effective, fast and efficient. Finally, since no pharmacopoeial method for determi- nation of PRG in bulk and pharmaceutical formulations have been reported yet, the proposed method could be useful and suitable for the determination of PRG in bulk and pharmaceutical formulations.

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High Performance Liquid Chromatography Quality Control

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A B S T R A C T

This review gives a general overview of High Performance Liquid Chromatography (HPLC) Method for the detection, the separation and the quantification of the active compounds from the organics matrices. A brief description of the instrumentation and the method devel- opment is provided. The principles of HPLC including different separation modes and detection methods for the quantitative analysis are summarized. Finely, the validation procedures in real samples are also described.

Keywords: HPLC; Analytical method; Validation; Development; Quality Control.

1. INTRODUCTION

Liquid chromatography was initially discovered as an analytical technique in the early twentieth century and was first used as a method of separating colored compounds. This is where the name chromatography chroma means color, graphy means writing, was derived. A Russian botanist named Mikhail S. Tswett used a rudimentary form of chromatographic separation to purify mixtures of plant pigments into the pure constituents. He separated the pigments based on their interaction with a stationary phase, which is essential to any chroma- tographic separation. The stationary phase he used was powdered chalk and aluminia, the mobile phase in his separation was the solvent [1].

High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture [2].

The enormous success of HPLC can be attributed to a number of inherent features associated with reproducibility, ease of selectivity manipulation, and generally high recoveries. The most significant feature is the excellent resolution that can be achieved under a wide range of conditions for very closely related molecules, as well as structurally quite distinct molecules [3], [4].

The principle is that a solution of the sample is injected into a column of a porous material (stationary phase) and a liquid (mobile phase) is pumped at high pressure through the column. The separation of sample is based on the differences in the rates of migration through the column arising from different partition of the sample between the stationary and mobile phase. Depending upon the partition behaviour of different components, elution at different time takes place. The sample compound with the greater affinity to the stationary layer will travel slower and for a shorter distance in comparison to compounds with less affinity which travel faster and for a longer distance [5].

Generally, the detector attached to the HPLC unit generates ultraviolet rays of particularly specified wavelength and the eluting sample is subjected to these ultraviolet rays. Molecules of the eluting species get excited by absorbing the energy of the ultraviolet rays, during the process of de-excitation energy is released which is being recorded by the detector [6], [7].

A signal proportional to the energy change is generated and recorded in the form of a graph called chromatogram. The absorption of the ultraviolet rays depends on the functional group of the eluting species, because every functional group requires a particular amount of energy for its excitation, which is carried by ultraviolet light of a certain wavelength. However, sometimes specifically reported costly apparatus is often not available in the research labs. To achieve the desired analysis on the available set of apparatus, certain parameters need to be tuned, this is called HPLC method development. The main goals of this review are to describing the instrumentation of the method, to explaining all parameters that influence the HPLC results and summarizing the validation procedures of developed method.

2. DIFFERENT TYPES OF HPLC

There are following variants of HPLC, depending upon the phase system (stationary) in the process:

Normal phase HPLC

This method separates analytes on the basis of polarity. NP-HPLC uses polar stationary phase and non-polar mobile phase. Therefore, the stationary phase is usually silica and typical mobile phases are hexane, methylene chloride, chloroform, diethyl ether, and mixtures of these.
Polar samples are thus retained on the polar surface of the column packing longer than less polar materials [8].

Reversed phase chromatography

Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC oper- ates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar ana- lyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent [9].

Ion exchange chromatography

In Ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. This form of chromatography is widely used in purifying water, Ligand-exchange chromatography, Ion-exchange chromatography of proteins, High-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc [10].

Size exclusion chromatography

Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides [11].

Bio-affinity chromatography

Separation based on specific reversible interaction of proteins with ligands. Ligands are covalently attached to solid support on a bio- affinity matrix, retains proteins with interaction to the column-bound ligands.

Proteins bound to a bioaffinity column can be eluted in two ways:

Biospecific elution: inclusion of free ligand in elution buffer which competes with column bound ligand. Aspecific elution: change in pH, salt, etc. which weakens interaction protein with column-bound substrate.

Because of specificity of the interaction, bioaffinity chromatography can result in very high purification in a single step (10 - 1000-fold) [12].

3. INSTRUMENTATION OF HPLC

The most important components of the HPLC instrument are: mobile phase/solvent reservoir, solvent delivery system, sample introduc- tion device, column, detectors, data collection and output [13] (Figure 1).

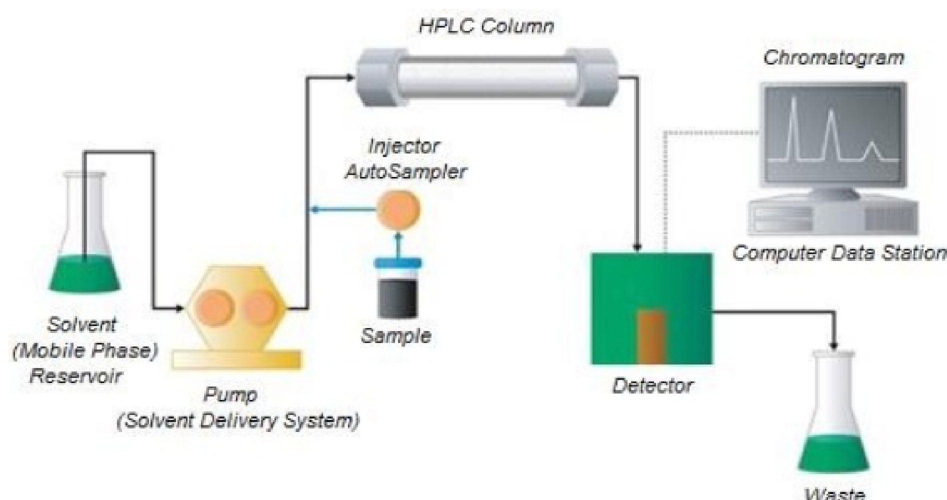

Fig. 1: High-Performance Liquid Chromatography HPLC System.

