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**RESEARCH ARTICLE**

# Quantification of Crystalline Linagliptin Form-A in Amorphous 5 mg Tablets using Calibration curve Method by using Powder X-Ray Diffraction (PXRD)

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## ABSTRACT

The ability to detect and quantify polymorphism of pharmaceutical compounds is critically important in ensuring weather the formulated product delivers the desired therapeutic properties or not because different polymorphic forms of a drug exhibit different solubilities, stabilities and bio-availabilities. Amorphous materials are often preferred in the pharmaceutical industry due to their enhanced dissolution rate and bioavailability. Since the amorphous state is metastable and thermodynamically less stable relative to the crystalline state, there is always a potential for unexpected crystallization in storage. Conversion of amorphous to crystalline state can be evaluated by stability studies. Accurate quantification of crystalline phase present in drug materials has become increasingly imperative, due to stringent regulatory concerns about polymorph characterization. In the present study, a quantification PXRD method has been developed to determine the amount of Linagliptin Form-A in Linagliptin amorphous 5 mg tablets. This method is capable of determining the amount of Linagliptin Form-A in Linagliptin amorphous drug substance and Linagliptin tablets. This study emphasizes on the importance of mathematical calculation for physical mixture preparation for polymorphic quantification in drug product. Validation of quantization method was carried out with respect to specificity, precision, ruggedness, linearity, robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ). This method can also be used at the manufacturing site to check the presence of crystalline phase of Linagliptin Form-A in amorphous drug substance and drug product.

**Key Words**: Linagliptin, Polymorphism, Powder X-Ray Diffraction (PXRD), Validation.

## INTRODUCTION

Crystalline solids normally require a significant amount of energy for dissolution due to their highly organized, lattice structures.[1] The energy required for a drug molecule to escape from a crystal is more than from an amorphous or a non-crystalline form.[2-5] It is known that the amorphous forms in a number of drugs exhibit different dissolution characteristics and in some cases dissimilar bioavailability patterns compared to the crystalline form.[6] For some therapeutic indications, one bioavailability pattern may be favoured over another.[7] An amorphous form of some of the drugs exhibit much higher bioavailability than the crystalline forms, which leads to the selection of the amorphous form as a final drug substance for pharmaceutical dosage from development.[8] Additionally, the aqueous solubility of crystalline form is lower than its amorphous form in some of the drugs,[9] which may result in the difference in their *in vivo* bioavailability.[10] Therefore, it is desirable to have the amorphous forms of drugs with high purity to meet the needs of regulatory agencies and also highly reproducible processes for their preparation.[11] Amorphous solids have attracted the interest of pharmaceutical scientists because of two major developments (i) a continuous increase in the number of insoluble developmental drug molecules because of the advent of novel methods of synthesis and screening and (ii) the growing attention in regulatory aspects of the pharmaceutical solids. The 'apparent solubility' and dissolution advantage offered by these systems is a vital approach to enhance the bioavailability of poorly water soluble drugs. However, limitations of amorphous systems such as physical instability and higher chemical

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reactivity, act as a hurdle in their extensive commercialization. [12-18] The use of amorphous materials in any field is associated some challenges. A very significant challenge is that the amorphous phase is thermodynamically less stable compared to any crystalline phase of the same material. During manufacturing operations and/or storage amorphous forms are likely to revert into the stable or a meta-stable crystalline form if they are not adequately stabilized. X-ray powder diffraction (XRPD) is a powerful tool in pharmaceutical industry for phase quantification.[19-20] Advantages of XRPD method are simplicity, measurement at room temperature and the non-destructive nature.[21] The Linagliptin is a DPP-IV inhibitor.[22-23] Linagliptin is useful for the prevention or treatment of diabetes mellitus, pre-diabetes or reduced glucose tolerance. The generic name Linagliptin is marketed by *Boehringer Ingelheim* under the brand name Tradjenta/Trajenta. The chemical name of linagliptin is 8-[(3R)-3-aminopiperidin-1-yl]-7-(but-2-yn-1-yl)-3-methyl-1-[(4-methylquinazolin – 2 - yl) methyl]-3,7-dihydro-1H-purine-2,6-dione. The molecular mass is 472.54 and molecular formula is $C_{25}H_{28}N_2O_2$. The structural formula is shown in **Figure 1**.

