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DETERMINATION OF RELATIVE POLYMORPHIC DISTRIBUTION BETWEEN VARIOUS POLYMORPHIC FORMS OF RIFAXIMIN DRUG IN RIFAXIMIN TABLETS USING POWDER X-RAY DIFFRACTION TECHNIQUE

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ABSTRACT

Rifaximin shows crystal polymorphism, and several polymorphs (α, β, γ, δ, ε) have been described. In vitro studies, it shows different dissolution and solubility rates for these polymorphs, and in vivo investigations in dogs found different PK patterns and polymorphs displaying the highest systemic bio-availability. There is a need to develop a sensitive method for the determination of relative polymorphic distribution of Rifaximin API in Rifaximin tablets. Powdered XRD is a preferred and extensively used technique for the determination of polymorphic mixtures, which has many advantages like uniqueness of X-ray powder pattern of the compounds, non-destructive nature, simplicity and measurement at room temperature of both the drug substance and product. To understand the polymorphic distribution in the sample a quantitative (%) XRD method was developed, wherein characteristic and specific peaks for each polymorph have been selected based on the XRD profile of individual polymorph and their distribution is estimated by the percentile calculation method. This PXRD method has its own advantage like minimum time interval, end user friendly, minimizes human errors and decreases sample preparation errors. In present study, PXRD method represents a convenient method to determine relative polymorphic distribution between various polymorphic forms of Rifaximin API in Rifaximin tablets. This method is capable in determining the amount of α, β, δ and ε polymorphs of Rifaximin API in Rifaximin tablets in single attempt. 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 also can be used in manufacturing site to check the relative polymorphic distribution between various polymorphs of Rifaximin API in Rifaximin Tablets.

KEYWORDS: Rifaximin tablets, Polymorphs (α, β, δ and ε), Powdered XRD, Validation, polymorphic distribution.

1. INTRODUCTION

The differences in physicochemical properties like dissolution rate, melting point, packing etc.[1] of the polymorphs have considerable impact on drug stability (physical and chemical) and Bio-Pharmaceutical performance.[2] The Phenomenon of Polymorphism and its impact are well recognized in the pharmaceutical industry. Many recent studies in solid state characterization and related areas lead to increased awareness and raising concern.[3,4] Therefore, regulatory bodies like FDA provide guidance which illustrates how to select a suitable drug polymorph or the alternatives for the product development, monitoring its stability and to have control, to ensure the quality.

Polymorphism is the ability of a compound to exist in more than one crystal form with different unit cell parameters.[5] An active pharmaceutical ingredient (API) can exist in different polymorphic forms with differences in properties like melting point, chemical reactivity, apparent solubility, dissolution rate, etc.[6] The importance of polymorphism in the pharmaceutical industry was well recognized in 1964.[7,8] However, the polymorphic transition that occurred in the commercial product known as Norvir (ritonavir) was the culminating point that drew the attention of the pharmaceutical industry and regulatory agencies on this issue, obliging them to review the quality control procedures implemented at that time.[9] Majority of the drugs are formulated and delivered in solid dosage forms like tablets and capsules. Many such drugs can display many polymorphic forms affecting the quality of the commercialized drug, especially when the dissolution rate is affected.[10]

Rifaximin (4-deoxy-4 –methylpyrido [1,2 -1,2] imidazo [5,4-c] rifamycin SV) is a synthetic derivative of rifamycin, with very low gastrointestinal absorption, but still displaying a broad spectrum of antibacterial activity.[11-13] Being virtually non-absorbed, its gastrointestinal bioavailability is high, with fecal concentrations largely exceeding minimum inhibitory concentrations against pathogenic enterobacteria, while its limited impact on extra-gastrointestinal sites minimizes the risk of antimicrobial resistance and systemic adverse

events.[14] With the appreciation of pathogenic role of gut flora in several gastrointestinal diseases, the use of Rifaximin has been extended from gastrointestinal infections to hepatic encephalopathy, small intestine bacterial overgrowth (SIBO), inflammatory bowel disease, and colonic diverticular disease.[15-16] The systematic name of Rifaximin is 2S,16Z,18E,20S,21S,22R,23R,24R,25S,26S,27S,28E)-5,6,21,23,25-pentahydroxy-27-methoxy-2,4,11,16,20,22,24,26-octa- methyl-2,7-(epoxypentadeca-[1,11,13] trienimino) benzofuro[4,5-e] pyrido[1,2-á]-benz- imidazole-1,15(2H)-dione,25-acetate. The molecular formula is $C_{43}H_{51}N_3O_{11}$ with molecular weight 785.9. The structure and crystal structure are shown in Fig. 1.

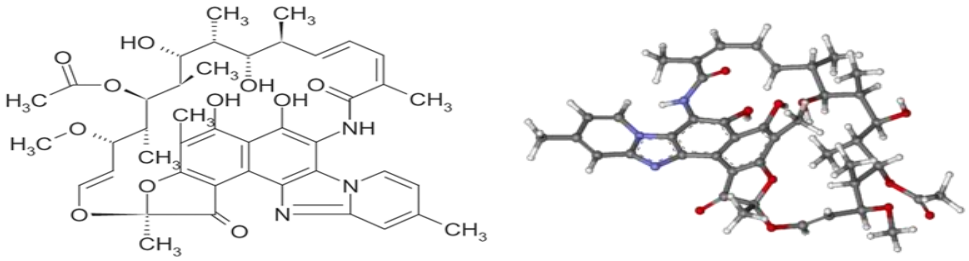


Figure. 1: Structure and crystal structure of Rifaximin.

According to the European Pharmacopoeia, Rifaximin shows crystal polymorphism,[17] and several polymorphs (α, β, γ, δ, ε) have been described.[17,18] In vitro studies, it shows different dissolution and solubility rates for these polymorphs, and vivo investigations in dogs found different PK patterns with polymorphs displaying the highest systemic bio-availability.[19] Previous PK studies in healthy volunteers and patients with inflammatory bowel disease or intestinal infections showed minimal absorption of Rifaximin after single and repeated doses. These studies were performed on Rifaximin polymorphism, while the current formulation contains only polymorph-α,[20] which is widely recognized as a poorly absorbed antibiotic.[21–24]

It can exist in various polymorphic forms with a significant difference in their pharmacological and toxicological properties along with variable bio-availability. It remains a challenge for a formulator to maintain the polymorphic integrity of the drug during the shelf-life, so that the end user consistently gets the same desired effect upon repeated administration. Also, it is required from the drug regulatory agencies to have the manufacturing methods of the drugs standardized and controlled in such a way that these forms give homogeneous results in terms of polymorphism. The importance of maintaining

the same polymorphic form becomes high in cases where there is a rapid conversion of one polymorphic form to another governed by numerous factors and where there exists a significant difference amongst the pharmacological and toxicological properties of the drug.

These polymorphic forms are susceptible to transform from one to another, even in the solid state at ambient conditions. The modification in the amounts of these different polymorphic forms in the finished pharmaceutical composition is highly critical as any variation in their amount during the shelf-life; the composition will directly affect the bio-availability of Rifaximin. Therefore, it is essential to prevent any modification of the polymorphic forms of Rifaximin during the shelf-life of the finished pharmaceutical composition. The present drug manufacturers have now developed a pharmaceutical composition of Rifaximin comprising a specific mixture of polymorphic forms of Rifaximin which shows good stability in the relative polymorphic distribution ratio of these polymorphs, and which provides uniform therapeutic effect, when administered. Hence there is a need to develop a sensitive method for determination of relative polymorphic distribution of Rifaximin API in Rifaximin tablets. Advantage like the uniqueness of X-ray powder pattern of the compounds, non-destructive nature, simplicity and measurement at room temperature of both the drug and product, PXRD the most preferred and extensively used technique for determination of polymorphic mixtures. One of the factors in developing any determination for solid-state forms is the generation of the authentic and validated methodology, which reproduces actual material that will be assayed in future. 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 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, identification of each polymorph, selection of characteristic and specific peaks of Alpha, Beta, Delta and Epsilon polymorphic forms and instrument parameters and (ii) development of an accurate, linear, precise, reproducible and robustness PXRD method for determination of relative polymorphic distribution of Rifaximin API in Rifaximin tablets.