Mobile phase/solvent reservoir

The reservoir that holds the mobile phase is often no more than a glass bottle. Often, the reagent bottle that holds our HPLC solvent can be used as a reservoir. Solvent is delivered from the reservoir to the pump by means of Teflon tubing -- called the "inlet line" to the pump. Some HPLC systems like the Agilent 1100 shown at the right have special compartments to hold one or more mobile phase reser- voirs. The reservoirs in these systems may have additional features that allow the mobile phase to be degassed and isolated from contact with air.

Solvent delivery system

The solvent delivery system is described like a deliver system of continuous pulse free flow of mobile phase to the HPLC regardless of the system back pressure.

Injection of the sample

The injection of a sample at atmospheric pressure into the system, at high pressure, represents a critical step in the chromatographic pro- cess. Sample injection valves, or switching valves, are used to introduce reproducible amounts of sample into the HPLC eluent stream without causing changes in pressure or flow.

Column

The column is the heart of a HPLC system, There are several types of matrices for support of the stationary phase, including silica, pol- ymers, and alumina. Silica is the most common matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems.

Detector

An overview of various HPLC detectors is provided with discussion of unique detector characteristics and a comparison of advantages and drawbacks between them. Focus is placed on the most common detectors, including UV/Vis absorbance, fluorescence, electrochemi- cal, conductivity, refractie index, and mass spectrometry detectors.

Data collection and output

The output is recorded as a series of peaks, each one representing a compound in the mixture passing through the detector and absorbing UV light (in the case of HPLC-UV/Vis). The area under the peak is proportional to the amount of substance, which is passed through detector, and this area can be calculated automatically by the computer linked to the display.

4. HPLC DEVELOPMENT METHOD

There are many factors to consider when developing methods. The initially collect the information about the analyte's physiochemical properties (pKa, log P, solubility) and determining which mode of detection would be suitable for analysis. The majority of the analytical development effort goes into validating a stability indicating HPLC–method. The goal of the HPLC-method is to try & separate quantify the main active compound, any reaction impurities, all available synthetic inter-mediates and any degradants [14].

Physicochemical properties of the active compound

Physicochemical properties of the active compound play an important role in method development. For method development one has to study the physical properties like solubility, polarity, pKa and pH of the molecule. Polarity is a physical property of a compound. It helps an analyst, to decide the solvent and composition of the mobile phase.

Selection of diluents is based on the solubility of analyte. The analyte must be soluble in the diluents and must not react with any of the diluent components. The diluent should match to the starting eluent composition of the assay to ensure that no peak distortion will occur, especially for early eluting components.

The acidity or basicity of a substance is defined most typically by the pH value. The pH value is defined as the negative of the logarithm to base 10 of the concentration of the hydrogen ion, pH = - log10

[H3O+]. Selecting a proper pH for ionizable analytes often leads to symmetrical and sharp peaks in HPLC. Sharp, symmetrical peaks are necessary in quantitative analysis in order to achieve low detection limits, low relative standard deviations between injections, and reproducible retention times.

The pKa is characteristic of a particular compound, and it tells how readily the compound gives up a proton.

An acid dissociation constant is a particular example of equilibrium constant. For the specific equilibrium between a monoprotic acid HA and its conjugate base A−.

It turns that the pKa of an acid is the pH at which it is exactly half dissociated. This can be shown by rearranging the expression for Ka: pH = pKa – log10([AH]/[A-])

At half-neutralization the pH is numerically equal to pKa. Conversely, when pH = pKa, the concentration of HA is equal to the concen- tration of A−.

The buffer region extends over the approximate range pKa ± 2, though buffering is weak outside the range pKa ± 1. At pKa ± 1, [A−]/[HA] = 10 or 1/10. If the pH is known, the ratio may be calculated. This ratio is independent of the analytical concentration of the acid. When the pKa and analytical concentration of the acid are known, the extent of dissociation and pH of a solution of a monoprotic acid can be easily calculated [15–18].

Column selection

The heart of a HPLC system is the column. Changing a column will have the greatest effect on the resolution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacted a chlorosilane with the hydroxyl groups present on the silica gel surface.

There are several types of matrices for support of the stationary phase, including silica, polymers, and alumina. Silica is the most com- mon matrix for HPLC columns. Silica matrices are robust, easily derivatized, manufactured to consistent sphere size, and does not tend to compress under pressure. Silica is chemically stable to most organic solvents and to low pH systems. One shortcoming of a silica solid support is that it will dissolve above pH 7. In recent years, silica supported columns have been developed for use at high pH [19].