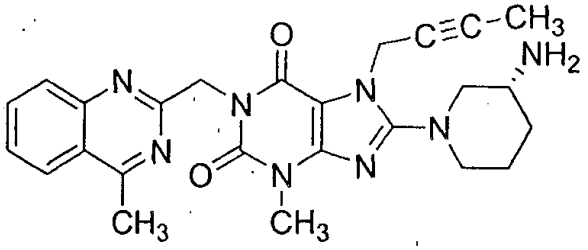

**Figure 1: Linagliptin Structure**

The linagliptin has more than thirty crystalline forms and stable amorphous form.[24-26] An amorphous Linagliptin is very stable, reproducible and so the amorphous Linagliptin is suitable for formulating as Linagliptin and there is possibility that the potential for unexpected crystalline Linagliptin form-A during manufacturing process and storage. Accurate quantification of crystalline phases present in drug materials has become increasingly imperative, due to stringent regulatory concerns about polymorph characterization. The scope of the present study was to demonstrate this application of PXRD in the quantification of low level crystalline content of Linagliptin Form-A in Linagliptin tablets 5 mg samples (Linagliptin amorphous API) as per the regulatory requirements.[27] Stable amorphous form of linagliptin is used in Linagliptin 5mg tablet formulation. International Conference on Harmonization (ICH) Q6A guidelines provides guidance on, when and how polymorphic forms should be monitored and controlled.[28] Hence there is a need to develop a sensitive XRD method for quantification of Linagliptin form-A in Linagliptin 5 mg Tablets. The PXRD quantification method development for Linagliptin 5 mg tablets has highly challenging because API has diluted by excipients hence XRD instrument become less sensitive, hence there is a need to develop a sensitive XRD method for quantification of Linagliptin form-A in Linagliptin tablets 5 mg, however, in this study we have achieved sensitivity in XRD method by the optimization of instrument parameters and sample preparation. This requires an accurate measurement of intensity, height and area of diffraction lines, but these decisive parameters are strongly influenced by potential source of errors due to inherent nature of the samples, instrument and sample preparation parameters. The latter two parameters can be optimized in order to minimize the errors associated with measurement of vital outputs. Various sample preparation parameters like type of sample holder, rotation of sample, powder packing, and preferred orientation effects, have been demonstrated to be critical. This study focuses on multiple objectives of (i) optimization of sample preparation, selection of characteristic and specific peaks of polymorphic form-A and instrument parameters and (ii) development of an accurate, linear, precise and reproducible and robustness for determination of Linagliptin of Form-A in Linagliptin 5 mg tablets.

## MATERIALS AND METHODS

### Materials

Linagliptin Form A, Linagliptin Amorphous polymorphic standards, placebo and Linagliptin tablets 5mg were gifted by Hetero drugs Ltd, Andhra Pradesh (India). All materials were used as received without any further purification.

### Sample Preparation and Measurement procedure

Take 5-6 tablets and carefully remove the tablets coating with the help of sharp blade. Grind the tablets sample to fine powder using mortar and pestle. Take required quantity of the sample in sample holder (stack). Press the sample in sample holder to prepare a smooth and flat surface of sample. Record the powder X-ray diffraction pattern(s) of the sample(s). Check for the

presence of a peak. If no peak is present, no further action is required. If a peak is present, calculate the percentage polymorphic form-A according to the established calibration curve. (See Mathematical Calculation).

**Reference mixture preparation (Spiked standard preparation)**

Linagliptin form A and Linagliptin amorphous are used as obtained. Placebo was prepared by physically mixing inactive ingredients as per the formulation. Average weight of a Linagliptin tablet is about 180 mg of which the drug strength is 5 mg. Prepare a series of reference mixtures containing 0.0, 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 % polymorphic form-A (expressed as % w/w versus the total amount of Linagliptin in tablets). These reference mixtures are made by mixing different amounts of % polymorphic form-A with grounded Linagliptin tablets 5 mg. It is recommended to pre-grind approximately 50 tablets in a mortar until a homogeneous powder mixture is obtained. The components are gently mixed using pestle and mortar, the components are added following a geometric series (1 part + 1 part; 2 parts + 2 parts …) and the mixing is made 3-dimensional using a scraper. First step 30% primary reference mixture is prepared then diluted to 25%, 20%, 15%, 10% and 5% by using pre-grind tablet powder. The actual weights of each component and the composition of the final spiked standard are given in **Table 1**.

**Table 1: Varying amounts of linagliptin form-A and amorphous tablet powder**

| %(w/w) Form-A | 30% | 25% | 20% | 15% | 10% | 5% | 0% |
|---|---|---|---|---|---|---|---|
| Form-A added (mg) | 59.56 | - | - | - | - | - | - |
| Tablet powder added (mg) | 5000.12 | 250.42 | 450.32 | 600.12 | 750.45 | 900.12 | Tablets powder |
| 30% Ref Mix added (mg) | - | 868.06 | 631.31 | 422.31 | 264.38 | 127.03 | - |

## MATHEMATICAL CALCULATION

Use the following equation to calculate the % polymorphic form-A of the 30.0 % reference mixture. [29-32]

**$C_r$ = [A/ (A + (5/180)*B)]*100 Equation-1**

Where:

$C_r$= concentration of polymorphic form-A (expressed as % w/w versus the total amount of Linagliptin)

**A= mg of form-A**

B=mg of ground Linagliptin tablet powder

Use following formula to calculate the coefficients K and L in order to calculate the exact concentrations of the 25.0, 20.0, 15.0, 10.0, and 5.0 % reference mixtures. [29-32]

**K= A/(A+B) Equation-2**

**L = ((5/180)*B+A)/(A+B) Equation-3**

K corresponds to the fraction of polymorphic form-A in the total mass of the physical mixture.