To understand the polymorphic distribution in the sample, a quantitative (%) XRD method was developed wherein characteristic and specific peaks for each polymorph have been selected based on the XRD profile of individual polymorph and their distribution is estimated by the percentile calculation method.

2. Experimental section

2.1 Materials: Rifaximin Alpha, Beta, Delta, Epsilon forms standards, placebo and Rifaximin tablets were gifted by Hetero drugs Ltd, Andhra Pradesh (India). All materials were used as received without any further purification.

2.2 Methods

2.2.1 Powder X-ray diffraction (PXRD)

PXRD patterns on samples were recorded at room temperature on PANalytical, X' Pert PRO MPD diffractometer (Netherlands) 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), antiscattering 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 (150 seconds) over scan range 3-30° 2θ. Suitable quantity of powder was loaded in a 16 mm 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 data was acquired in data collector software and diffractograms obtained were analyzed with High score (plus) software.

2.2.2 Optimization of sample preparation and instrumental parameters

Rifaximin tablets are available in two strengths i.e. 200 mg and 550 mg. The theoretical average weight is 990 mg for 550mg coated tablets and theoretical average weight is 360 mg for 200 mg coated tablets. Both strengths are dose proportional, so higher strength tablet is selected for this study. Different polymorphs of Rifaximin API are reported. Alpha, Beta, Delta and Epsilon are pseudo polymorphs and may change to each other depending on

different humidity, drying and vacuum conditions. Take 3 or 4 tablets and carefully remove the coatings of the tablets, grind gently to fine powder using mortar and pestle and stack in 16 mm sample holder using back loading technique and placebo also grind gently to fine powder using mortar and pestle and stack in 16 mm sample holder using back loading technique. For the optimization of sample parameters, all studies were carried for all the forms of Rifaximin, samples, Rifaximin tablets and placebo. Each sample is analyzed. Based on the optimization of sample preparation parameters following precautions should be taken during the analysis.

The sample submitted in triple laminated pack should be used for analysis; Relative humidity should be maintained below 45% by keeping activated silica gel inside XRD instrument if XRD instrument is ambient. XRD and Sample stack should be freshly prepared before analysis and should be exposed as minimum as possible prior to analysis.

2.2.2.1 Preparation of Tablets and Placebo

In this work 55.56% of Rifaximin (mixture of polymorphs) was used and the proportions of excipients were: microcrystalline cellulose (31.69%), sodium starch glycolate (4.17%), colloidal silicon dioxide (0.28%) glyceryl distearate (5.00%), Talc (0.28%), magnesium stearate (0.51%) and Opadry (2.53%). Each 550mg strength tablet has a total mass of 990 mg, where 440 mg are excipients and 550 mg are Rifaximin polymorphs and each 200mg strength tablet has a total of mass 360 mg, where 160 mg are excipients and 200 mg are Rifaximin polymorphs. The placebo was prepared using quantities of excipients usually present in a tablet. The placebo preparation was carried out by physical mixing of excipients.

The polymorphs were added in each tablet according to a ternary design, totaling 6 samples, according to Table 1. All the samples were homogenized using a mortar and pressed using a 13 mm evocable die and a pressure of 1 ton.

Table. 1: Amount of Rifaximin in mg of each polymorph for samples used in the study.

Relative polymorphic distribution ratio of Rifaximin Alpha to Beta form	Alpha form(α) (in mg)	Beta form(β) (in mg)
Mixture of Alpha and Beta form (15:85)	82.5	467.5
Mixture of Alpha and Beta form (30:70)	165.0	385.0
Mixture of Alpha and Beta form (40:60)	220.0	330.0
Mixture of Alpha and Beta form (60:40)	330.0	220.0
Mixture of Alpha and Beta form (70:30)	385.0	165.0
Mixture of Alpha and Beta form (85:15)	467.5	82.5

2.2.2.2 Effect of temperature and humidity on conversion of one polymorphic form to another: All the four polymorphic form standards of Rifaximin and tablets contains different relative polymorphic distribution ratios of Rifaximin Alpha to Beta form (about 15:85 to about 85:15, about 30:70 to about 70:30 and about 40:60 to about 60:40) were stored at a relative humidity of 75% and temperature 40°C for a period of one, two and three months and analyzed for relative polymorphic distribution ratio of Form α and Form β through X-ray powder diffraction method. The results of the analysis are presented in Table 3.

2.2.2.3 Optimization of instrument parameters: The divergence and anti-scatter slits varied into monitoring the peak sharpness, soller slits can be placed in both the incident and diffracted beam path so that we have varied soller slits to obtain the required resolution and intensity. The step size and time per step varied to alter the scan rate of sample. Voltage and current varied up and down to monitor the peak intensities.

The instrument parameters have been optimized in the Rifaximin tablets containing 40% form-α and 60% form-β and finally these optimized parameters have been used in the determination of relative polymorphic distribution of Rifaximin API in Tablets. The slit optics for Bragg-Brentano geometry has been optimized. The smaller slits give higher resolution and lower intensity and vice versa. Therefore, the final method keeps a balance between peak resolution and intensities. The degree of precision in the measurement of the decisive parameters (area, height and intensity) determines the accuracy of quantitative PXRD. Several instrumental parameters have been reported to critically affect the area of diffraction peaks. Among these parameters, Time per steps(s), step size increments have a direct impact on the counting statistics. Therefore, final experimental method should keep a balance between the peak resolution and recording time. Counting time per step 150 sec with about 34 min recording time was the fastest offering resolution of maximum peaks and was thus selected for further experiments.

2.2.3 Identification of Rifaximin polymorphs and its characteristic peak positions

Identification of each polymorph is required for calculation of % relative polymorph distribution. The characteristic peak positions of each polymorph have been selected based on highest diffraction peak intensities are shown in figure 2-9. The characteristic and specific peaks of Alpha, Beta, Delta and Epsilon are given in Table 2.

Table. 2: The characteristic peak position of Rifaximin polymorphs.

S. No.	Polymorphs	Peak position (±0.2° 2θ)
1	Alpha(α)	5.8, 10.5,11.6, 19.6
2	Beta(β)	5.3, 7,0, 18.3
3	Delta(δ)	5.6, 6.6, 17.0
4	Epsilon(ε)	8.2, 14.5, 16.3

2.2.4 Preparation of Alpha and Beta polymorphic mixture with respectively tablet label claim: Rifaximin α and β forms were physically mixed in various relative polymorphic distribution ratios (from about 15:85 to about 85:15, about 30:70 to about 70:30 and about 40:60 to about 60:40) with placebo by geometric mixing in a controlled environment (25 ± 2 °C, 35±5% RH). Samples were made in triplicate and powder mixtures were loaded on sample holder. The graphs were plotted between the average area and % relative polymorphic distribution ratios of form α and form β. Limit of detection (LOD) and Limit of quantization (LOQ) were determined with the help of this calibration curve.

i. Impact of grinding energy on sample preparation

Take 10 tablets and carefully remove the coating of the tablets, grind gently to fine powder then tablet powder is divided in five portions. The first portion is gently mixed in a mortar; the second is mixed while applying force for 2 minutes, the third is mixed while applying force for 4 minutes, the fourth is mixed while applying force for 6 minutes. The fifth portion is mixed while applying force for 10 minutes. Five different sample holders are filled and analyzed according to the description in the test method.