Shape and particle size effect

Generally, Smaller particle results in a greater number of theoretical plates, or increased separation efficiency. However, the use of smaller particles also results in increased backpressure during chromatography and the column more easily becomes plugged [20].

Common stationary phases

The Common stationary phases are C4 (butyl), C8 (octyl), C18 (octadecyl), nitrile (cyanopropyl), and phenyl (phenyl propyl) columns. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide greater retention of non-polar analytes.

Commonly used reverse phase columns and their uses are listed below. Propyl (C3), Butyl (C4), and Pentyl (C5) columns are useful for ion- pairing chromatography. Examples: include Zorbax SB-C3,

YMC-Pack C4, and Luna C5. These columns are generally less stable to hydrolysis than columns with longer alkyl chain. Octyl (C8) columns have wide applicability. This phase is less retentive than the C18 phases, but is still quite useful for pharmaceuticals. Example: include (Zorbax SB-C8, Luna C8 and YMC-Pack-MOS). Octadecyl (C18, ODS) columns are the most widely used and tend to be the most retentive for non-polar analytes. Examples include Zorbax SB-C18, YMC- Pack ODS and Luna C18. Xterra RP-C18 and Zorbax Extend-C18 columns have been formulated to tolerate high pH systems (pH >7, normally up to pH 11).

Phenyl (Ph) columns offer unique selectivity from the alkyl phases and are generally less retentive than C8 or C18 phases. Phenyl col- umns are commonly used to resolve aromatic compounds. Examples include Zorbax SB-Phenyl, YMC-Pack Phenyl and Luna Phenyl- Hexyl. Nitrile (CN or cyano) columns are polar and can be used for both reverse and normal phase applications. This phase is often used to increase retention of polar analytes. Examples include Zorbax SB-CN, Luna-CN, and YMC-Pack CN.

The type of column chosen for a particular separation depends on the compound and the aim of analysis 21-27 .

Column temperature

Column temperature control is important for long-term method reproducibility as temperature can affect selectivity. A target temperature in the range of 30–40 °C is normally sufficient for good reproducibility. Use of elevated temperature can be advantageous for several reasons. First, operating at a temperature higher than ambient reduces the viscosity of the mobile phase and thus the overall backpressure on the column. Lower system pressures allow for faster flow rates and thus faster analyses. The temperature may also affect selectivity patterns because analytes will respond dissimilarly to different temperatures [28].

Mobile phase solvent type

Acetonitrile (ACN), methanol (MeOH) and tetrahydrofuran (THF) are commonly used solvents in RP-HPLC having low UV cut-off of 190, 205 and 212nm respectively. These solvents are miscible with water. Mixture of acetonitrile and water is the best initial choice for the mobile phase during method development.

The mobile phase effects resolution, selectivity and efficiency. In reverse phase chromatography, the mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed will affect the analysis of the molecule.

Selection of the mobile-phase and gradient conditions is dependent on the ionogenic nature of the analyte and the hydrophobicity of the analytes in the mixture respectively. Acidic analytes in buffers of sufficiently low pH will remain unchanged, increasing retention. Con- versely, at higher pH neutral basic compounds will be more retained, and ionized acidic compounds will elute earlier. Peak splitting may be observed if the pKa of a compound is similar to the pKa of the buffer, and the analyte elutes as both a charged and uncharged species. The pH of a buffer will not greatly affect the retention of non-ionizable sample components [29].

Isocratic elution

A separation that employs a single solvent or solvent mixture of constant composition.

Gradient elution

Here two or more solvent systems that differ significantly in polarity are employed. After elution is begun; the ratio of the solvents is varied in a programmed way, sometimes continuously and sometimes in a series of steps. Separation efficiency is greatly enhanced by gradient elution.

Buffer selection

Choice of buffer is typically governed by the desired pH. It is important that the buffer has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pKa value < 2 units of the desired mobile phase pH (Table 1).

Table 1: HPLC Buffers, pKa Values and Useful pH Range

Buffer	pKa	Useful pH Range
Trifluoroacetic acid (TFA)	<2	1.5-2.5
KH2PO4/K2PO4	7.2	6.2-8.2
KH2PO4/ phosphoric acid	2.1	1.1-3.1
Ammonium acetate	4.8	3.8-5.8
	9.2	8.2-10.2
Ammonium formate	3.8	2.8-4.8
	9.2	8.2-10.2
Ammonium hydroxide/ ammonia	9.2	8.2-10.2
Potassium formate / formic acid	3.8	2.8-4.8
Potassium Acetate/ acetic acid	4.8	3.8-5.8
Borate (H3BO3/Na2B4O7 10H2O)	9.2	8.2-10.2

Generally, a buffer concentration of 10-50 mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC. Phosphate buffers can be replaced with sulfate buffers when analyzing organophosphate compounds [30].

General considerations for buffer selection:

- TFA can degrade with time, is volatile, absorbs at low UV wavelengths.
- Phosphate is more soluble in methanol/water than in acetonitrile/water or THF/water.
- At pH greater than 7, phosphate buffer accelerates the dissolution of silica and severely shortens the lifetime of silica-based HPLC columns. If possible, organic buffers should be used at pH greater than 7.
- Ammonium bicarbonate buffers usually are prone to pH changes and are usually stable for only 24 to 48 hours. The pH of this mo- bile phase tends to become more basic due to the release of carbon dioxide.
- Ammonium salts are generally more soluble in organic/water mobile phases.
- Some salt buffers are hygroscopic. This may lead to changes in the chromatography (increased tailing of basic compounds, and pos- sibly selectivity differences).