L corresponds to the fraction of Linagliptin (Linagliptin form-A + Amorphous Linagliptin) in the total mass of the physical mixture.

Use the following equation to calculate the % crystalline Linagliptin form-A in the physical mixtures for 25.0, 20.0, 15.0, 10.0, and 5.0 % reference mixtures [29-32]:

**$C_r$ = [(K*X)/(L*X + (5/180)* B)]*100**

**Equation 4**

Where:

$C_r$=concentration of polymorphic form-A (expressed as % w/w versus the total amount of Linagliptin)

X=mg of 30.0 % reference mixture

B=mg of ground Linagliptin tablet powder

**Calculation for Sample**

Calculate the correction factor (f) which compensates for the difference in intensity of the X-ray source, using the following formula [29-32]:

**$f = NA_{calibration\ curve}/NA_{sample}$ Equation-5**

Where

$NA_{calibration\ curve}$ = net area (counts°) of the silicon peak at the time the calibration curve was established

$NA_{sample}$ = net area (counts°) of the silicon peak at the time of the current analyses.

Integrate the net area (counts°) of the diffraction peak of polymorphic form-A in the sample scan (if present).

Convert the integrated net area to percentage of form-A in Linagliptin tablet (expressed as % w/w versus the total amount of Linagliptin) using a calibration curve (points: 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 %w/w) [29-32].

$C_i = (f*r_i - b)/a$ **Equation-6**

Where:

$C_i$=concentration of polymorphic form-A (expressed as %w/w versus Linagliptin label claim)

$r_i$=net area of the diffraction peak

b=intercept on the y-axis of the calibration curve

a=slope of the calibration curve

**X-Ray Powder Diffraction**

**For Sample**

PXRD patterns on samples were recorded at room temperature on PANalytical, X' Pert PRO MPD diffract meter (Netherland) Cu Kα X-ray tube radiation (1.54A°), at 45Kv, 40mA passing through nickel filter with programmable divergence slit (irradiated length 10 mm), soller slit (0.02 rad), beam mask (10mm), ant scattering slit(1°) with beam knife, in diffracted beam path long anti scatter shield (5mm), soller slit(0.02 rad) and detector(X' celerator, line detector). The diffractometer with Bragg-Brentano geometry, vertical diffractometers use the θ/θ mode and was calibrated for linearity peak positions with silicon pellet(NIST 640), drift aging test with silicon pellet(NIST 640), alumina disc(NIST 1976) and sensitivity v/s 2θ angle with alumina(NIST1976). Samples were subjected to X-ray powder diffraction analysis in continuous mode with a step size of 0.02° and time per step (2000 Seconds) over scan range 6- 9° 2θ. Suitable quantity of powder was loaded in a 16mm sample loader using back loading technique and pressed by a clean glass slide to ensure co-planarity of the powder surface with the surface of the holder. The sample holder was rotated with spinner resolution time (1 sec) during the measurement. Samples acquisition in data collector soft ware and obtained diffractograms were analyzed with High score (plus) soft ware.

**Procedure for Calibration curve**

Perform a scan of the 0.0 % reference mixture using the same experimental conditions as described in X-Ray powder diffraction for sample. Perform a scan of the 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 % reference mixtures using the same experimental conditions as described in X-Ray powder diffraction for sample. Integrate the net area (counts°) of the diffraction peak corresponding to Linagliptin polymorphic form-A at approximately 7.6° 2θ. Subtract the net area of the 0.0% reference mixture from the net area of each reference mixture. Plot the percentage polymorphic form-A of the 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0% reference mixtures (expressed as % w/w versus Linagliptin label claim) versus the subtracted net area. Calculate the correlation coefficient (r). Acceptance criteria for the calibration curve: $r \geq 0.99$.

**For System suitability (Silicon disc)**

The source intensity of the XRD-instrument is monitored by measurement of a Silicon disc (Zero-Background holder) prior to the analysis of a sample. All the instrument parameters are same as above except few parameters like programmable divergence slit (irradiated length 11.5 mm) and time per step (120 seconds) over scan range 50- 60° 2θ. The net area (counts°) of the Silicon peak is integrated from 54.5° to 58° 2θ **Figure 2**. If this net area (counts°) is lower than 9000 (counts°), no more sample analyses may be performed using this method.