2.2.6 Validation of analytical method: The analytical method developed for the determination of % relative polymorphic distribution ratios of Rifaximin API in Rifaximin tablets was checked for validation parameters like specificity, precision, ruggedness, linearity, accuracy, LOD & LOQ and robustness.

3. RESULTS AND DISCUSSION

3.1 Identification of Rifaximin polymorphs, its characteristic peak positions and Calculations: Identification of each polymorph is required for calculation of % relative polymorph distribution. The characteristic and specific peaks of Alpha, Beta, Delta and Epsilon are given in Table 2. Based on the straight review the XRD diffractogram of the sample and identify each polymorph present in the sample with the help of following approaches. Compare the XRD profile of the sample with the XRD profile of polymorph

standards used in these studies and as given in patent (Figure 2-9 and Table 2). Compare the XRD profile of the sample with the representative samples (having different polymorph mixtures) diffractograms (Figure 10-12). These can be used as references for identification and determination of relative polymorphic distribution in the sample.

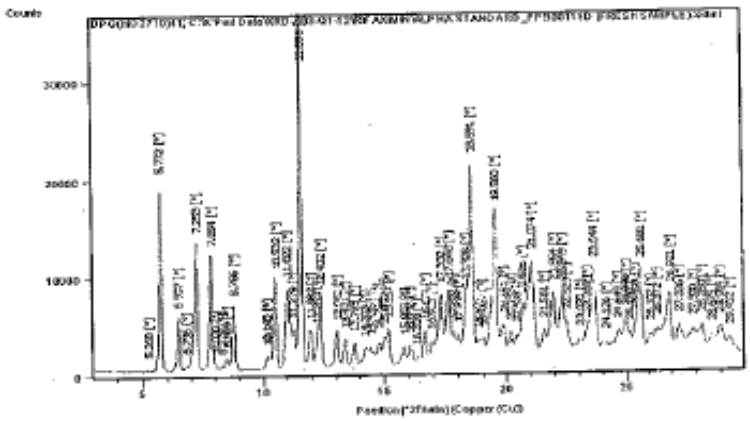


Figure. 2: XRD Diffractogram of Alpha polymorph.

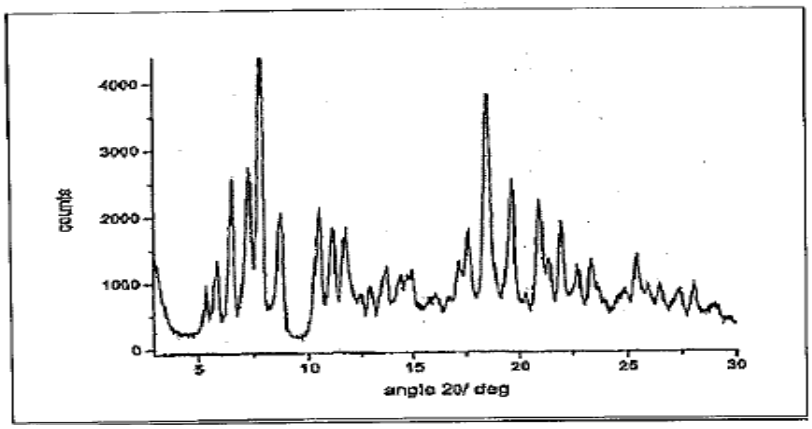


Figure. 3: XRD Diffractogram of Alpha Standard polymorph given in the patent.

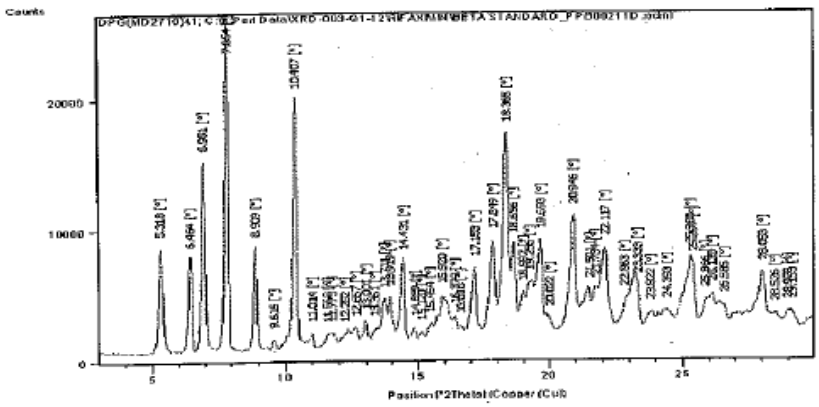


Figure. 4: XRD Diffractogram of Beta polymorph.

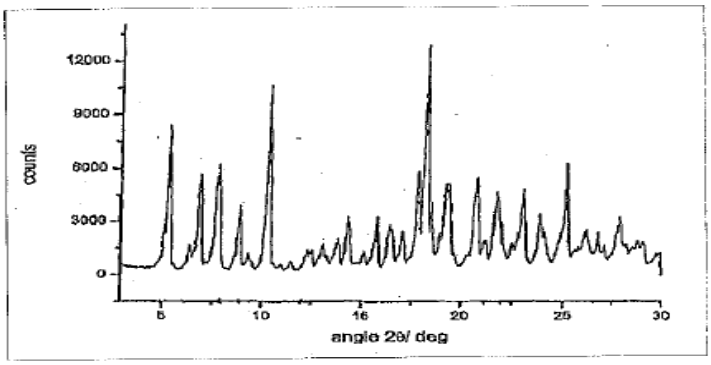


Figure 5: XRD Diffractogram of Beta Standard polymorph as given in patent.

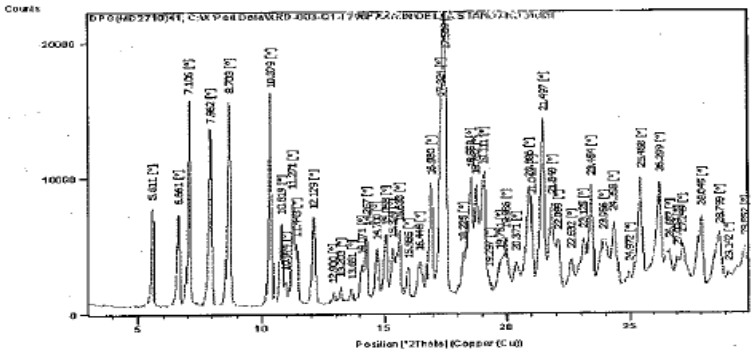


Figure. 6: XRD Diffractogram of Delta polymorph.

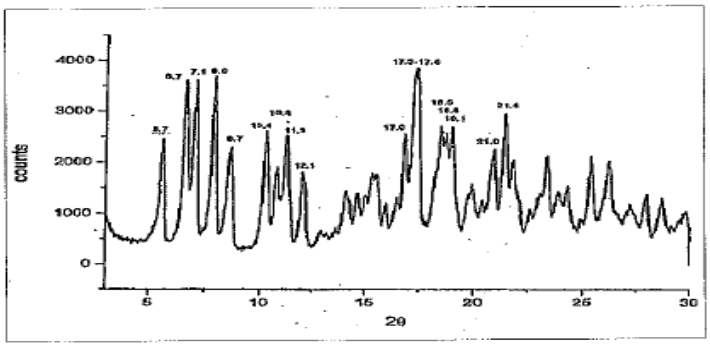


Figure. 7: XRD Diffractogram of Delta as standard polymorph given in patent.