- Microbial growth can quickly occur in buffered mobile phases that contain little or no organic modifier. This growth will accumu- late on column inlets and can damage chromatographic performance.
- After buffers are prepared, they should be filtered through a 0.2-μm filter.
- Mobile phases should be degassed.

Selection of detectors

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analyses, potential interference, limit of detection required, availability and/or cost of detector (Table 2).

Table 2: HPLC Detector Choice

Detector	Type of compound can be detected
UV-Visible &Photodiode array	Compounds with chromophores, such as aromatic rings or multiple alternating double bonds.
Fluorescence detector	Fluorescent compounds, usually with fused rings or highly conjugated planer system.
Conductivity detector	Charged compounds, such as inorganic ions and organic acid.
Electrochemical detector	For easily oxidized compounds like quinines or amines
Refractive Index detector & Evaporative light scattering detector	Compounds that do not show characteristics usable by the other detectors, eg.polymers, saccharides.

UV-visible detector is versatile, dual wavelength absorbance detector for HPLC. This detector offers the high sensitivity required for routine UV-based applications to low-level impurity identification and quantitative analysis. Photodiode Array (PDA) Detector offers advanced optical detection for Waters analytical HPLC, preparative HPLC, or LC/MS system solutions. Its integrated software and op- tics innovations deliver high chromatographic and spectral sensitivity. Refractive index chromatographic and spectral sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi- wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds [31], [32].

Preparation of sample solutions for method development

The components being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. The sample solution should be filtered, the use of a 0.22 or 0.45 μm pore-size filter is generally rec- ommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analyses [31–34].

Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants /insoluble components without leaching undesirable artifacts into the filtrate. If any additional peaks are observed in the filtered samples, then the diluent must be filtered to determine if a leachable component is coming from the syringe filter housing/filter.

Optimization method

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, sample amounts, Injection volume and diluents solvent type [35].

5. VALIDATION METHOD

Validation of an analytical procedure is the process by which it is established, by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines [36].

The main typical analytical performance characterstics which may be tested during methods validation are System Suitability, Accuracy, Precision, Repeatability, Intermediate precision, Linearity, Detection limit, Quantification limit, Specificity, Range, Robustness, System suitability determination, Forced degradation studies and Solution stability studies.

System suitability

System suitability testing originally believed by the industry of pharmaceuticals to decide whether a chromatographic system is being utilized day today in a routine manner in pharmaceutical laboratories where quality of results is most important which is suitable for a definite analysis.

The parameters used in the system suitability tests (SST) report are: Number of theoretical plates or Efficiency (N), Capacity factor (K), Separation or Relative retention (α), Resolution (Rs), Tailing factor (T), Relative Standard Deviation (RSD) [37–40].

Number of theoretical plates/efficiency (N)

In a specified column, efficiency is defined as the measurement of the degree of peak dispersion and it should have the column character- istics. The efficiency is conveyed in terms of number of theoretical plates. The formula of calculation of N is illustrated bellow in the following Sigma/tangential method (USP method) With the help of sigma/tangential method N is calculated which is shown in the following figure 2 duly noting the formula for calculation of N.

Fig. 2: Sigma/Tangential Method Relating to Determination of N.

$$N = 16\left(\frac{t_R}{W_b}\right)^2 = 5.54\left(\frac{t_R}{W_{\frac{1}{2}}}\right)^2$$

N = Number of theoretical plates.
t_R = Retention time or retention distance.
h = Peak height.
w_b = width of the peak at the base line.

The plate number depends on column length. Theoretical plate number is the measure of column efficiency. As stated by plate theory, the analyte will be in instant equilibrium with stationary phase and column has to be divided into number of hypothetical plates and each plate consists of a fixed height and analyte spends finite time in the plate. Height equivalent to theoretical plate (HETP) is given by following formula:
HETP = L/N,
Where, L = length of column. N = plate number

Capacity ratio or capacity factor (K')

The capacity factor (k') is a means of measuring the retention of an analyte on the chromatographic column.

$$K' = \frac{t_R - t_M}{t_M}$$

The above said capacity factor sometimes is called as a retention factor which has no dimension and independent from flow rate of mo- bile phase as well as column dimensions which is the measure of extent of retention relating to an analyte relative to an un-retained peak. Where tR implies retention time of the sample peak and retention time of an un-retained peak is tM. k' = 0 means no compound is left in the column. Generally the value of k' is > 2 [41].

Relative retention or separation factor (α)

The selectivity (or separation) factor (α) is the ability of the chromatographic system to 'chemically' distinguish between sample compo- nents.

