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INVESTIGATION OF PHYSIOCHEMICAL AND ANTI-MYCOBACTERIAL ACTIVITY OF NOVEL CO-CRYSTAL OF ISONIAZID

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ABSTRACT

The objective of the present research work aims to apply crystal engineering for the selection of API and co-former with primary amide and to investigate the preparation and characterization of co-crystal preparation. Isoniazid (INH, Pyridine-4-carboxyhydrazide) is used as a first line anti-tubercular agent, in combination with other anti-tubercular drugs for the effective treatment of active diseases and also used for prevention of tuberculosis in individuals who have been exposed to active disease. The supra molecular interactions of isoniazid with carboxylic acid resulted in co-crystal based on the nature of carboxylic acid.co-crystals of (1:1 stoichiometric ratio) isoniazid with para amino salicylic acid was reported. Preparation and characterization of co-crystal preparation methods include Analytical method optimization, Preformulation studies, Preparation of novel multicomponent crystal forms of isoniazid, Characterization of prepared novel multicomponent crystal forms of isoniazid, Crystal structure analysis by Single crystal XRD, Solubility studies, Micrometric characterization, Invitro evaluation and Anti-tubercular activity. These prepared crystal forms I resolve the poor micrometric problems of isoniazid and shows improved flow and compaction property than isoniazid. From the anti-tubercular test performed it has confirmed that the co-crystal forms of PAS and INH (S4, S5, S6) had shown increased activity compared to the pure drug.

Key Words: Isoniazid, supra molecular interactions, anti-tubercular activity.

INTRODUCTION

The drugs therapeutic efficiency depends upon the bioavailability i.e. the amount of drug reaches the systemic circulation. Two main factors influence the bioavailability are Solubility, Permeability. Many other factors influence these bioavailability such as chemical stability, poor dissolution rate, purity, compaction behaviour of crystal, moisture uptake and crystal habit etc. Usually market value of a drug significantly lowered by these factors.

Various solid state modifications have been done to improve the bioavailability of drug without hanging its pharmacological activity. These approaches are described such as polymers, solvates, hydrates, salts, co-crystals, and amorphous solids which involves non covalent interactions. Usually developers and regulatory authorities prefer these types of crystal forms, because highly pure products that are superior with respect to reproducibility and scalability were afforded by crystallisation.

The physicochemical properties of each form differ individually which can influence the manufacturability, bioavailability, purification and stability of drugs. Among this the widely used approach is salt formation. However this salt formation has major limitation i.e. a suitable ionisable site should be possessed by a drug (acidic or basic drug). In comparison, the co-crystal formation (freely reversible multi component assemblies) may potentially be employed with all drugs, including acidic, basic and non ionisable molecules.

Isoniazid (INH,Pyridine-4-carboxyhydrazide)is used as a 1st line anti tubercular agent, in combination with other anti-tubercular drugsfor the effective treatment of

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active diseases and also used for prevention of tuberculosis in individuals who have been exposed to active disease. The supra molecular interactions of isoniazid with carboxylic acid resulted in co-crystal based on the nature of carboxylic acid.co-crystals of (1:1 stoichiometric ratio) isoniazid with para amino salicylic acid was reported [1-3].

The scope of present research work aims to apply crystal engineering for the selection of API and co-former with primary amide and to investigate the co-crystal preparation and characterization of the same.

MATERIALS AND METHODS

Following materials were used for the present study,

1. Isoniazid (Loba chemie pvt. Ltd, Mumbai)
2. 4-amino salicylic acid (Hi media pvt . ltd, Mumbai)
3. methanol, ethanol & Potassium dihydrogen phosphate (Merck Pvt. Ltd, Mumbai)

Following Equipments were used for the present study,

1. Electronic Analytical Balance (Shimadzu,Japan),
2. UV-Visible spectrophotometer UV-1700 (Shimadzu, Japan),
3. FT-IR Spectrophotometer IR 200 (Thermo electron corporation)
4. Differential Scanning Calorimetry (NETZSCH DSC 204)
5. Scanning Electron Microscope (ZEISS Electron Microscope, EVO MA 15)
6. Single Crystal X-Ray Diffractometer (Enraf Nonius CAD4-MV31)

ANALYTICAL METHOD OPTIMIZATION

Number of analytical methods is available for quantification of Isoniazid such as ultra-violet spectroscopy, liquid chromatography with UV detection, gas chromatography and mass spectroscopy. The following method was optimized for further studies.

Standard Curve of Isoniazid (INH) with 0.1N HCl

10 mg of Isoniazid was dissolved in 100 ml of 0.1 N HCl, and further dilutions were made by using 0.1 N HCl to obtain concentrations ranging from 2 to 10 µg/ml. The absorbance of solution was measured at 266.5 nm using UV –Visible Spectrophotometer. The readings obtained are shown in figure no. 1.

Standard Curve of Isoniazid with pH 6.8 Phosphate buffer

10 mg of Isoniazid was dissolved in 100 ml of 6.8 Phosphate buffer, and further dilutions were made by using 6.8 Phosphate buffer to obtain concentrations ranging from 5 to 25 µg/ml. The absorbance of solution was measured at 262.5 nm using UV –Visible Spectrophotometer. The readings obtained are shown in figure no.2.

Standard Curve of Para amino salicylic acid (PAS) with 0.1N HCl

10 mg of Para amino salicylic acid was dissolved in 100 ml of 0.1 N HCl, and further dilutions were made by using 0.1 N HCl to obtain concentrations ranging from 2 to 10 µg/ml. The absorbance of solution was measured at 266.5 nm using UV –Visible Spectrophotometer. The readings obtained are shown in figure no.3

Standard Curve of Para amino salicylic acid (PAS) with pH 6.8 Phosphate buffer:

10 mg of para amino salicylic acid was dissolved in 100 ml of 6.8 Phosphate buffer, and further dilutions were made by using 6.8 Phosphate buffer to obtain concentrations ranging from 5 to 25 µg/ml. The absorbance of solution was measured at 262.5 nm using UV –Visible Spectrophotometer. The readings obtained are shown in figure no. 4.

PREFORMULATION STUDIES

Selection of drug and Coformers

Isoniazid

It is the principal ingredient in "triple therapy" used to effectively treat tuberculosis from 1952 onwards and on the basis of previous literature studies it was evident that a potentially versatile supramolecular reagent to prepare novel supramolecular complexes. The pyridine ring of INH is excellent hydrogen bonding acceptor for carboxylic acids and the possible attaching point for any heterosynthons. The carbohydrazide group of INH has both good hydrogen bonding acceptor (O and N atoms) and donor (3 H atoms) functionality. Hence, it is a potentially versatile supramolecular reagent to prepare cocrystals. Therefore it was selected for further studies.

Coformers

Depend upon the functional group and possible supramolecular synthon of the Isoniazid, GRAS coformers which having carboxylic acid functional group like para amino salicylic acid was selected to prepare novel multicomponent crystals of isoniazid by carboxylic acid pyridine heterosynthon. The solid state properties of Isoniazid, Para amino salicylic acid was determined by FTIR, DSC, SEM and XRD.

Infrared spectroscopy (FTIR)

IR spectroscopy was conducted using a FTIR Spectrophotometer (Thermo-IR 200) and Potassium bromide pellet method was employed and background spectrum was collected under identical conditions. The spectrum of INH, Para amino Salicylic acid was recorded in the wavelength region of 4000–400 cm−1 [4].