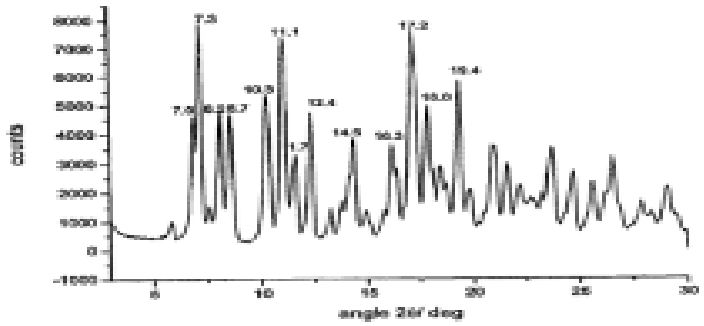


Figure. 8: XRD Diffractogram of Epsilon.

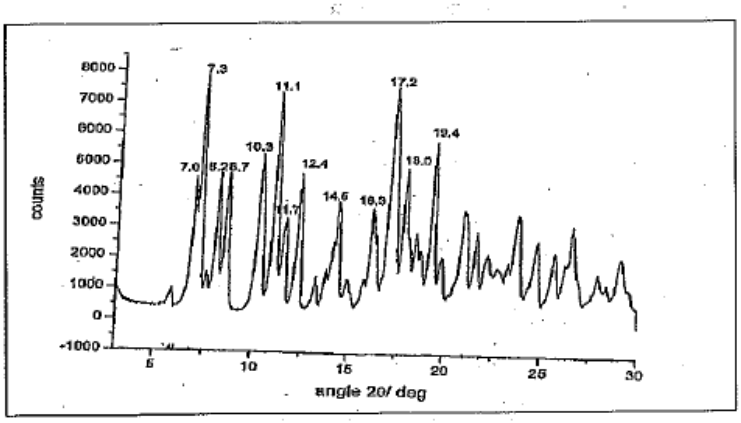


Figure. 9: XRD Diffractogram of Epsilon Standard polymorph as given in patent.

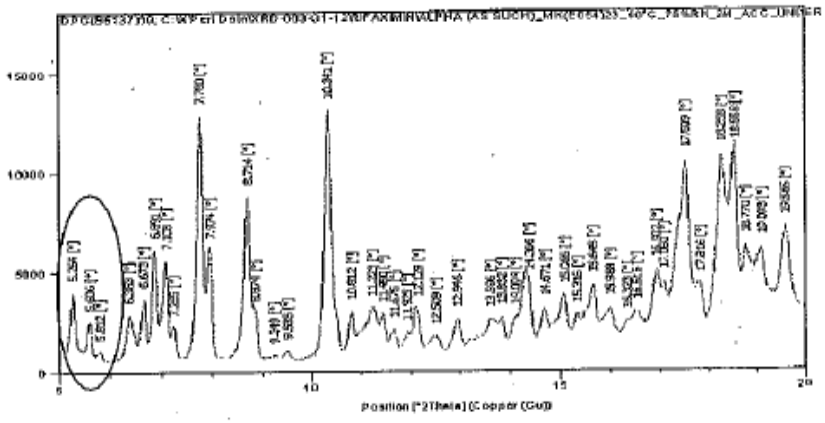


Figure. 10: XRD Diffractogram of sample having Alpha, Beta and Delta polymorphs (Peak due to Beta, Delta and Alpha appears at 5.3°, 5.6° and 5.8° 2 θ respectively).

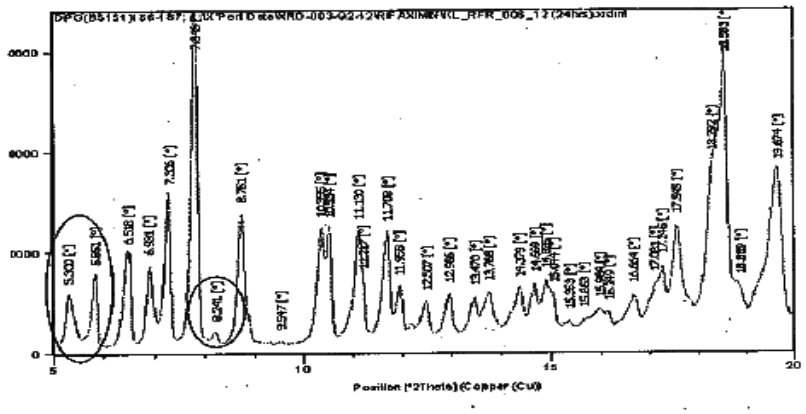


Figure. 11: XRD Diffractogram of sample having Alpha, Beta and Epsilon polymorphs (Peak due to Beta, Alpha and Epsilon appears at 5.3°, 5.8° and 8.2° respectively).

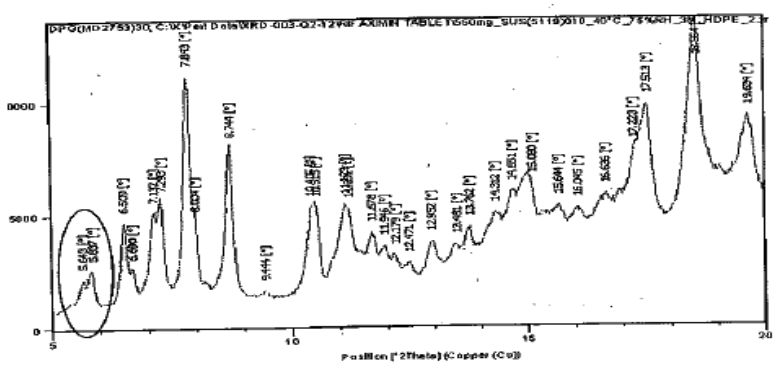
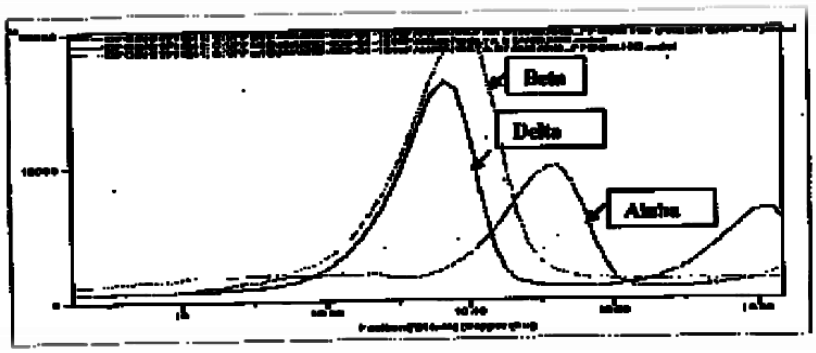


Figure. 12: XRD Diffractogram of sample having Alpha, and Delta polymorphs (Peak due to Alpha and Delta appears at 5.8° and 5.6° respectively).