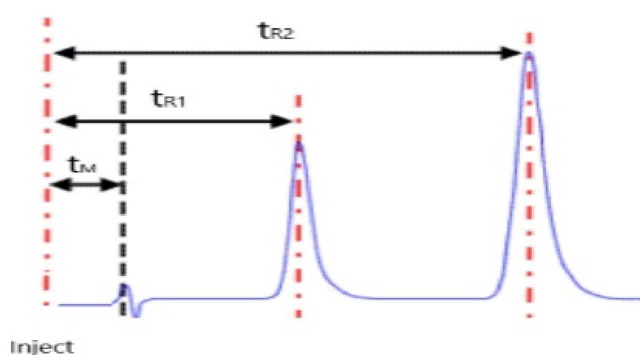

Fig. 3: Determination of Separation Factor (A).

$$\alpha = \frac{(t_{R2} - t_M)}{(t_{R1} - t_M)}$$

t_1= the retention time from the point of injection of reference peak defined. (Suppose no reference peak is found, value would be zero).
t_2= Retention time calculated from point of injection.
t_M= Unretained peak time (Retention time (t_R) of an inert component not retained by the column).

Resolution (Rs)

Resolution is the capability of the column to separate 2 components in 2 individual peaks or chromatographic zones and it is improved by enhancing column length, reduction of particle size and rising temperature, altering the eluent or stationary phase. It can be told in terms of ratio of separation of the apex of two peaks by the tangential width average of the peaks. By using the following formula resolution is calculated.

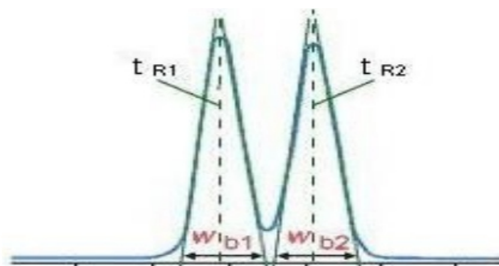

Fig. 4: Determination of Resolution between Two Peaks.

$$Rs = \frac{(t_{R2} - t_{R1})}{(0.5(t_{wb1} - t_{wb2}))}$$

t_{R1} and t_{R2} are the retention times for the two peaks of components. twb1 and twb2 At the baseline lies between tangents drawn to the sides of the peaks. (Tangents are drawn at 0.6 times the peak height). If the peaks are correctly symmetric, provided the valley between the two peaks should touch the baseline Rs is 1.5. Generally good value of resolution is Rs ≥2 should be adequate and preferred normally [42].

Resolution factor (R)

Resolution is a function of capacity factor, function of selectivity and a function of efficiency (or) number of theoretical plates (N). In order to separate any two peaks you must have right capacity factor ideally between 2 and 10, but appropriate selectivity is required i.e., ideally 1.2 and enough efficiency i.e., number of theoretical plates (more than 2000 theoretical plates). Resolution should be ≥ 1.5. 1.5 defines baseline resolution.

$$R = \frac{1}{4}\sqrt{N}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{K'}{K' + 1}\right)$$

Tailing factor or asymmetry factor

Chromatographic peak assumed to have a Gaussian shape under ideal conditions. However in practical conditions, there is always a deviation from normal distribution which indicates non- uniform migration and non-uniform distribution process. The asymmetry factor and tailing factor are roughly same and rarely accurate and equal in most cases. Values should normally between 1.0-1.5 and values greater than 2 are unacceptable. The peak asymmetry is computed by utilizing the following formula [43].

As = B/A Where:

As = peak asymmetry factor.

B = distance from the point at peak midpoint to the trailing edge (measured at 10 % of peak height). A = distance from the leading edge of peak to the midpoint. (Measured at 10 % of peak height).

Table 3: System Suitability Parameters are shown in the Following.

Parameter name	Acceptance criteria
Number of theoretical plates or Efficiency (N)	> 2000
Capacity factor (K)	< 1
Separation or Relative retention (α)	> 1
Resolution (Rs)	> 1.5
Tailing factor or Asymmetry(T)	< 2
Relative Standard Deviation (RSD)	< 2

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typical- ly these might include impurities, degradants, matrix, etc.

Accuracy

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conven- tional true value or an accepted reference value and the value found.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements ob- tained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra- assay precision.

Intermediate precision

Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardization of methodology).

Detection limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not neces- sarily quantified as an exact value.

Quantification limit

The quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low levels of com- pounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

Linearity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample.

Range

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Forced degradation studies

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical method's ability to measure an active ingredient and its degradation products, without interference, by generating potential degradation products. During validation of the method, the substance are exposed to acid, base, heat, light and oxidizing agent to produce approximately 10% to 30% degradation of active substance. The studies can also provide information about the degradation pathways and deg- radation products that could form during storage [44].

Solution stability studies

During validation the stability of standards and samples is established under normal conditions, normal storage conditions, and some- times in the instrument to determine if special storage conditions are necessary, for instance, refrigeration or protection from light [45].

6. CONCLUSION

It can be concluded from the entire review that HPLC is a versatile, reproducible chromatographic technique for the estimation of each products. It has wide applications in different fields in term of quantitative and qualitative estimation of active molecules.

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Method Development and Validation of Clobazam in Bulk and Pharmaceutical Dosage Forms by using High Performance Thin Layer Chromatographic Method

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ABSTRACT

In the present research a simple, accurate, precise and cost-effective High-performance thin layer chromatographic method for the estima- tion of clobazam, in bulk and pharmaceutical dosage form was illustrated. The RF value of the drug was found to be 0.74 in the mobile phase, acetone: toluene: formic acid (1: 1: 0.05 v/v/v). A linear response was observed in the range of 100-700 ng with a regression coefficient of 0.999. Validation parameters were carried out as per the guidelines of International Conference for Harmonization (ICH). This method can be used in the industries for determination of clobazam to analyze the quality of formulation without interference of the excipients.