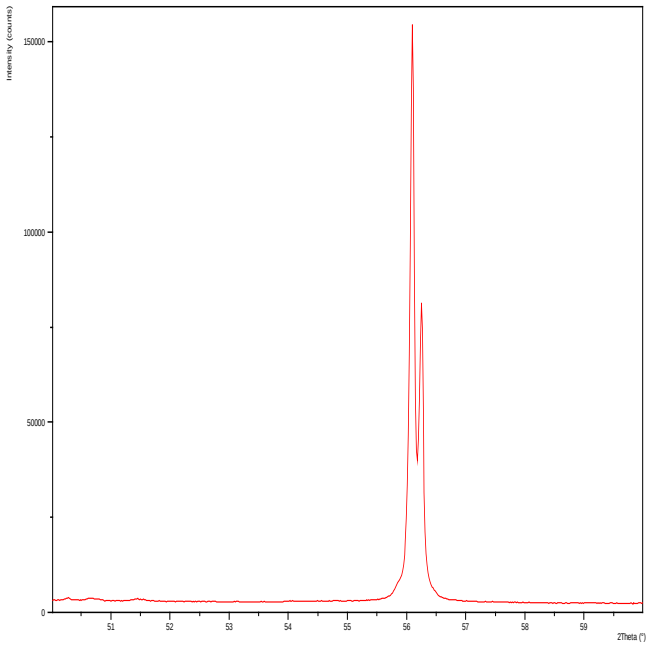


**Figure 2: Silicon Standard (NIST 640)**

**Method Development**

Purpose of the XRPD method is to demonstrate that determination of Linagliptin form A in Linagliptin amorphous 5 mg tablets. A "low" limit of quantization is desired, with the eventual desire to relate degree of crystalinity, to product performance, such as dissolution rate. The method, including interpretation of the powder patterns, must be able to run and analyze reproducibly by several scientists, and possibly may need to be transferred to a manufacturing site. The test samples are monitored for the amount of Linagliptin form A in Linagliptin 5mg tablets. There is a possibility that some of the samples may also contain amorphous phase that has transformed to crystalline phase. Thus, the expected PXRD pattern will be an amorphous, sometimes with peaks due to crystalline phase. The limit of detection and Limit of quantization was determined by spiking the standard crystalline phase at different levels in the amorphous phase under development following variations are studied.

## Optimization of sample preparation

Grind gently tablets containing 20% Linagliptin form A without removing the coating material of the tablets to fine powder using mortar and pestle and fill the same sample in 16 mm holders and analyzed six times. Carefully remove the coating material of tablets containing 20% Linagliptin form A, grind gently to fine powder with mortar and pestle and fill the same sample in 16 mm holders and analyzed six times. In PXRD quantification method, optimization of robust sample preparation and handing sample analysis are very crucial part. Hence it should be borne in mind that the day-to-day error for which the sample was removed daily from the instrument is a composite including variability resulting from sample re-positioning and possibly from sample disturbance on re-analysis. The effect of position of sample holder in the PXRD auto sampler was determined. Variation due to crystal orientation was investigated by re-packing a single sample six times and recording the diffraction pattern after each preparation. The results are given in **Table 2**.

**Table 2: Results for the sample parameters**

| S, No | Parameters | %RSD | Acceptance criterion |
|---|---|---|---|
| 1 | Removed coating material of tablets | 3 | % RSD should not be more than 10 |
| 2 | Without remove coating material of tablets | 11 | |
| 3 | Sample re-positioning | 7 | |
| 4 | Re-packing | 12 | |
| 5 | Changing time per step (500sec) | 8 | |
| 6 | Changing time per step (1000sec) | 5 | |
| 7 | Changing time per step (1500sec) | 4 | |
| 8 | Changing time per step (2000sec) | 2 | |

## Optimization of instrument parameters

The divergence and anti scatter slits varied in order to monitor the peak sharpness, soller slits can be placed in both the incident and diffracted beam path so that it has been varied soller slits in order to monitor on the required resolution and intensity. The step size and time per step varied in order to alter the scan rate of sample. Voltage and current varied up and down in order to monitor the peak intensities.

## RESULTS AND DISCUSSION

## Identification of polymorphs by XRPD

The characteristic XRPD profiles of two polymorphs are compared in **Figure 3, 4 &5** with placebo's. Characteristic Peaks of Linagliptin Form-A at 7.6° 2θ respectively are chosen for quantitative measurements. As a result the selected range for quantitative measurements is from 6 to 9° 2θ.