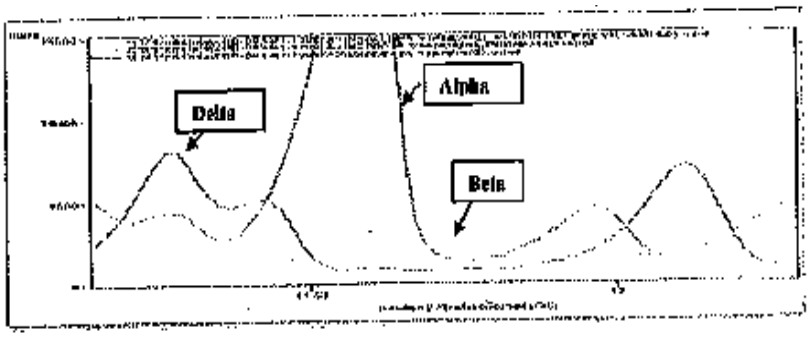
3.1.1 Peak integration for Rifaximin tablets (Figure 13-22)

Integration of the following peaks (peak top to be considered in the specified range) of each polymorph after suitable background corrections and record the sum of net intensities (in cps)

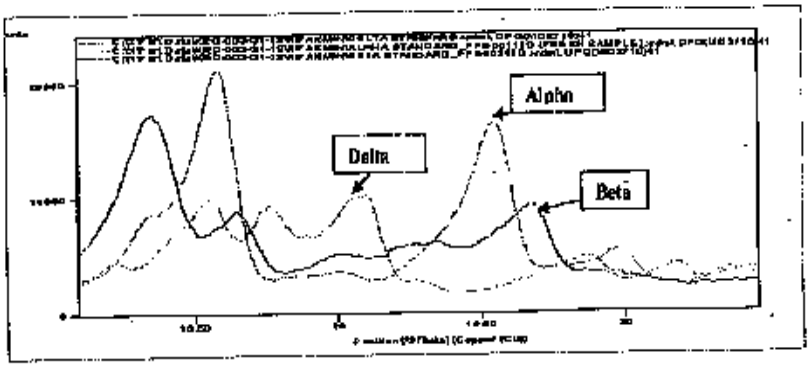
a Alpha polymorph: 1 Peak at 10.5 2θ (peak top range: 10.46 – 10.58 2θ). Representative Figure 13 (overlay of polymorph standard) given below for reference purpose.



2 Peak at 11.6 2θ (peak top range: 11.56 – 11.77 2θ). Representative figure 14 (overlay of polymorph standard) given below for reference purpose.

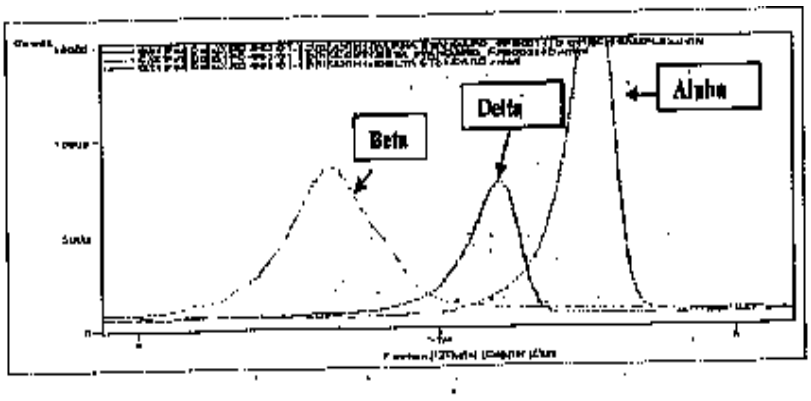


3 Peak at 19.6 2θ (peak top range: 19.48 – 19.72 2θ). Representative figure 15 (overlay of polymorph standard) given below for reference purpose.

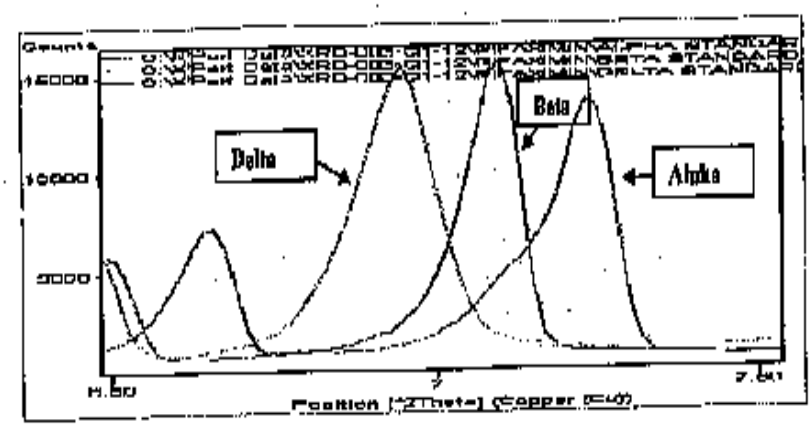


b Beta polymorph

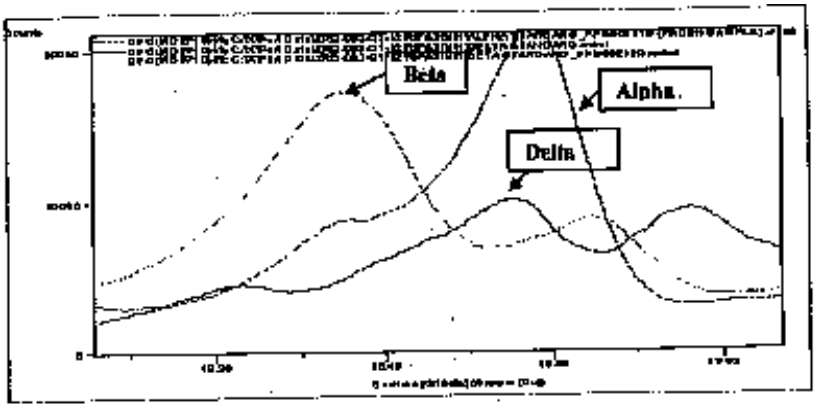
1. Peak at 5.3 2θ (peak top range: 5.20 – 5.47 2θ). Representative **figure16** (overlay of polymorph standard) given below for reference purpose.



2. Peak at 7.0 2θ (peak top range: 6.87 – 7.09 2θ). Representative **figure 17** (overlay of polymorph standard) given below for reference purpose.

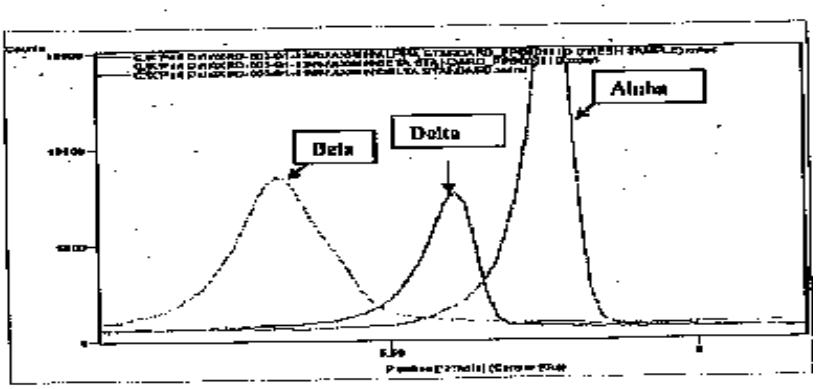


3. Peak at 18.3 2θ (peak top range: 18.20 – 18.49 2θ). Representative **figure 18** (overlay of polymorph standard) given below for reference purpose.