Keywords: Clobazam; Anti-Epileptic; λ max; ICH; High Performance Thin Layer Chromatography.

1. INTRODUCTION

Clobazam is an antiepileptic drug belonging to the benzodiazepine series coming under the class of Anticonvulsant drugs and chemically called as 7-chloro-1,5-dihydro-1-methyl-5-phenyl-1,5-benzodiazepine2,4(3H)-dione (Fig 1). Clobazam is a long-acting 1,5-benzodiaze- pine with uses similar to those of diazepam as 1,4-benzodiazepine. It is used in the treatment of epilepsy in association with other antiepi- leptics. It is also used in the short-term treatment of acute anxiety [1-2]. Clobazam belongs to the 1,5-benzodiazepine class with a pka value of 6.65. Clobazam bulk powder is a white crystal with molecular weight of 300.7. the drug is slightly soluble in water and soluble in organic solvents [3]. The reference of Clobazam is not found in majority of pharmaceutical and chemistry books. clobazam is official in British and Indian pharmacopoeia 2007 [4-5]. Today majority of marketed antiepileptic dosage forms are of clobazam e.g. Frisium, Urbanyl, etc., There are several research papers which illustrates the method for estimation of clobazam by colorimetry and HPLC in bulk and pharma- ceutical dosage form [6-7]. Also, there are several bio-analytical methods developed for clobazam in biological fluids containing clobazam, like serum and plasma [8]. There have been very a smaller number of analytical methods developed for estimation of clobazam in pure bulk form and in dosage form. In the present study method development and validation was carried out by HPLC method [9].

7-chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4(3H,5H)-dione

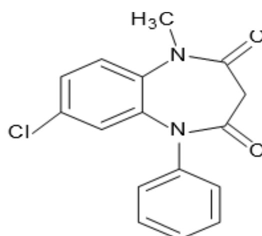

Fig. 1: Structure of Clobazam

2. EXPERIMENTAL

Chemicals

Clobazam was obtained from Sanofi-Aventis, Ltd. Goa, India. Acetone, toluene and formic acid were purchased from Qualigens fine chemicals, India. Chemicals and reagents were of AR-grade.

Chromatography

Analysis was performed on 10 × 10 cm aluminium backed silica gel F254 HPTLC plates (E-Merck, Darmstadt, Germany). Before using, the plates were predeveloped with methanol and then dried in an oven at 60 °C for 5 min. Standard solution and sample solution were applied to the plate as 6 mm bands by means of a Camag Linomat-V (Muttenz, Switzerland) sample applicator equipped with 100 µl syringe (Hamilton, Reno, Nevada, USA); the distance between the bands was 11.6 mm. Camag twin trough chamber used as development chamber previously saturated for 20 min with acetone: toluene: formic acid (1: 1: 0.05 v/v/v) as mobile phase. The average development time is 20 min. After development, the plate was dried at 110 °C in an oven for 10 min. Densitometric scanning at 254 nm was then performed with a Camag TLC Scanner equipped with Win-Cat software, version 1.3.0 using a Deuterium light source. The slit dimensions were 6.00 mm × 0.20 mm.

Optimization and detection of UV wavelength

The sensitivity of HPTLC method that uses UV/VIS detection depends upon the proper selection of detection wavelength. An ideal wave- length is one that gives good response for the drug that is to be analyzed. In the present study, by appropriate dilution of each stock solution, various concentrations of clobazam were prepared. Each solution was scanned in the spectrum mode and their spectra were observed. The wavelength selected for the analysis was 254 nm at which clobazam showed significance absorbance.

Preparation of stock solutions

Standard clobazam 10 mg was weighed and transferred to a 10 ml volumetric flask. 5 ml of methanol was added to dissolve the drug. The flask was shaken and volume was made up to the mark with methanol to give a solution containing concentration of 1000 µg/ml (stock solution A). From this stock solution, pipette out 1 ml and place it in 10 ml volumetric flask. The volume was made up to mark with methanol to give a solution containing concentration of 100 µg/ml (stock solution B).

Method validation [10]

Linearity

Standard solution equivalent to 100, 200, 300, 400, 500, 600 and 700 ng per band of clobazam were applied to a pre-developed HPTLC plate. The plate was developed, dried and scanned as described above. The chromatograms were obtained and peak area was determined for each concentrations of drug solution. A calibration plot was constructed by plotting peak area against amount of clobazam (ng). The linearity of response for clobazam was assessed in the concentration ranges 100-700 ng per band; the slope, intercept, and correlation coefficient were also determined.

Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation of clobazam were determined by using standard deviation of the response and slope approach as defined as in International Conference on Harmonization (ICH) guidelines.

The LOD and LOQ were calculated by using following formulae LOD = 3.3 × S.D of intercept/ Average of slope.

LOQ = 10 × S.D of intercept/ Average of slope.

Precision

Precision was evaluated by using standard solutions containing clobazam at concentration covering the 100, 200 and 300 ng per band. The precision of the method in terms of intra-day precision (% R.S.D) was determined by analyzing clobazam standard solution in the range (100-300 ng per band) three times on the same day. The inter-day precision (% R.S.D) was assessed by analyzing these solutions (100-300 ng per band) on three different days over a period of one week.