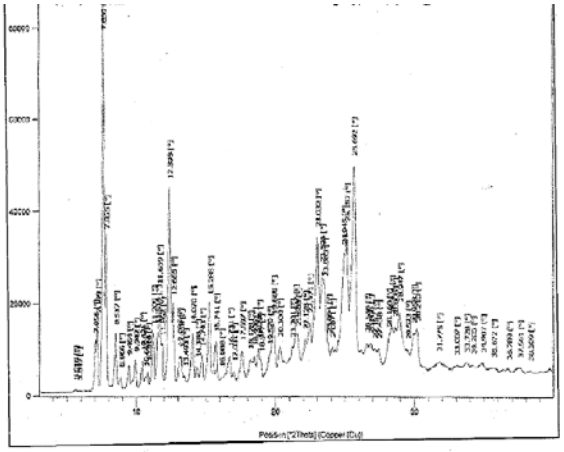

**Figure 3: Linagliptin Form-A polymorphic standard**

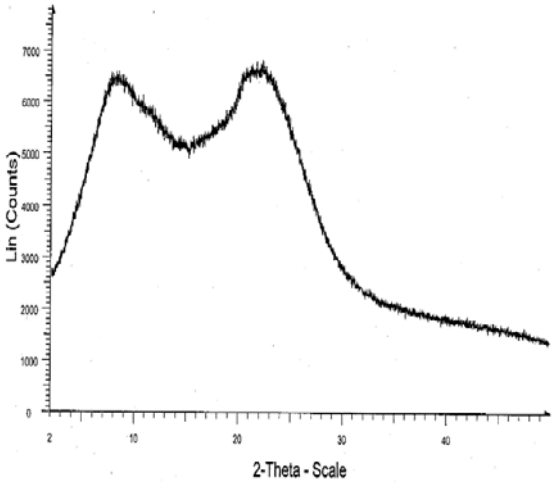


**Figure 4: Linagliptin Amorphous Drug substance**

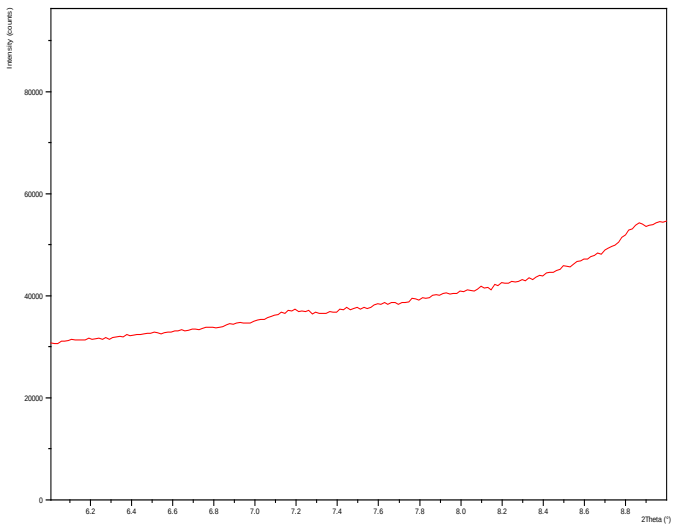

**Figure 5: Placebo for Linagliptin tablets 5mg**

## Method development

The results from the sample analysis with various method parameters like change in Sample preparation with and without coating material, sample position changing, repacked sample analysis and important parameter changing the

time per step are compiled in Table 2. The amount (% weight / weight) of polymorph Linagliptin form- A in Linagliptin tablets calculated for 20% spiked standard using equations (4) and (5). The variations observed for without removing of coating material of tablets, sample re-positioning, re-packing and time per step at 500 sec. Based on the PXRD diffraction response.

## Specificity

Linagliptin Form-A, Linagliptin Amorphous standard, Linagliptin 5 mg tablets and placebo for Linagliptin tablets have been scanned as per method. Specificity is shown in **Figure 6 & 7** by qualitative comparison of the diffraction patterns of placebo, tablets and polymorphic standards.