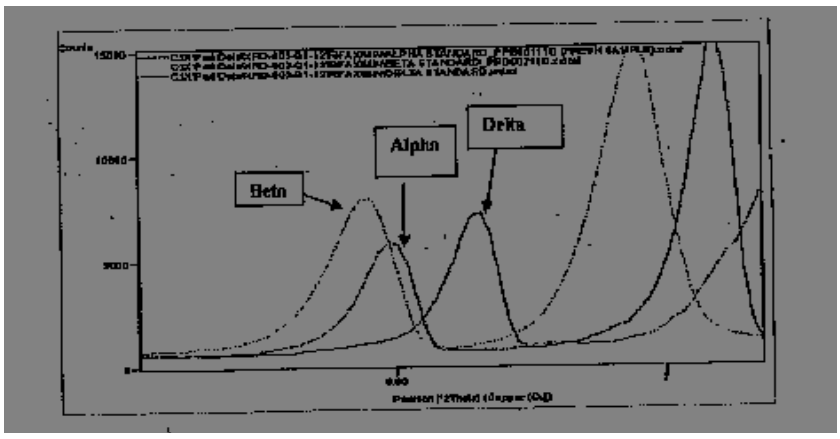


C. Delta polymorph

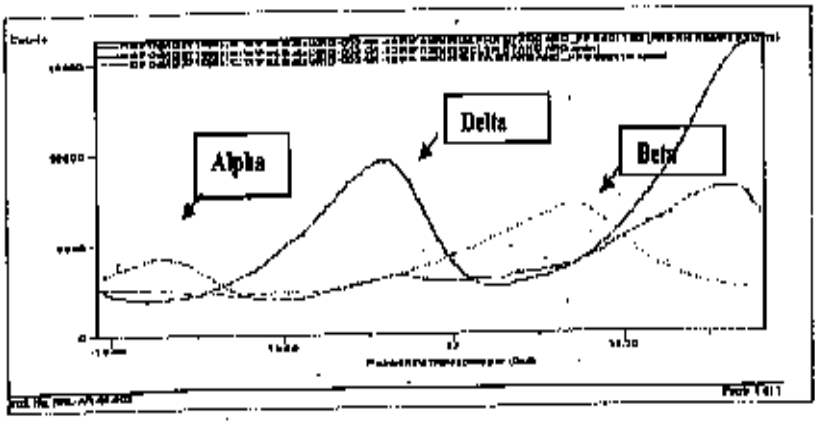
1 Peak at 5.6 2θ (peak top range: 5.58 – 5.66 2θ). Representative **figure 19** (overlay of polymorph standard) given below for reference purpose.



3... Peak at 6.6 2θ (peak top range: 6.62 – 6.71 2θ). Representative **figure 20** (overlay of polymorph standard) given below for reference purpose.



3... Peak at 17.0 2θ (peak top range: 16.84 – 16.96 2θ). Representative **figure 21** (overlay of polymorph standard) given below for reference purpose.



d Epsilon polymorph

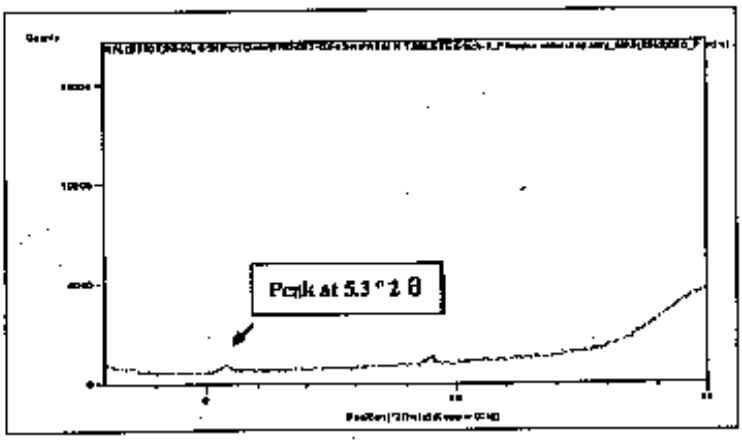
The peak integration must be performed at the positions given below.

Peak at 8.2 2θ (peak top range: 8.18- 8.30 2θ)

Peak at 16.3 2θ (peak top range: 16.28- 16.35 2θ)

Peak at 14.5 2θ (peak top range: 14.50- 14.59 2θ)

Peak integration for placebo Representative **figure 22** given below for reference purpose Integrate peak at 5.3 2θ (Peak top range 5.20 -5.47 2θ) after suitable background corrections and record the sum of net intensities (in cps).



3.1.2 CALCULATIONS

3.1.2.1 Relative polymorph distribution of sample (A^1)

Step 1: A (Form α) = Sum of net intensities (in cps) for peak at 10.5° 2θ + Sum of net intensities (in cps) for peak at 11.6° 2θ+ Sum of net intensities (in cps) for peak at 19.6° 2θ

B (Form β) = Sum of net intensities (in cps) for peak at 5.3° 2θ + Sum of net intensities (in cps) for peak at 7.0° 2θ+ Sum of net intensities (in cps) for peak at 18.3° 2θ.

C (Form δ) = Sum of net intensities (in cps) for peak at 5.6° 2θ + Sum of net intensities (in cps) for peak at 6.6° 2θ+ Sum of net intensities (in cps) for peak at 17.0° 2θ

D (Form ε) = Sum of net intensities (in cps) for peak at 8.2° 2θ + Sum of net intensities (in cps) for peak at 14.5° 2θ+ Sum of net intensities (in cps) for peak at 16.3° 2θ

Step 2: X (Sum of net intensities (in cps) = A+ B + C + D.

Step 3:

Relative polymorph distribution of Alpha (%) = A/X x 100

Relative polymorph distribution of Beta (%) = B/X x 100

Relative polymorph distribution of Delta (%) = C/X x 100

Relative polymorph distribution of Delta (%) = D/X x 100

Relative polymorph distribution for calculation of Placebo (B^1)

Calculate the relative polymorph distribution of sample as defined in A^1, wherein B is as follows

B = [Sum of net intensities (in cps) for peak at 5.3° 2θ for Rifaximin tablets - Sum of net intensities(in cps) for peak at 5.3° 2θ for the placebo + Sum of net intensities (in cps) for peak at 7.0° 2θ+ Sum of net intensities (in cps) for peak at 18.3° 2θ]

Placebo interference (%) = 100 – (Beta polymorph calculated in B^1/ Beta polymorph calculated in A^1 x 100).

Case 1: If placebo interference is less than or equal to 3.0%, report the Relative polymorph distribution (A^1)

Case 2: If placebo interference is more than to 3.0%, report the Relative polymorph distribution (B^1)

3.2 Optimization of sample preparation

The optimization of sample preparation parameters was performed by studying the effect of sample parameters on area, height and resolution on characteristic intense peaks. Large variations and fluctuations have been observed when the tablets were used without removing the coating material, grind them gently to fine powder and fill the same sample in 16 mm holders and analyzed. When coatings of the tablets were carefully removed, and same sample powder was analyzed. No variations and fluctuations of characteristic peak intensities were

found. However, coatings of the tablets should be carefully removed before grinding to fine powder. Results are given in Table 3.

Table. 3: Results for the tablets without removing the coating and with removing the coating for Rifaximin tablets containing 40% Alpha and 60% Beta form.