Accuracy

Accuracy is the closeness of the test results obtained by the method to the true value. To study the Accuracy, twenty tablets of each formulation were weighed and powdered and also analysis of the same was carried out. Recovery studies were carried out using standard addition method by adding known amount of standard drug solution (50%, 100% and 150%) to the sample solution and % recovery was calculated.

Reproducibility

The reproducibility of sample application was assessed by spotting drug solution (4 µl) six times on a HPTLC plate, then development of plate and recording the peak AUC.

Analysis of the marketed formulation

Twenty tablets [Frisium 5, Sanofi-Aventis, Ltd.] were weighed and finely powdered. The powder equivalent to 10 mg of clobazam was accurately weighed and transferred to 10 ml volumetric flask containing 5 ml methanol. The flask was shaken and volume was made up to the mark with methanol. The solution was filtered through Whatmann filter paper (No. 41) to give a solution of concentration 1000 µg/ml. From the above solution pipette out 1 ml and make up the volume to 10 ml with methanol to give a solution containing 100 µg/ml. From this solution, appropriate volume was injected to the TLC plate. The analysis was carried out by two analysts.

3. RESULTS AND DISCUSSION

Optimization of the procedure

The pure drug was applied to the TLC plates and chromatographed with different mobile phases. Initially ethyl acetate: chloroform (4: 6 v/v) and acetone: toluene (1: 1 v/v) were tried. Addition of 0.05 ml formic acid to these mobile phases improved the characteristics of the bands. Finally, the mobile phase acetone: toluene: formic acid (1: 1: 0.05 v/v/v) was found to enable good resolution with a sharp and symmetrical peak of RF 0.74. Well defined bands were obtained when the chamber was saturated with mobile phase for 20 min at room temperature (Fig.2)

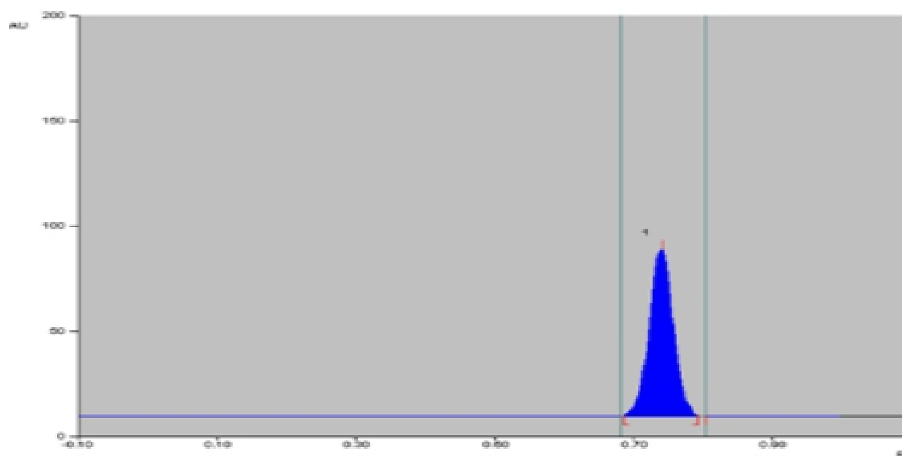

Fig. 2: Densitogram Obtained from Clobazam Standard by HPTLC Method.

3.2.1. Linearity

The calibration graph was linear, i.e. the system adhered to Beer's law, over the range 100-700 ng per band. Linearity was evaluated by duplicate analysis of seven standard working solutions equivalent to 100-700 ng per band of clobazam. The regression data showed linearity was good over the concentration range investigated; this was apparent from the high value of the correlation coefficient. Typical linearity data are given in Table 1.

The calibration curve (Fig. 3) and 3D view (Fig. 4) shows good correlation between clobazam concentrations and peak areas (Fig. 5).

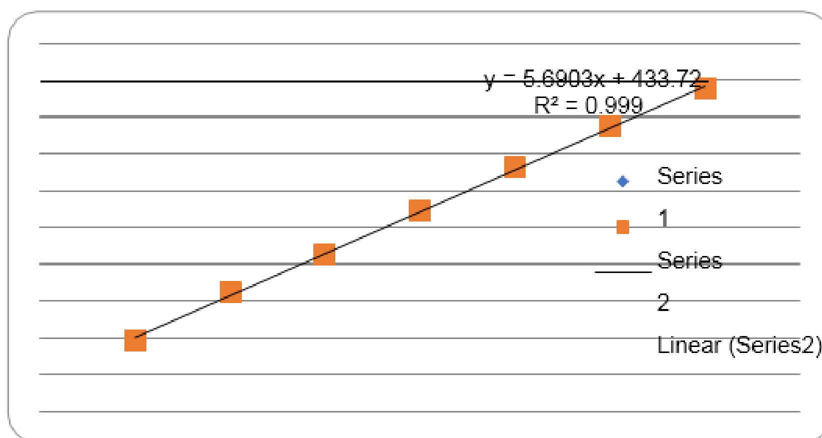

Fig. 3: Calibration Curve of Clobazam by HPTLC Method.