Differential scanning calorimetry (DSC)

Thermal analysis of INH, Para amino Salicylic acid was recorded on a DSC (NETZSCH DSC 204). The temperature axis and cell constant of DSC were previously calibrated with indium. A heating rate of 100C/min was

employed with nitrogen pursing. Powder samples (15- 30 mg) was weighed into an aluminum pan and analyzed as sealed with pin holes and an empty aluminum pan was used as reference [5].

Scanning electron microscopy (SEM)

The surface characteristics of INH, Para amino Salicylic acid was studied by SEM (ZEISS Electron Microscope, EVO MA 15). The specimens were scanned with an electron beam of acceleration potential of 20 kV and the images were collected as secondary electron mode [6].

Powder X-Ray Diffraction (P-XRD)

The pXRD were undertaken to investigate the crystalline nature of INH and Para amino Salicylic acid. The study was carried out using X-Ray Diffractometer using Cu kα radiation. The tube operated at 45 kV, 9mA and data was collected over an angular range from 0 to 500 2θ of the diffraction angle in continuous scan mode using a step size of 0.050 2θ and a time of 0.1 s [7].

Anti-Tubercular activity

The anti-mycobacterial activity of compounds were assessed against M. tuberculosis using microplate Alamar Blue assay (MABA). In this method, 200µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of untreated drug and prepared cocrystal were made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.

RESULT AND DISCUSSION

ISONIAZID (INH)

FTIR (Fourier Transform Infra-red Spectroscopy) Studies

The FT-IR spectrum of INH showed a strong C=O stretch (Amide) band around 1666.3 cm−1, free NH2 at 1221.3 cm−1, N-H bend at 1634.2 cm−1 and 1411.8 cm−1of pyridine. IR Spectrum and Interpretation of Isoniazid have shown in Figure no.4.

Differential scanning calorimetry (DSC)

DSC thermo grams of isoniazid shows sharp endothermic peak at 172.6C. This indicates pure crystal form. A DSC thermo gram of Isoniazid was shown in figure no.6.

Scanning electron microscopy (SEM)

SEM photography of Isoniazid demonstrates small rod like crystals. SEM photographs are shown in Figure no.7.

Powder X-Ray Diffraction

X-ray powder diffractometry (XRPD) is a powerful technique for the identification of the crystalline solid phases. Every crystalline solid phase has a unique XRPD pattern, which can form the basis for its identification. The X-ray powder diffraction (XRD) spectra of INH (figure no.8) shows characteristic peak at 19.7^0 (100%), 16.75^0, 15.6^0, 14.35^0 and 12^0 indicates pure Isoniazid.

PARA AMINO SALICYLIC ACID (PAS)

FTIR Studies

The FT-IR spectrum of PAS showed a strong C=O stretch (Amide) band around 1658.7 cm−1, free NH2 at 3494.4 cm−1, O-H str. vibration at 3387.1 cm−1 and 1619.4 cm−1of C=C. IR Spectrum and Interpretation of Para Amino Salicylic acid has shown in Figure no.9 and table no.2

Differential scanning calorimetry (DSC)

DSC study of Para amino salicylic acid shows endothermic peak at 135.28^0C, A DSC thermo gram of salicylic acid was shown in figure no.10.

Scanning electron microscopy (SEM):

SEM photography of PAS shows irregular rough surfaced particles. SEM photographs are shown in Figure no11.

Powder X-Ray Diffraction

X-ray powder diffractometry (XRPD) is a powerful technique for the identification of the crystalline solid phases. Every crystalline solid phase has a unique XRPD pattern, which can form the basis for its identification. The X-ray powder diffraction (XRD) spectra of Para amino salicylic acid (figure no.12) shows characteristic peak at 26.391^0 (100%), 27.35^0, 25.62^0, 16.67^0 and 14.32^0 indicates pure Para amino salicylic acid.

Preparation of novel multicomponent crystal forms of isoniazid

Crystallization can happen from the melt or from the solution, or from the vapor phase. Crystallization can produced from the solution by lowering the temperature, removing the solvent from solution (this is also called evaporation), by an anti-solvent addition method (drowning out), by reactive crystallization (precipitation), or by altering the solution pH (iso-electric precipitation method). In this study solution crystallization (slow evaporation) method has followed to prepare following crystal forms of isoniazid.

Preparation of Crystal form 1: INH-Para amino salicylic acid (1:1) co-crystal by solvent evaporation

Isoniazid (0.137g, one m.mol) and Para amino salicylic acid (co-crystal former, 0.153g, one m.mol) were dissolved separately in 5 ml of methanol with warming and mixed together. Solution was cooled to room temperature and kept for slow evaporation for 6 h. The crystals were isolated by filtration through a membrane (0.45 μm) and dried in the air (102).

Preparation of Crystal form 2: INH-Para amino salicylic acid (1:1) co-crystal by solvent drop method

INH(0,137g, one m.mol) and Para amino salicylic acid (co-crystal former, 0.153g, one m.mol) were taken in glass motor and pestle and grounded up to 10 min. then add solvent(ethanol) few drops in drop wise. And again grounded for 10 min. and keep it for drying.

Preparation of Crystal form 3: INH-Para amino salicylic acid (1:1) co-crystal by co- grinding method

INH(0,137g, one m.mol) and Para amino salicylic acid (co-crystal former, 0.153g, one m.mol) were taken in glass motor and pestle and grounded up to 1 hr. and keep it for drying.

CHARACTERIZATION NOVEL MULTI COMPONENT CRYSTAL FORMS OF ISONIAZID

INH- Para amino Salicylic acid (1:1) co-crystals in 3 methods and INH and Methyl paraben(1:1) molecular complexs in 3 methods were characterized by FTIR, DSC, SEM and XRD studies with previously mentioned methods. And also by Saturation solubility studies, Tablet Crushing Strength, flowability and packability studies, Invitro dissolution and antitubercular activity.

Single Crystal X-Ray Crystallography

Single crystal diffraction data were collected on Enraf Nonius CAD4- MV31 single crystal X-ray diffractometer equipped with a Apex CCDdetector with Mo-Ka radiation (λ= 0.71073 Å, graphite monochromator, mono-capillary collimator) for crystal form **1** and **2** at 293 K. It consists of an FR 590 generator, a goniometer, CAD4F interface and a microVAX3100 equipped with a printer and plotter. The detector is a scintillation counter. Integration, data reduction and cell refinement were carried out using SAINT. Space group assignment was made using XPREP. Furthermore, an empirical absorption correction was applied. The structure was solved in the WinGX suite of programs by direct methods using SHELXTL and refined using full-matrix least squares/difference Fourier techniques using SHELXTL. All non-hydrogen atoms were refined anisotropically. Thereafter, all hydrogen atoms attached to N and O atoms were located in the difference Fourier map and their coordinates refined freely with isotropic parameters 1.5 times those of the heavier atoms to which they are attached. All C–H hydrogen atoms were placed at idealized positions and refined as riding atoms with isotropic parameters 1.2 times those of the heavy atoms to which they are attached. Diagrams and publication material were generated using Mercury 2.3 (Build RC4) software [8-13].

Drug content

For the determination of drug content, prepared supramolecular crystals (100 mg) were dissolved in 100 ml distilled water and the solution was analyzed spectrophotometrically at 262 nm (λmax) for drug content, after sufficient dilution with distilled water. The study was performed in triplicate [14].

Saturation Solubility

Saturation solubility studies of INH were performed in triplicate according to method reported by Higuchi and Connors. In this saturation solubility study, an excess quantity of Isoniazid was placed in the vials containing 10 ml of different pH Medias. The vials were agitated in incubator shaker (100 agitations / min) for 4 hr at room temperature. The solution was then filtered through a membrane (0.45μm) and the amount of the drug dissolved was analyzed spectrophotometrically (UV-1700, Shimadzu, Japan) at 266 nm (0.1N HCl), and 262 nm (Distilled water and pH 6.8 phosphate buffer) [15].