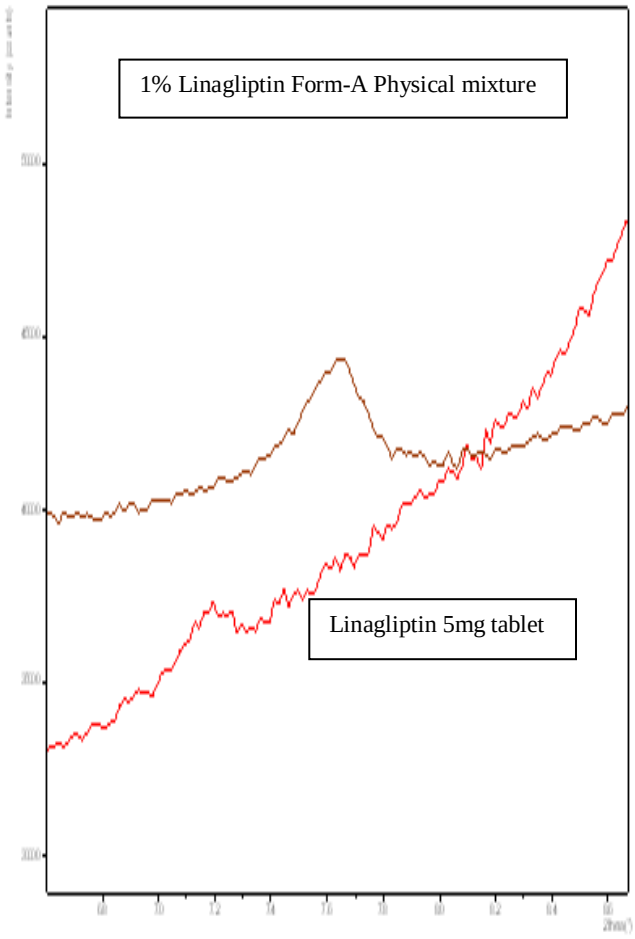


**Figure 6: Linagliptin 5mg tablet and 1% Linagliptin form-A Physical mixture (LOD solution)**

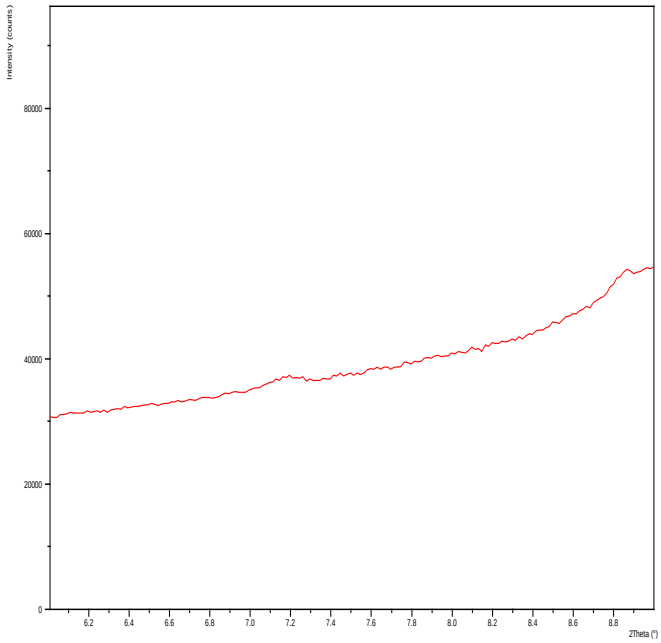

**Figure 7: Placebo for Linagliptin tablets 5mg**

## Precision and Ruggedness

The repeatability of the analysis expresses the precision of this method over a short interval of time and intermediate precision was performed by two different analysts on two different days. The results are provided in **Table 3**. All the results are in the acceptable range and prove the suitability of the method for a precise determination of Linagliptin Form A in Linagliptin 5mg tablets.

**Table 3: results for precision and ruggedness for 20% Form-A spiked sample**

| | Precision | Ruggedness | Acceptance criterion |
|---|---|---|---|
| Average for six runs | 20.1 | 20.3 | % RSD should not be more than 10 |
| SD | 0.8134 | 0.8091 | |
| %RSD | 4 | 4 | |
| Overall %RSD | 4 | | |

## Linearity

The linearity check proves the ability to obtain test results which are directly proportional to the concentration of analyte in the sample. Linearity is demonstrated using seven determinations covering the whole range. Linearity is evaluated by visual inspection of a plot and by a mathematical estimation of the degree of linearity and shown in the **Figure 8 & 9**

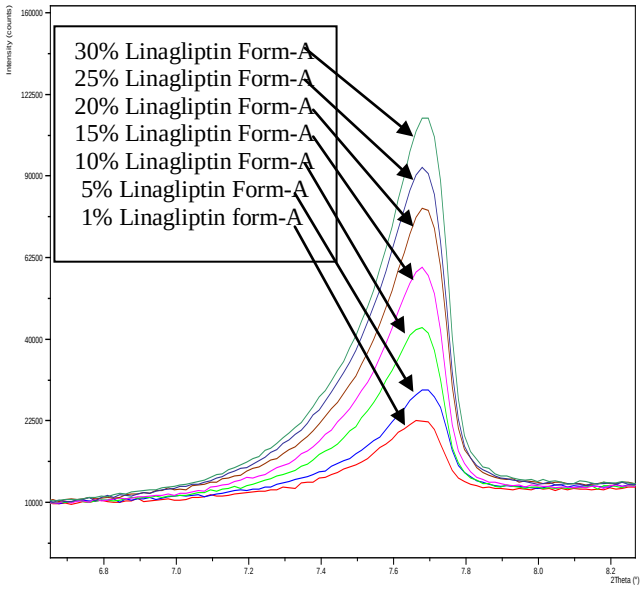


**Figure 8: Overlaid diffractogram representing linear change in the area under peak at about 7.6°2θ, for Linagliptin Form-A (1%, 5%, 10%, 15%, 20%, 25% and 30% of linagliptin form-A with respectively Linagliptin label claim)**

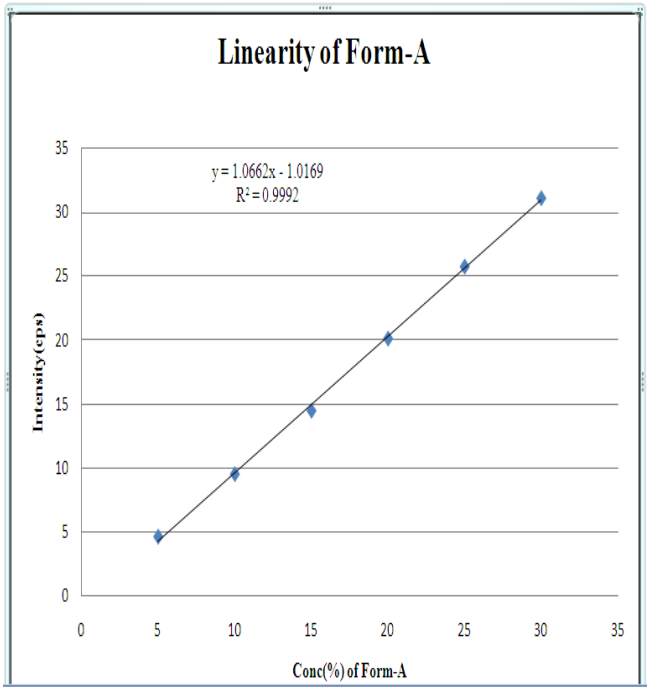


**Figure 9: Linearity Curve of Linagliptin Form A**

Correlation coefficient is 0.9996, where all relevant acceptance criteria are met, demonstrating the acceptable linearity of the method.