Without removing the coating of tablets			**Removing coating of tablets**		
Preparation	**α polymorph (%)**	**β polymorph (%)**	**Preparation**	**α polymorph (%)**	**β polymorph (%)**
Preparation-1	43	57	Preparation-1	39	61
Preparation-2	36	64	Preparation-2	39	61
Preparation-3	45	55	Preparation-3	38	62
Preparation-4	41	59	Preparation-4	38	62
Preparation-5	37	63	Preparation-5	39	61
Preparation-6	45	55	Preparation-6	40	60
Average	41	59	**Average**	39	61
SD	3.9	3.9	**SD**	0.8	0.8
%RSD	10	7	**%RSD**	2	1

3.2.1 Effect of temperature and humidity on conversion of one polymorphic form to another: Polymorphic forms of Rifaximin are designated as Form α, Form β, Form δ and Form ε. It is also known that the formation of these polymorphic forms could depend upon various factors, such as the presence of water within a crystallization solvent, the temperature at which the product is crystallized, and the amount of water present in the product at the end of the drying process. It is further identified that the presence of water in Rifaximin in the solid state is reversible, such that the water absorption and/or release can take place under specific ambient conditions leading to a change in the polymorphic forms. Therefore, Rifaximin is susceptible to transition from one form to another form even in the solid state, irrespective of the process involving the steps of dissolution and crystallization. This also stresses the fact that during the phase of preservation of the final Rifaximin product, special care need to be taken so that the ambient conditions do not change the water content of the product. The modification in the amounts of these different polymorphic forms in the finished pharmaceutical composition is highly critical as any variation in their amount during the shelf-life of the composition will directly affect the bioavailability of Rifaximin to patients. Therefore, it is essential to prevent any modification of the polymorphic forms of Rifaximin during the shelf-life of the finished pharmaceutical composition. The present inventors have now developed a pharmaceutical composition of Rifaximin comprising a specific mixture of polymorphic forms of Rifaximin which shows good stability in the relative polymorphic distribution ratio of these polymorphs, which provides uniform therapeutic effect when

administered to the patients. Wherein the relative polymorphic distribution ratio of Form α to Form β is from about 15: 85 to about 85: 15, 30: 70 to about 70: 30 and 40: 60 to about 60: 40 and wherein the said ratio remains substantially unchanged in the pharmaceutical composition after exposure to a relative humidity of 75% and a temperature of 40°C for at least three months. The relative polymorphic distribution ratio of Form α to Form β is from about 15: 85 to about 85: 15, 30: 70 to about 70: 30 and 40: 60 to about 60: 40 of tablets mixtures were stored at a relative humidity of 75% and a temperature of 40°C for a period of three months and analyzed for relative polymorphic distribution ratio of Form α and Form β determined through present X-ray powder diffraction method. The results of the analysis are represented in Table 4.

It is clear from Table 4 that the relative polymorphic distribution ratio of Form α and Form β remained substantially unchanged for a period of three months, which shows that the pharmaceutical compositions prepared accordingly remained stable for three months at Relative Humidity of 75% and a Temperature of 40°C. The term "relative polymorphic distribution ratio", as used herein, refers to the amount of Form α and Form β relative to each other in the pharmaceutical composition. The distribution ratio of the present manufacturing tablets is expected to remain substantially unchanged after the manufacturing process through the entire shelf- life of the pharmaceutical composition.

Table. 4: Results of the Stability Study of the relative polymorphic distribution ratio of Form α to Form β is from about 15: 85 to about 85: 15, 30: 70 to about 70: 30 and 40: 60 to about 60: 40.

Condition	Ratio of α to β (15:85)		Ratio of α to β (85:15)		Ratio of α to β (30:70)	
	Form α	Form β	Form α	Form β	Form α	Form β
Initial	14.5	85.5	83.6	16.4	31.9	68.1
40°C/75%RH/1M	15.1	84.9	85.2	14.8	29.2	70.8
40°C/75%RH/2M	13.9	86.1	84.1	15.9	32.1	67.9
40°C/75%RH/3M	14.8	85.2	84.8	15.2	31.2	68.8
Condition	**Ratio of α to β (70:30)**		**Ratio of α to β (40:60)**		**Ratio of α to β (60:40)**	
	Form α	**Form β**	**Form α**	**Form β**	**Form α**	**Form β**
Initial	72.5	27.5	38.4	61.6	58.4	41.6
40°C/75%RH/1M	69.8	30.2	41.2	58.8	61.6	38.4
40°C/75%RH/2M	71.1	28.9	39.5	60.5	58.9	41.1
40°C/75%RH/3M	68.9	31.1	38.1	61.9	59.6	40.4

3.3 Impact of grinding energy on sample preparation: Based on results given below in Table 5, the results pass their acceptance criteria, demonstrating gentle or harsh manipulation of a powdered material, has no impact on the intensity of the diffraction peak of interest. The results of the analysis are represented in Table 5.

Table. 5: Results for Impact of grinding energy on Rifaximin tablets containing 40% form-α and 60% form-β.

Preparation	Form α (%)	Form β (%)	Acceptance criterion
Initial	42	58	% RSD should not be more than 10
Grinding for 2 min	39	61	
Grinding for 4 min	41	59	
Grinding for 6 min	43	57	
Grinding for 10 min	40	60	
Average	41	59	
SD	1.6	1.6	
%RSD	4	3	

3.4 Validation of the analytical method: Any analytical method before being successfully utilized for quantification needs to be validated [25]. The development method was found to be specific, accurate, precise, linear, rugged and robust.

3.4.1 Specificity: Rifaximin form-α, form- β, form- δ, form- ε, Rifaximin tablets and placebo for Rifaximin tablets have been scanned as per method. Specificity is shown (Figure 2-22) by qualitative comparison of the diffraction patterns of placebo, tablets and polymorphic standards. Based on that comparison, specificity demonstrated by resolution and interference between Rifaximin polymorphs Form –α, β, δ, and form-ε were well resolved from each other and no interference with any other diffraction peak.

3.4.2 Method precision and intermediate precision (Ruggedness)

The analysis repeatability expresses the precision of the analytical 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 6. These results are in the acceptable range and prove the suitability of the method for a precise determination of relative polymorphic distribution of Rifaximin API in Rifaximin tablets.

Table. 6: The results for method precision and intermediate precision (sample batch containing 40% Alpha form and 60% Beta form).

Overall % RSD data (Acceptance criteria: % RSD should not be more than 10)					
Method precision	**Form α (%)**	**Form β (%)**	**Ruggedness**	**Form α (%)**	**Form β (%)**
Method prec-1	43	57	Ruggd-1	39	61
Method prec-2	45	55	Ruggd-2	39	61
Method prec-3	41	59	Ruggd-3	38	62
Method prec-4	41	59	Ruggd-4	38	62
Method prec-5	40	60	Ruggd-5	39	61
Method prec-6	42	58	Ruggd-6	40	60
Average	42	58	**Average**	39	61
SD	1.8	1.8	**SD**	0.8	0.8
%RSD	4	3	**%RSD**	2	1
Overall % RSD of Form α and Form β					
	Form α		Form β		
Average	40		60		
SD	2.1		2.1		
%RSD	5		4		

3.4.3 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 five 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 23. All relevant acceptance criteria are met (Acceptance criteria: $R^2 \geq 0.99$) demonstrating the acceptable linearity of the method. The actual versus predicted content (%, w/w) of Alpha and beta forms were plotted (Fig. 23), and a linear curve with R^2 values of 0.9994 & 0.9996 were obtained. This further indicated that the method developed was rugged.

3.4.4 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. LOD & LOQ for Alpha and Beta Forms are 0.7% & 2.0% and 1.6% & 5.0% respectively.

3.4.5 Accuracy: The accuracy of an analytical method expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and the value found. The accuracy of the method is assessed using nine determinations covering the specified range at three concentration levels. The accuracy is calculated and reported as the mean recovery and the individual recoveries. The results are tabulated in Table 7 & 8.