Measurement of flowability

The bulk density (BD) and tapped bulk densities (TBD) were investigated in triplicate by using Density apparatus. The Carr's index (%) and the Hausner's ratio (HR) were calculated by using BD and TBD. The angle of repose of Isoniazid was assessed by fixed funnel method. The Carr's index represents the compressibility of the Isoniazid, and there is a correlation between the compressibility index and the flowability of the pure drug.

$$Bulk\ density = \frac{Mass\ (gm)}{V\ (ml)}$$

$$Tapped\ density = \frac{mass\ (gm)}{V\ (ml)}$$

$$cl = \frac{Tapped\ density - bulk\ density}{Tapped\ density} \times 100$$

$$HR = \frac{Tappe\ density}{Bulk\ density}$$

$$\theta = tan^{-1}\ (h|r)$$

Where V_O = Initial volume, V_n = Final volume after n_{th} tapping, CI = Carr's index, HR = Hausner's ratio, θ= angle of repose, h = height of pile and r = radius.

In vitro dissolution studies

The in vitro dissolution studies were carried out in triplicate using eight-station USP type II dissolution apparatus (Lab India, Model Disso 2000 Tablet dissolution test apparatus, Mumbai, India). Dissolution studies were carried out using 900mL of 0.1N HCl, distilled water and pH 6.8 phosphate buffer at 37 ± 0.5°C and 50 rpm. 5 mL of sample was withdrawn after suitable time interval and replaced each time with 5mL fresh medium. The solutions were immediately filtered through 0.45 mm membrane filter, diluted and the concentration of Isoniazid was determined spectrophotometrically at respective λmax (0.1N HCl - 266 nm, distilled water – 262 nm and pH 6.8 phosphate buffer - 262.5 nm).

Crystal form I: INH-Para amino salicylic acid (1:1) co-crystal (solvent evaporation) :

FTIR Studies

The IR spectra and interpretation for isoniazid, para amino salicylic acid and crystal form **1** were presented in figure no. 13.

Differential scanning calorimetry (DSC)

DSC experiments were carried out to study the thermal behavior of the crystal form 1 in relation to the individual components. DSC thermal data are shown in figure no. 14. DSC study of INH and Para aminosalicylic acid shows endothermic peak at 172.60^0C and 135.28^0C while DSC study of prepared cocrystal shows sharp endothermic value at 143.61^0C, the sharp endothermic values of crystal form 1 and the individual components agreed with the measured melting range in the melting point determination. The thermal profile of crystal form 1 was distinct, with a different melting transition from that seen with either of the individual components. This indicates the formation of novel crystal phase: crystal form 1 of INH with Para amino salicylic acid (1:1 molar ratio). This single endothermic transition indicates the absence of any unbound or absorbed solvent or water and also demonstrates the stability of the phase until the melting point.

Scanning electron microscopy (SEM)

SEM photography of prepared cocrystal shows uniform block like crystals while Isoniazid shows small rod like crystals and Para amino salicylic acid shows.. SEM photographs of isoniazid, Para amino salicylic acid and crystal form **1** shown in figure no.15

The X-ray powder diffraction (XRD) spectra of INH and PAS co-crystal shows characteristic peak at 25.753^0 which is 100% relative intensity which is different from individual components of INH and PAS also at 28.388^0, 31.00^0, 42.8^0 new peaks were appeared. This indicates formation of new crystalline phase.

Crystal form II: INH-Para amino salicylic acid (1:1) co-crystal (solvent drop)

FTIR Studies

The IR spectra and interpretation for isoniazid, para amino salicylic acid and crystal form **II** were presented in figure no.17.

Differential scanning calorimetry (DSC)

DSC experiments were carried out to study the thermal behavior of the crystal form II in relation to the individual components. DSC thermal data are shown in figure no. 18. DSC study of INH and Para aminosalicylic acid shows endothermic peak at 172.60^0C and 135.28^0C while DSC study of prepared cocrystal shows sharp endothermic value at 141.82^0C, the sharp endothermic values of crystal form II and the individual components agreed with the measured melting range in the melting point determination. The thermal profile of crystal form II was distinct, with a different melting transition from that seen with either of the individual components. This indicates the formation of novel crystal phase: crystal form II of INH with Para amino salicylic acid (1:1 molar ratio). This single endothermic transition indicates the absence of any unbound or absorbed solvent or water and also demonstrates the stability of the phase until the melting point.

Scanning electron microscopy (SEM)

SEM photography of prepared co-crystal shows reduction in size while Isoniazid and Para amino salicylic acid shows small rod like crystals shows small rod like crystals. SEM photographs of isoniazid, Para amino salicylic acid and crystal form II shown in figure no.19.

The X-ray powder diffraction (XRD) spectra of INH and PAS co-crystal shows characteristic peak at 25.494^0 which is 100% relative intensity which is different from individual components of INH and PAS also at 16.98^0, 27.20^0, 29.15^0 new peaks were appeared. This indicates formation of new crystalline phase (Fig 20).

Crystal form III: INH-Para amino salicylic acid (1:1) co-crystal (co- grinding)

FTIR Studies

The IR spectra and interpretation for isoniazid, para amino salicylic acid and crystal form II were presented in figure no.21.

Differential scanning calorimetry (DSC)

DSC experiments were carried out to study the thermal behavior of the crystal form II in relation to the individual components. DSC thermal data are shown in figure no. 22. DSC study of INH and Para aminosalicylic acid shows endothermic peak at 172.60^0C and 135.28^0C while DSC study of prepared cocrystal shows sharp endothermic value at 141.11^0C, the sharp endothermic values of crystal form III and the individual components agreed with the measured melting range in the melting point determination. The thermal profile of crystal form III

was distinct, with a different melting transition from that seen with either of the individual components. This indicates the formation of novel crystal phase: crystal form III of INH with Para amino salicylic acid (1:1 molar ratio). This single endothermic transition indicates the absence of any unbound or absorbed solvent or water and also demonstrates the stability of the phase until the melting point.

Scanning electron microscopy (SEM)

SEM photography of prepared co-crystal had shown flake like crystals while Isoniazid and Para amino salicylic acid shows small rod like crystals shows small rod like crystals. SEM photographs of isoniazid, Para amino salicylic acid and crystal form III shown in figure no.23.

The X-ray powder diffraction (XRD) spectra of INH and PAS co-crystal shows characteristic peak at 25.65^0 which is 100% relative intensity which is different from individual components of INH and PAS also at 17.103^0, 27.05^0, 29.31^0 new peaks were appeared. This indicates formation of new crystalline phase (Fig 24).

Drug content

Drug content of prepared crystal forms were determined in triplicate by spectrophotometrically. The practical yield was found satisfactory and ranged from 88.4% to 96.4% for those in pH 6.8 phosphate buffer and 74.8% to 100% for those in 0.1 N HCl. The values of prepared crystal forms were shown in table no.3.

Saturation Solubility

The solubility studies of Isoniazid and prepared novel multicomponent crystal forms in 0.1N HCl distilled water and pH 6.8 phosphate buffer were shown in figure 25. This indicates that pure drug shows high solubility compare with prepared crystal forms of isoniazid. In the prepared crystal forms, Crystal forms 2 have least solubility in 0.1N HCl distilled water and pH 6.8 phosphate buffer.