## Limit of Detection (LOD) and Limit of Quantization (LOQ)

The Limit of Quantization (LOQ) and Limit of Detection (LOD) of the test method were determined through Linearity curve. Linagliptin Form-A LOD & LOQ values are 1% & 5% respectively.

## Accuracy

The accuracy of the method is assessed using nine determinations covering the specified range at three concentration levels. The accuracy is calculated as the mean recovery and the individual recoveries are reported. The results are tabulated in **Table 4**. All the results pass their acceptance criteria and prove the suitability of the methods for an accurate determination of Linagliptin Form-A in Linagliptin tablets.

**Table 4: Accuracy results for spiked sample at 5%, 15% and 30% of Form-A**

| Actual conc. (%) | Mean Recovery | %RSD | Acceptance criteria |
|---|---|---|---|
| 5.0 | 99.5 | 5 | Recovery should be in between 90.0% - 110.0% |
| 15.0 | 98.1 | 5 | |
| 30.0 | 97.6 | 4 | |

## Robustness

### Linearity of X-Ray source intensity evaluation

A series of physical mixture of tablet samples G002 spiked with polymorphic form-A in the range from 0 to 30% w/w is prepared and measured at different source intensities to evaluate the effect of a decrease of the source radiation on the linearity of the calibration curve. The decrease of the source radiation was simulated by decreasing the amperage of the instrument varying from 45kV-40mA down to 45kV-12mA, which corresponds to a Si net area of approximately 9000 counts°. The results are tabulated in Table 5. All the results are in the acceptable criteria range, demonstrating that the linearity of the calibration curve obtained is even when the source intensity is reduced to the minimum allowed Si net area of approximately 9000 counts° and shown in the **Figure 10**.