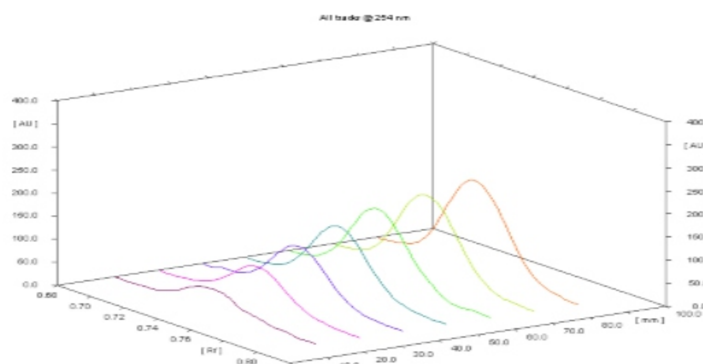

Fig. 4: 3D View of Clobazam by HPTLC Method.

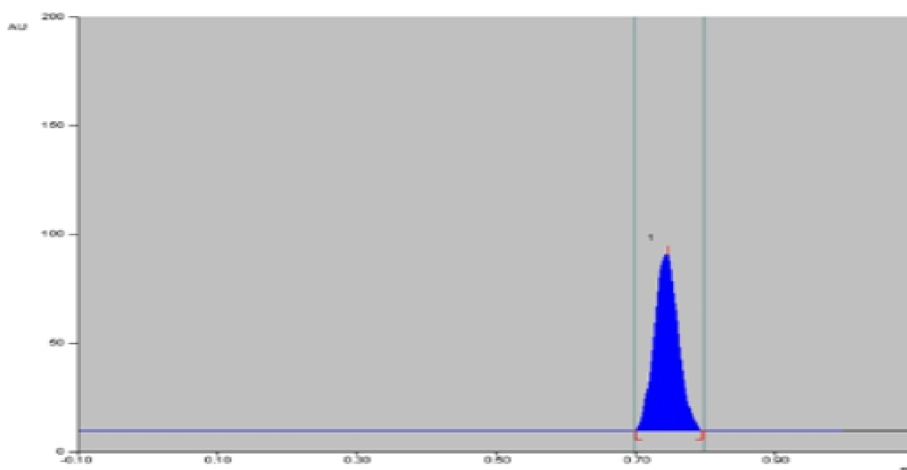

Fig. 5: Chromatogram of 400ng of Sample Solution of Clobazam by HPTLC Method.

Table 1: Linear Regression Data for the Calibration Plots

Regression data Value
Linear range 100-700 ng per spot r^2 0.999
Slope 5.690
Intercept 433.7

3.2.2 Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ for the proposed method were found to be 20.98 and 63.56 ng per band, respectively.

Precision

Repeatability of sample application of peak area, as % R.S.D, was determined for concentrations 100, 200 and 300 ng. % R.S.D for interday and intraday analysis was <2%. The values are shown in Table 2.

Table 2: Results of the Measurement of Intra and Interlay Precision for Clobazam by HPTLC Method

Concentration (ng per spot)	Intra-day precision Mean ± S. D**	% R. S. D	Inter-day Precision Mean ± S. D**	% R. S. D
100	943.3 ± 15.42	1.635	945.26 ± 0.66	0.07
200	1618.6 ± 9.77	0.639	1658 ± 17.74	0.949
300	2199.3 ± 24.54	1.116	2215.4 ± 40.61	1.833

****Average of Three determinations.**

Accuracy

When the method was used for subsequent analysis of clobazam in Pharmaceutical dosage form spiked with 50, 100 1nd 150 % extra drug, recovery was 98-102 % of clobazam as bulk and in dosage form (Table 3).

Table 3: Results of Accuracy Studies of Clobazam by HPTLC Method

Level of recovery (%)	Amount of drug added (mg)	Amount of drug recovered (mg)*	% Recovery ± S. D*
50	5	5.05	101.92 ± 0.479
100	10	9.95	99.55 ± 0.286
150	15	14.8	98.68 ± 0.917

***Average of three determinations**

Reproducibility

The % R.S.D was calculated for peak area and RF value with repeated determination for the same concentration. The studies were carried out using 400 ng as a target concentration. The reports are listed in Table 4.

Table 4: Reproducibility Results of Clobazam by HPTLC Method

Sl. No	Area	Rf
1	2749.8	0.74
2	2780.8	0.75
3	3 2734	0.74
4	2822.8	0.74
5	2839.8	0.74
6	2829.4	0.75
Mean	2792	0.74
S. D	44.49	0.0051
% R.S. D	1.593	0.6947

3.3. Analysis of marketed formulation

A single band at RF 0.74 was observed in the densitogram of drug samples extracted from tablets. There was no interference from excipients commonly present in the tablets. The drug content was found to be 99.84% and 99.44 for analyst 1 and analyst 2, respectively. The results are reported in Table 5.

Table 5: Applicability of the Proposed HPTLC Method for Analysis of Commercial Tablets

Sample	Label claim (mg)	Analyst I		Analyst II	
		Amount found (mg)	% Recovery ± S.D**	Amount found (mg)	% Recovery ± S.D**
Frisium	5	4.99	99.84 ± 1.04	4.97	99.44 ± 1.271

****Average of three determinations**

4. CONCLUSION

This HPTLC method for quantitative analysis of clobazam in Pharmaceutical formulations is cost effective, precise, accurate and repro- ducible without interference from the excipients. The method was validated in accordance with ICH guidelines. The method reduces anal- ysis time compared with other methods and seems to be suitable for routine analysis of Pharmaceutical formulations in quality-control laboratories, where economy and speed are essential.

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