Measurement of flowability and compressibility

In order to achieve uniformity in tablet weight, the feed crystals must flow smoothly into the die cavity of the tablet machine. Therefore, it is an essential purpose to improve the flow properties of powders.

The micromeritic properties such as angle of repose, Carr's index and Hausner's ratio were calculated in triplicate for pure drug and prepared novel multi component crystal forms. The results of these micromeritic properties were given in table no. 4.

Pure drug isoniazid exhibited poor flowability and compressibility as indicated by Carr's index (0.84%), Hausner's ratio (1.31) and angle of repose (32.37). This could be due to the rod shape and small size of powder with stickiness, which put hurdles in the uniform flow of powder from the funnel. The prepared crystal form 2 showed poor flowability and compressibility as showed by low value of Carr's index (0.136%), Hausner's ratio (1.15) and angle of repose (43.4) when compared to pure drug. The crystal form 1 has shown improvement in the flowability with angle of repose showing 28.39 with Carr's index 0.20% and Hausner's ratio 1.20.

In vitro dissolution studies

Invitro dissolution studies were done in triplicate for isoniazid and prepared novel multicomponent crystal forms of isoniazid in 0.1 N HCl and pH 6.8 phosphate buffer. The powder dissolution profiles for Isoniazid, Crystal form 1, 2, 3, 4, 5 and 6 were shown in figure no.26 and 27 respectively. Dissolution data was shown in table no.5 and 6.

Anti-Tubercular activity

96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilution of compounds were made directly on plate. The final drug concentrations tested were 100 to 0.2 µg/ml. Plates were covered and sealed with parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink.