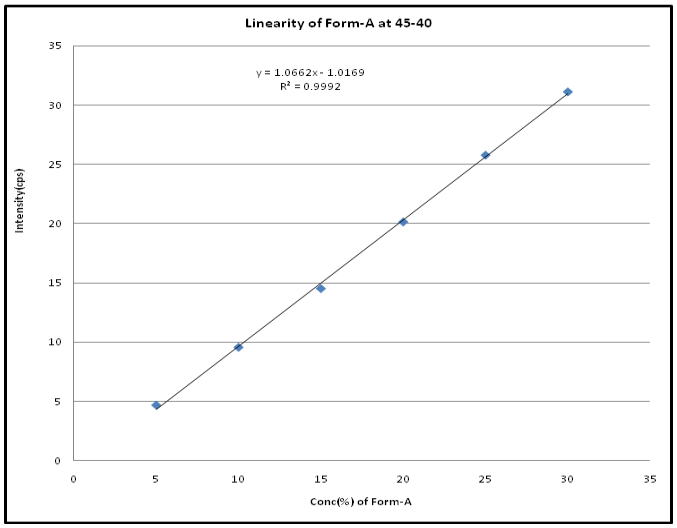


**Figure 10 a: Calibration Curve of Linagliptin at Voltage (kV): Current (mA) -45Kv:40 mA**

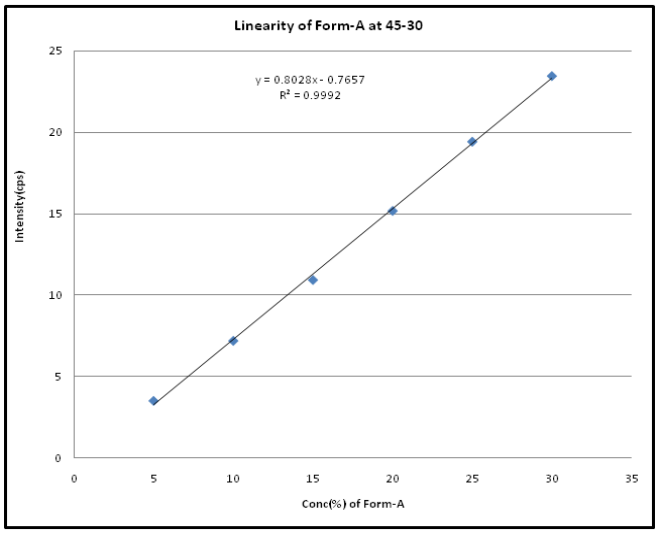


**Figure 10 b: Calibration Curve of Linagliptin at Voltage (kV): Current (mA) -45Kv:30 mA**

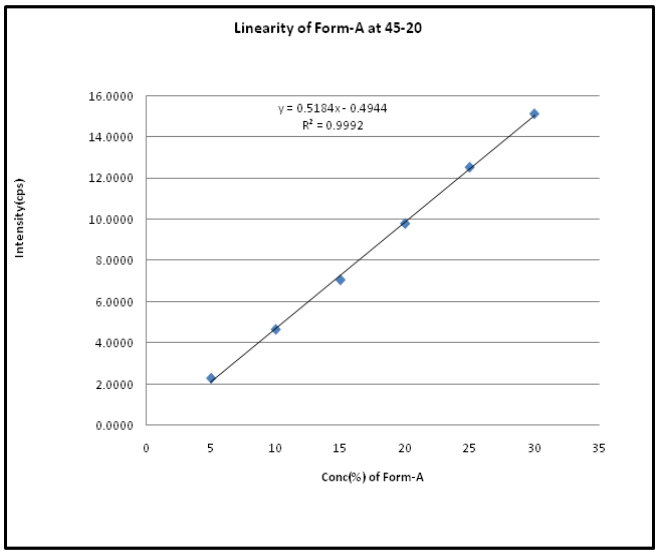


**Figure 10 c: Calibration Curve of Linagliptin at Voltage (kV): Current (mA) -45Kv:20 mA**

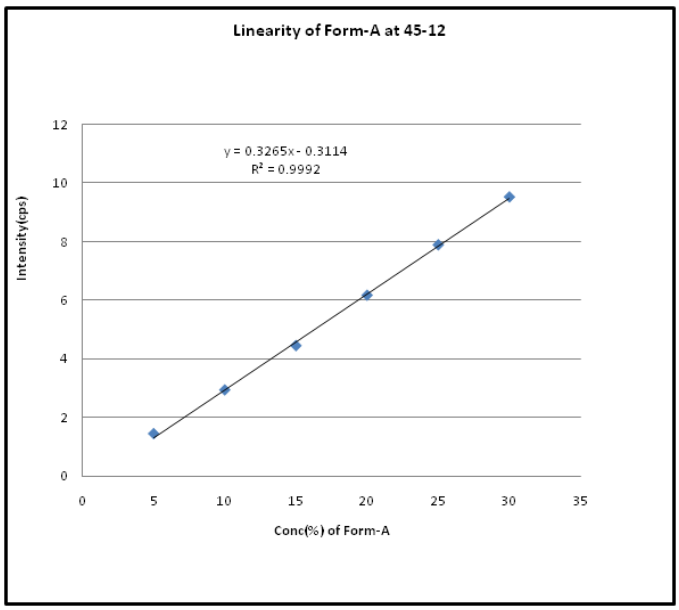


**Figure 10 d: Calibration Curve of Linagliptin at Voltage (kV): Current (mA) -45Kv:12 mA**

**Table 5: Robustness – linearity of x-ray source intensity evaluation**

| Voltage (Kv: mA) | Correlation coefficient (r) | Net area accounts Silica(°) | Acceptance criteria |
|---|---|---|---|
| 45:40 | 0.9996 | 25621.5 | r ≥ 0.99. |
| 45:30 | 0.9996 | 19292.6 | |
| 45:20 | 0.9996 | 12456.8 | |
| 45:12 | 0.9996 | 7845.6 | |

## Impact of grinding energy

A mixture containing 30% of Form A is divided into three portions. The first portion is gently mixed in a mortar, the second is mixed while applying force for 5 minutes and the third portion is mixed while applying force for 10 minutes. Three different sample holders are filled and analyzed according to the description in the test method and % RSD (2%) was obtained. The results are good and acceptable; demonstrating gentle or harsh powdering of material, has no impact on the intensity of the diffraction peak of interest.

## CONCLUSION

A quantification PXRD method has been developed to determine the amount of Linagliptin Form-A in Linagliptin amorphous 5 mg tablets. This method is capable of determining the amount of Linagliptin Form-A in Linagliptin amorphous drug substance and Linagliptin tablets. This study emphasizes on the importance of mathematical calculation for physical mixture preparation for polymorphic quantification in Drug product. In order to minimize the errors associated with the quantification and to obtain an accurate method, sample preparation, sample handling and instrument parameters were optimized. The PXRD quantification method development for drug product especially lower strength has highly challenges because API has diluted by excipients hence XRD instrument become less sensitive, however, in this study the sensitivity has been achieved in XRD method by the optimization of instrument parameters and sample preparation. Regulatory organizations such as the FDA and ICH are pressing the pharmaceutical industry to adopt methodologies and innovative analytical techniques like PXRD that should provide better understanding of the polymorphism phenomenon for drug under development, and enable quality control departments to adequately evaluate the solid state of batches produced. Another advantage of this method is a calibration curve can be prepared when an instrument is used for the first time only, no need to prepare the

calibration curve for every analysis. The correction factor (f) which is introduced compensates for the difference in intensity of the X-ray source for day to day analysis. Validation of quantization method was carried out with respect to specificity, precision, ruggedness linearity, robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ). This method may also be used at the manufacturing site to check the presence of crystalline phase of Linagliptin Form-A in amorphous drug substance and drug product. This PXRD method has its own advantage like non destructive sample analysis, end user friendly, minimizes human errors and minimizes sample preparation errors. This PXRD method represents a convenient method to determine the accurate content of Linagliptin Form-A in Linagliptin tablets.

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