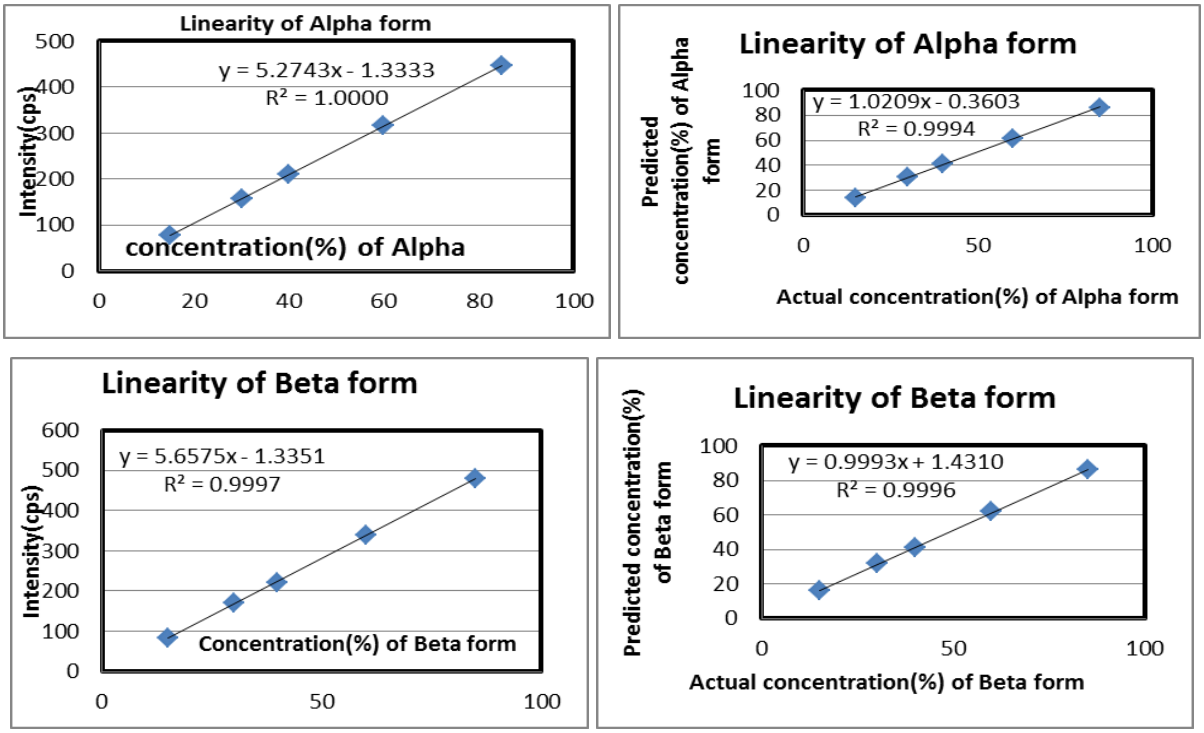


Figure. 23: Linearity Graphs for 15% – 85%, of Alpha form and Beta form (15%, 30%, 40%, 60% & 85%) (Concentration (%) v/s intensity (cps) and actual concentration (%) v/s predicted concentration (%)).

Table. 7: Accuracy at 15, 60 and 85% of Alpha form.

Actual Conc. (%) Form-α	**Predicted Conc. Form-α**	**% Recovery**	**Acceptance criteria**
15.0	14.5	96.7	90.0% ≤ Recovery ≤ 110.0%
15.0	15.8	105.3	
15.0	13.9	92.7	
Mean Recovery		98.2	
60.0	58.5	97.5	90.0% ≤ Recovery ≤ 110.0%
60.0	61.7	102.8	
60.0	59.4	99.0	
Mean Recovery		99.8	
85.0	83.8	98.6	90.0% ≤ Recovery ≤ 110.0%
85.0	86.2	101.4	
85.0	84.1	98.9	
Mean Recovery		99.6	
Actual Conc. (%) Form-β	Predicted Conc. Form-β	% Recovery	Acceptance criteria
15.0	16.2	108.0	90.0% ≤ Recovery ≤ 110.0%
15.0	13.8	92.0	
15.0	15.9	106.0	
Mean Recovery		102.0	
60.0	62.1	97.5	90.0% ≤ Recovery ≤
60.0	58.2	102.8	

60.0	61.2	99.0	110.0%
Mean Recovery		100.8	
85.0	85.5	100.6	90.0% ≤ Recovery ≤ 110.0%
85.0	84.2	99.1	
85.0	86.1	101.3	
Mean Recovery		100.3	

All the results pass their acceptance criteria and prove the suitability of the methods for an accurate determination of relative polymorphic distribution of Rifaximin API in Rifaximin tablets.

3.4.5 Robustness

The impact of the intensity of the X-ray source will be evaluated during robustness testing. This evaluation is made to make sure that upon aging of the instrument, an accurate determination of relative polymorphic distribution of Rifaximin API in Rifaximin tablets is still possible. Since evaluation of the presence of relative polymorphic distribution of Rifaximin API in a sample is based on a comparison of % of relative polymorphic distribution of Rifaximin samples with a method precision average value. Robustness is assessed using three different sample holders containing a representative sample batch containing 40% Alpha form and 60% Beta form at different X-ray tube voltage and current like 50kV- 40 mA, 40kV- 40 mA, 45kV- 45mA and 45kV- 35 mA. The results are given in Table 8.

Table. 8: Results for Robustness analysis.

Robustness data (Acceptance criteria: % relative mean differences between precision and Robustness value should not more than 10)					
(50kV : 40mA)	**Form α (%)**	**Form β (%)**	**(40kV: 40mA)**	**Form α (%)**	**Form β (%)**
Mean of Robustness	41	59	Mean of Robustness	40	60
Average value of Precision	42	58	Average value of Precision	42	58
%Relative mean difference	1	1	%Relative mean difference	2	2
(45kV : 45mA)	Form α (%)	Form β (%)	(45kV: 35mA)	Form α (%)	Form β (%)
Mean of Robustness	40	60	Mean of Robustness	41	59
Average value of Precision	42	58	Average value of Precision	42	58
%Relative mean difference	2	2	%Relative mean difference	1	1

All relevant acceptance criteria are met; hence the results demonstrated that this technique is robust.

4. CONCLUSION

A quantification PXRD method has been developed to determine the amount of α, β, δ and ε - forms of Rifaximin API in Rifaximin tablets with 550 mg strength. This method can determine the amount of α, β, δ and ε - polymorphs of Rifaximin API and Rifaximin tablets in a single shot and is suitable for determining the relative polymorphic distribution of Rifaximin drug substance and drug products also. To minimize the errors associated with the quantification and to obtain an accurate method, sample preparation, sample handling and instrument parameters were optimized. The characteristic peak identification of different polymorphs of Rifaximin and its quantitative calculation procedure were highly significant part of this method. The PXRD quantification method development for drug product has challenges because API was diluted by excipients, hence XRD instrument become less sensitive, however, in this study we have achieved sensitivity 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 Rifaximin drug under development and enable quality control departments to adequately evaluate the solid state of batches produced. This study also showed the diversity in the polymorph monitoring of Rifaximin tablets available in the market, providing information on the relative polymorphic distribution of the Rifaximin samples to improve the quality control and reproducibility of the Rifaximin formulations. 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 technique can also be used at the manufacturing site to check the relative polymorphic distribution between various polymorphs of Rifaximin API in Rifaximin Tablets. This PXRD method has its own advantage like small interval of time (34 minutes runtime), end user friendly, minimizes human errors and minimizes sample preparation errors. It also represents a convenient method to determine relative polymorphic distribution between various polymorphic forms of Rifaximin API in Rifaximin tablets.

Complience with Ethical Standards Conflict of Interest

The authors declare that they have no conflict of interest. This work and this article do not contain any studies with animals or human participants performed by the authors.

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