Fig 1. Standard Curve of Isoniazid with 0.1N HCl

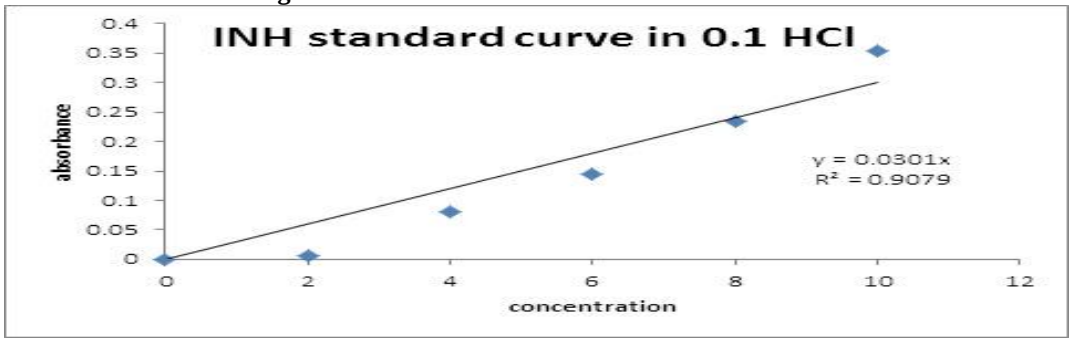


Fig 2. Standard Curve of Isoniazid with pH 6.8 Phosphate buffer

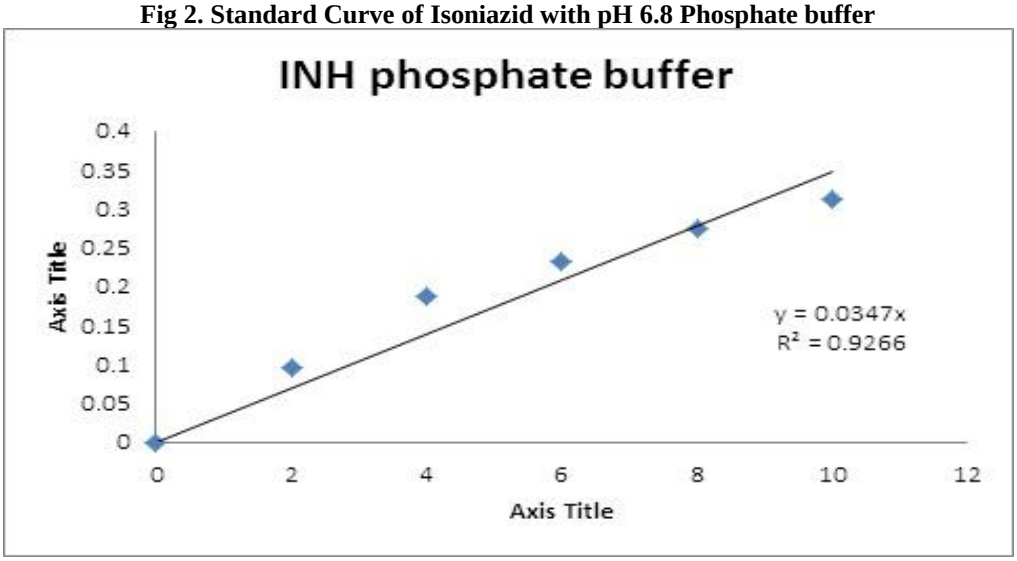


Fig 3. Standard Curve of PAS with 0.1N HCl

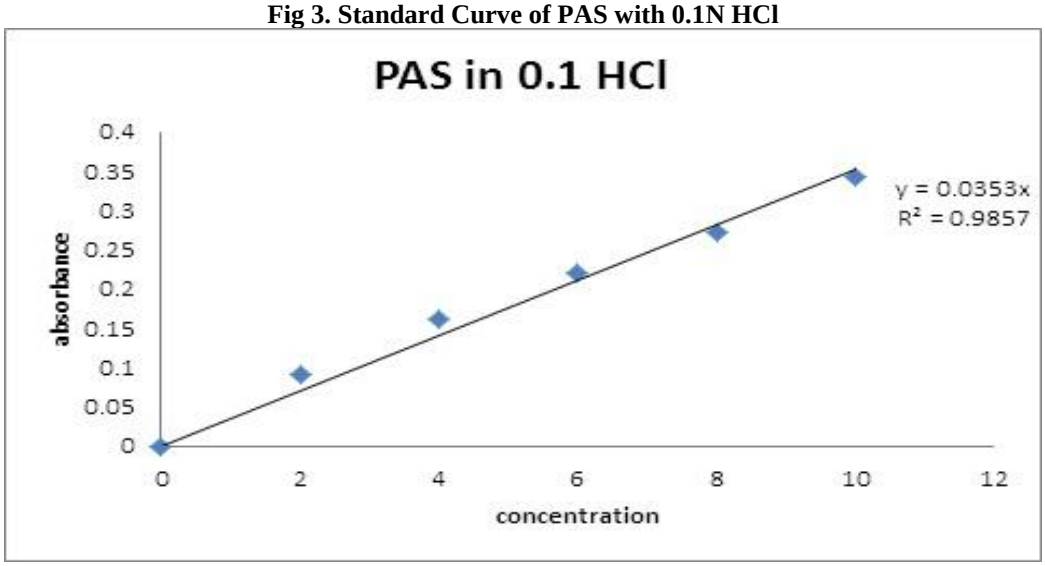


Fig 4. Standard Curve of PAS with pH 6.8 Phosphate buffer

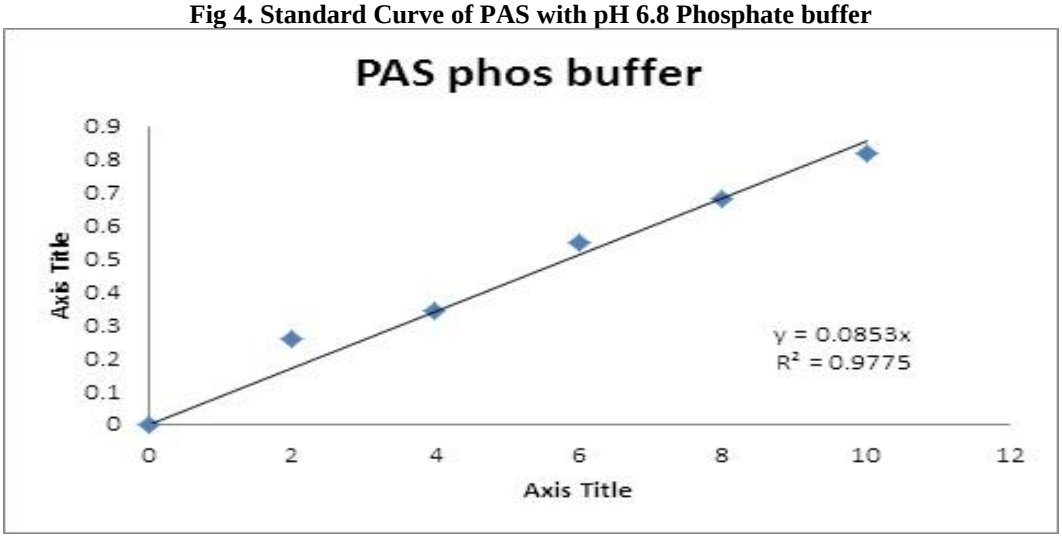


Fig 5. FTIR spectra of INH

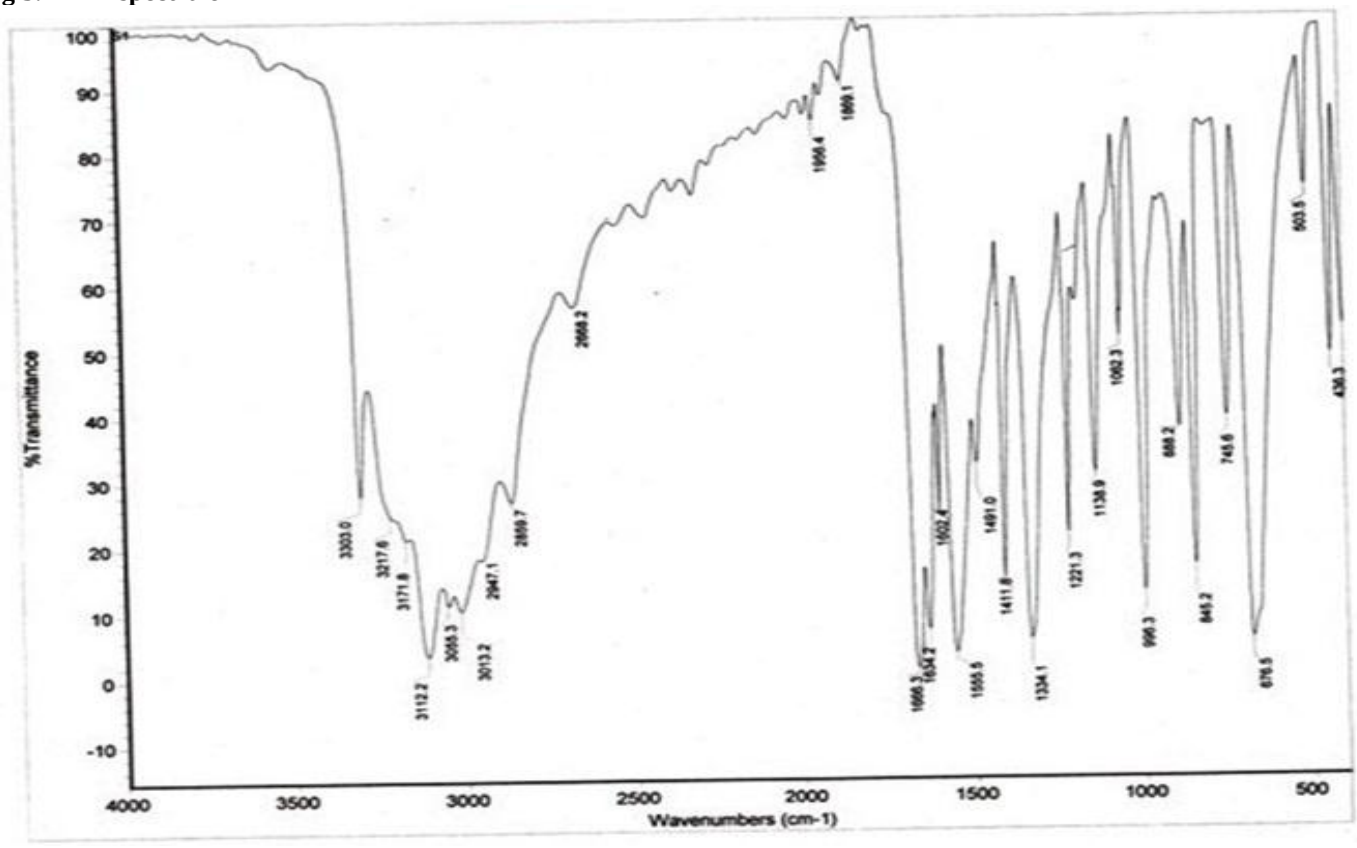


Fig 6. DSC thermo gram of INH

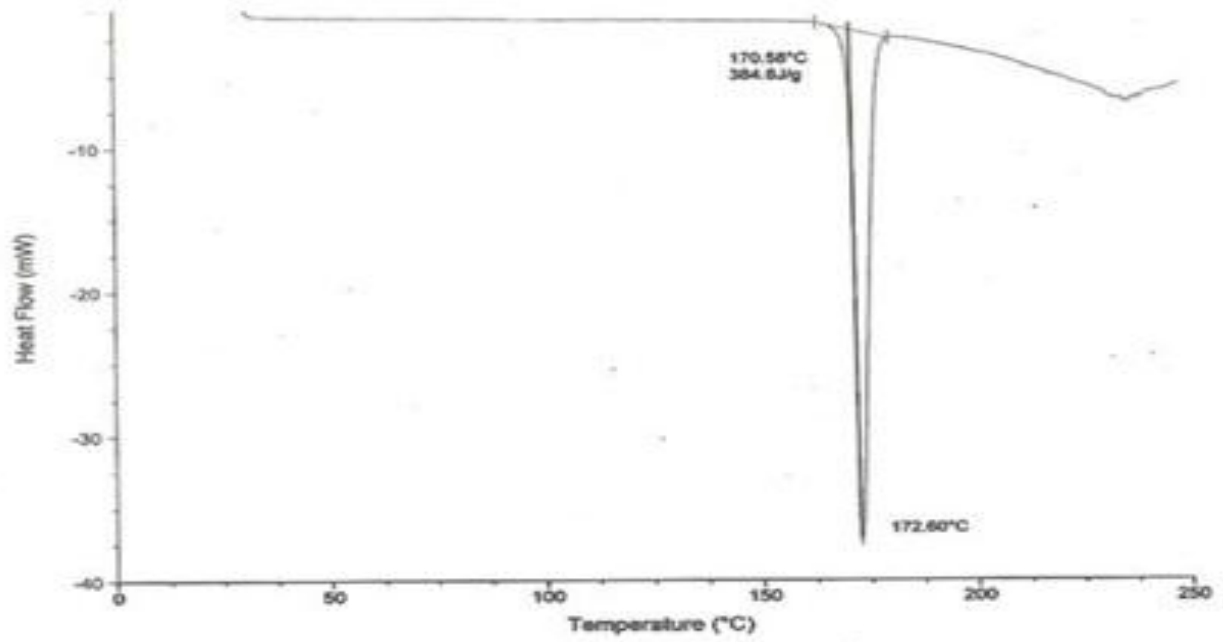


Fig.7 SEM photographs of INH

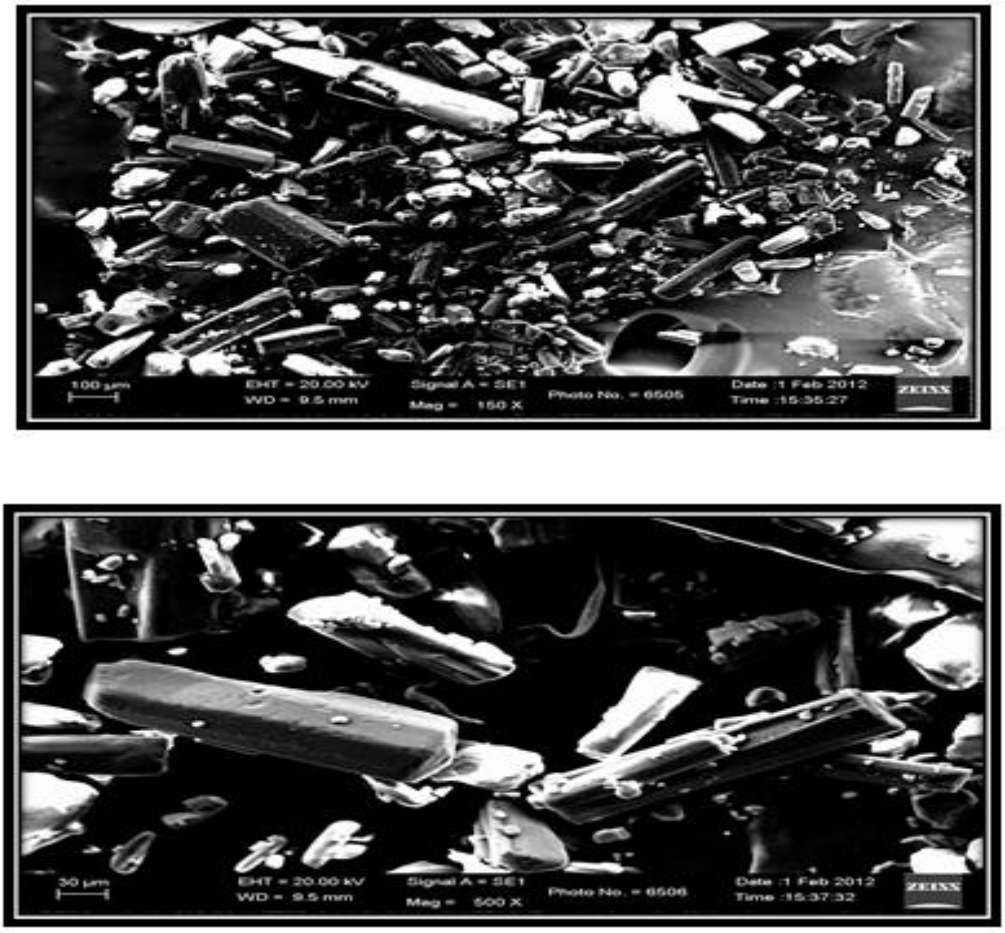


Fig 8. XRD of INH

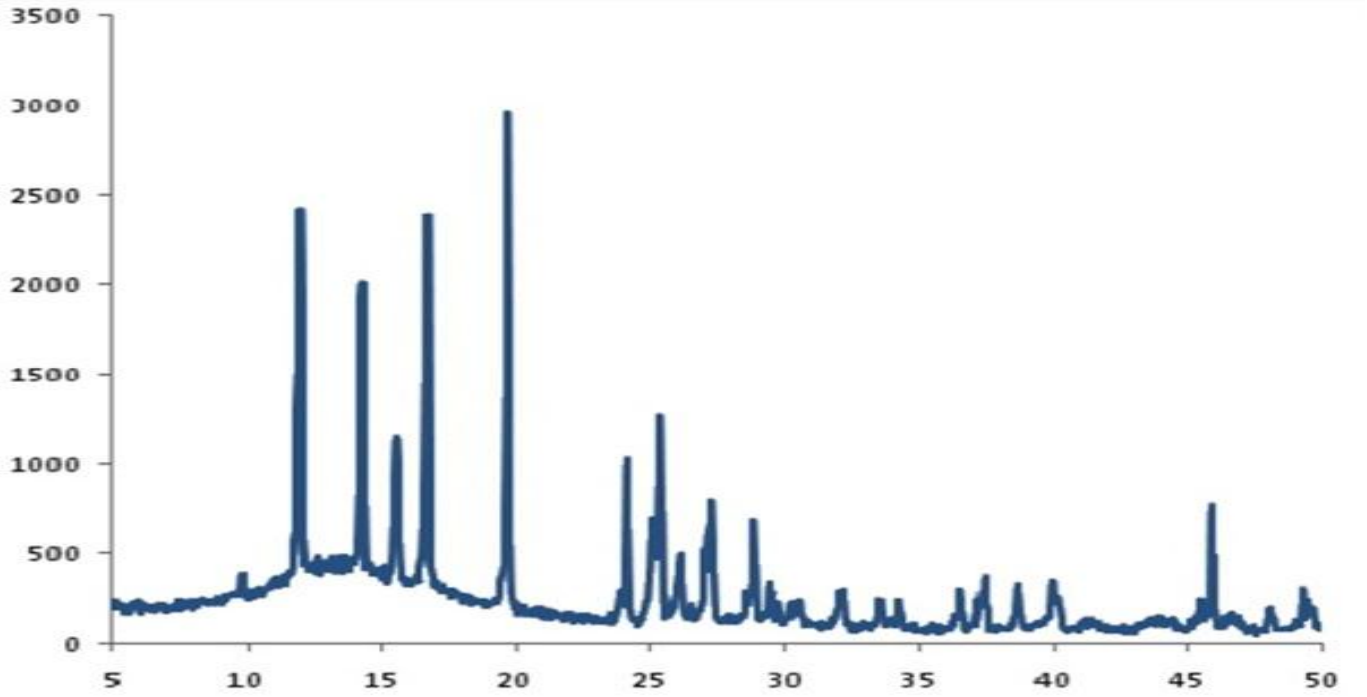


Fig 9. FTIR spectrum of PAS

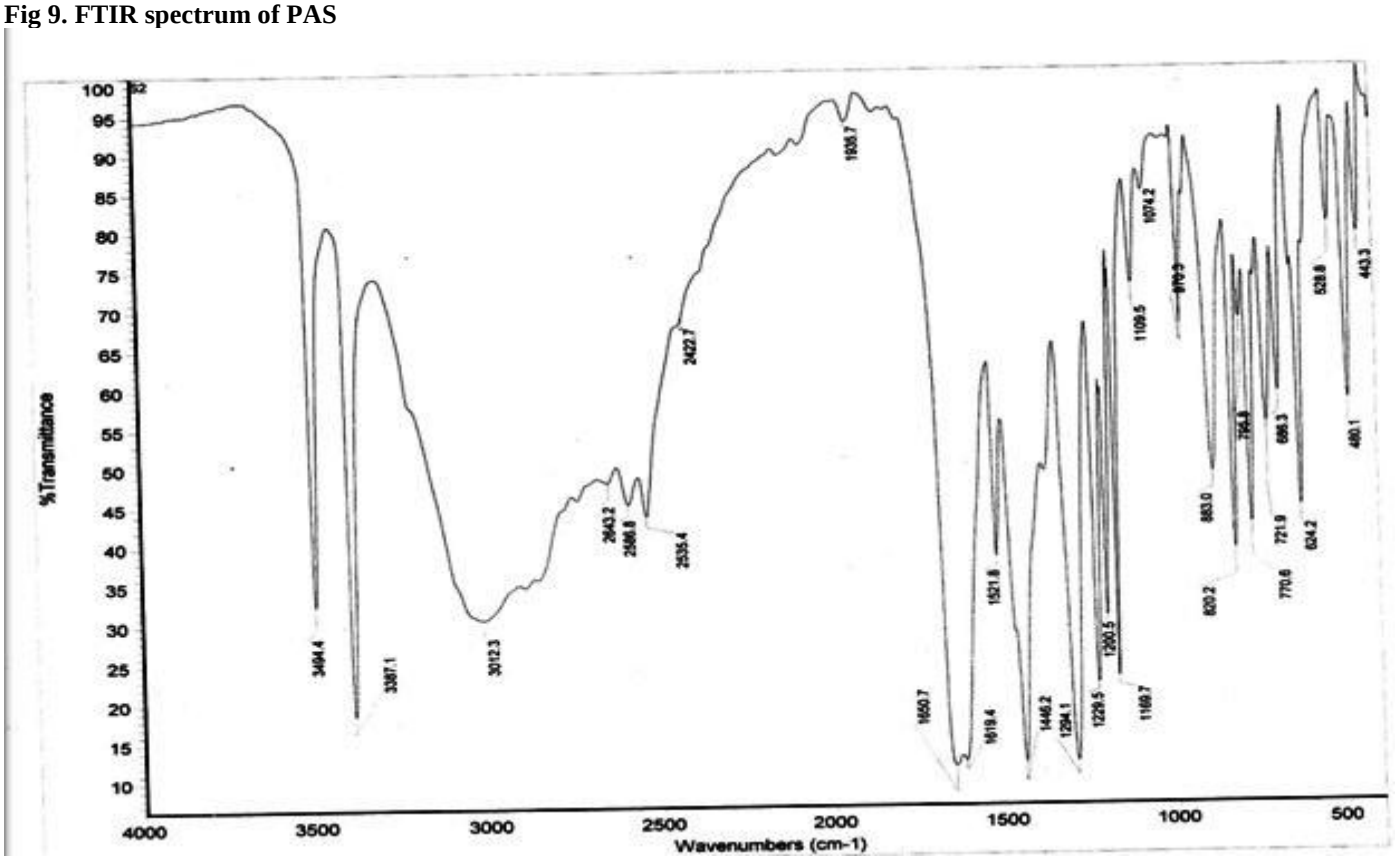


Fig 10. DSC thermo gram of PAS

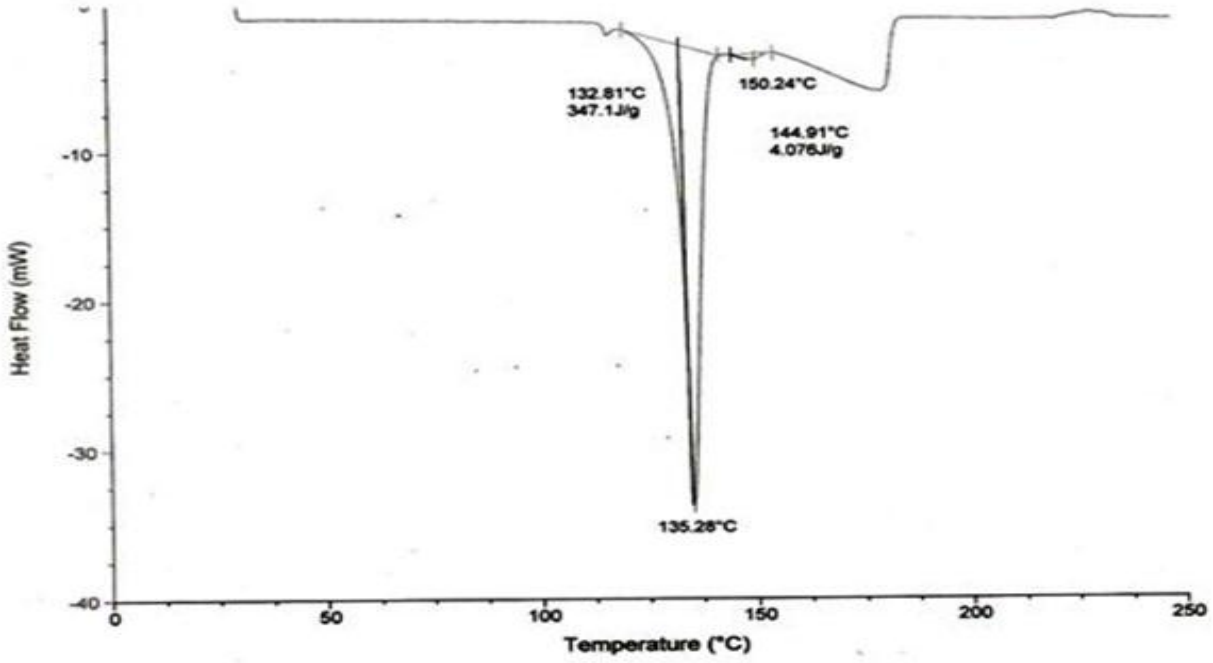


Fig.11. SEM photographs of PAS

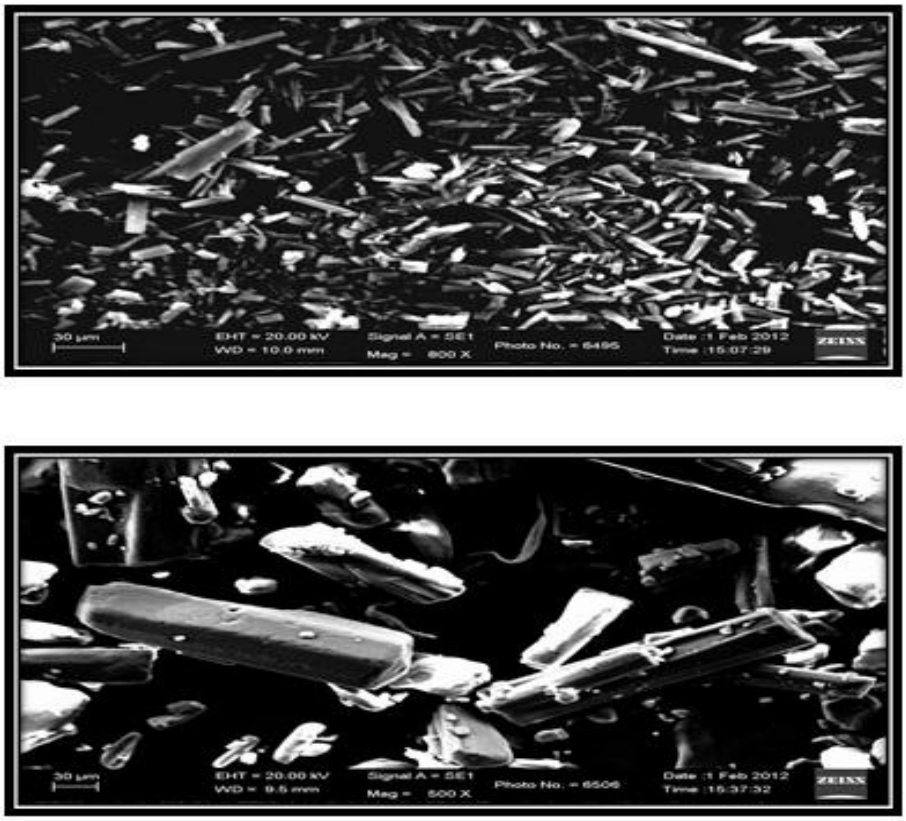


Fig. 12. XRD Pattern of PAS

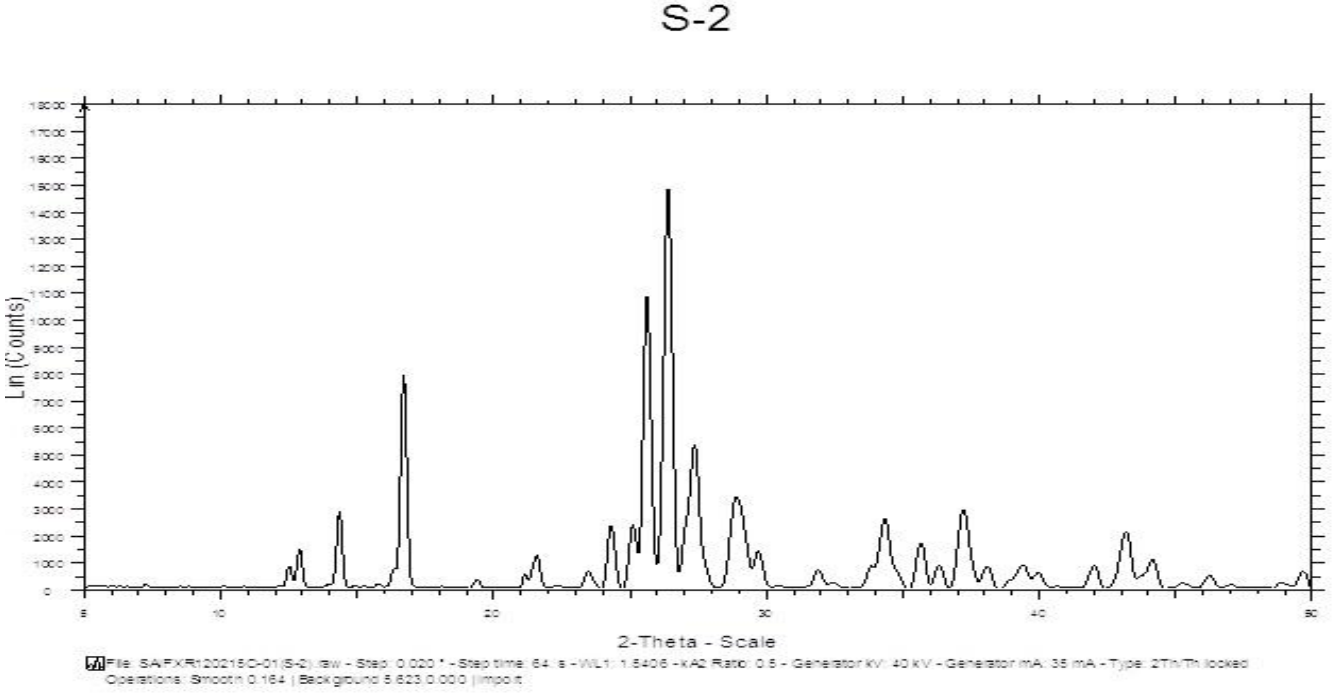


Figure 13. IR spectrum of (a) Isoniazid, (b) Para amino salicylic acid and (c)Crystal form I

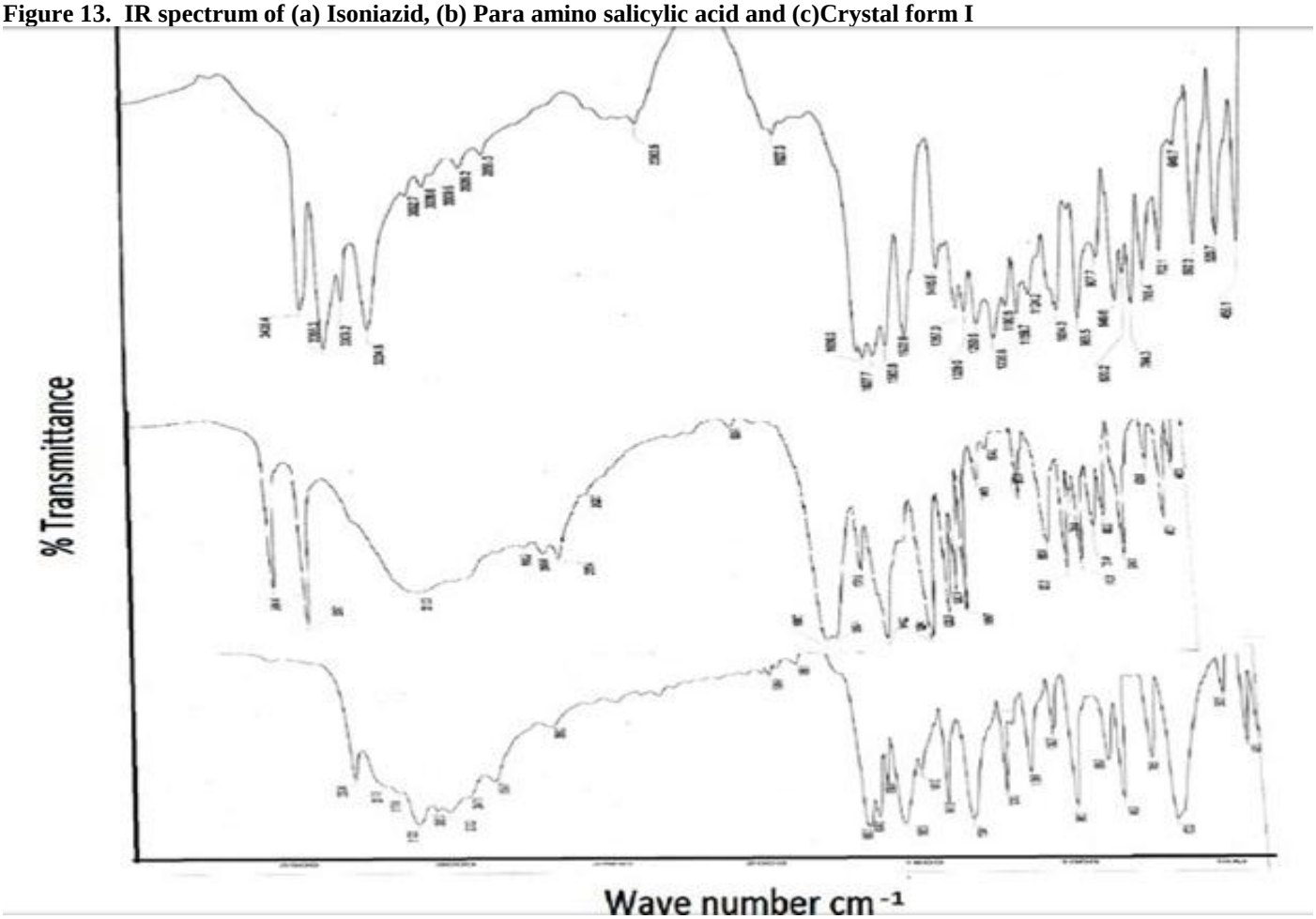


Fig 14. DSC of (a) Isoniazid, (b) Para amino salicylic acid and (c)crystal form I

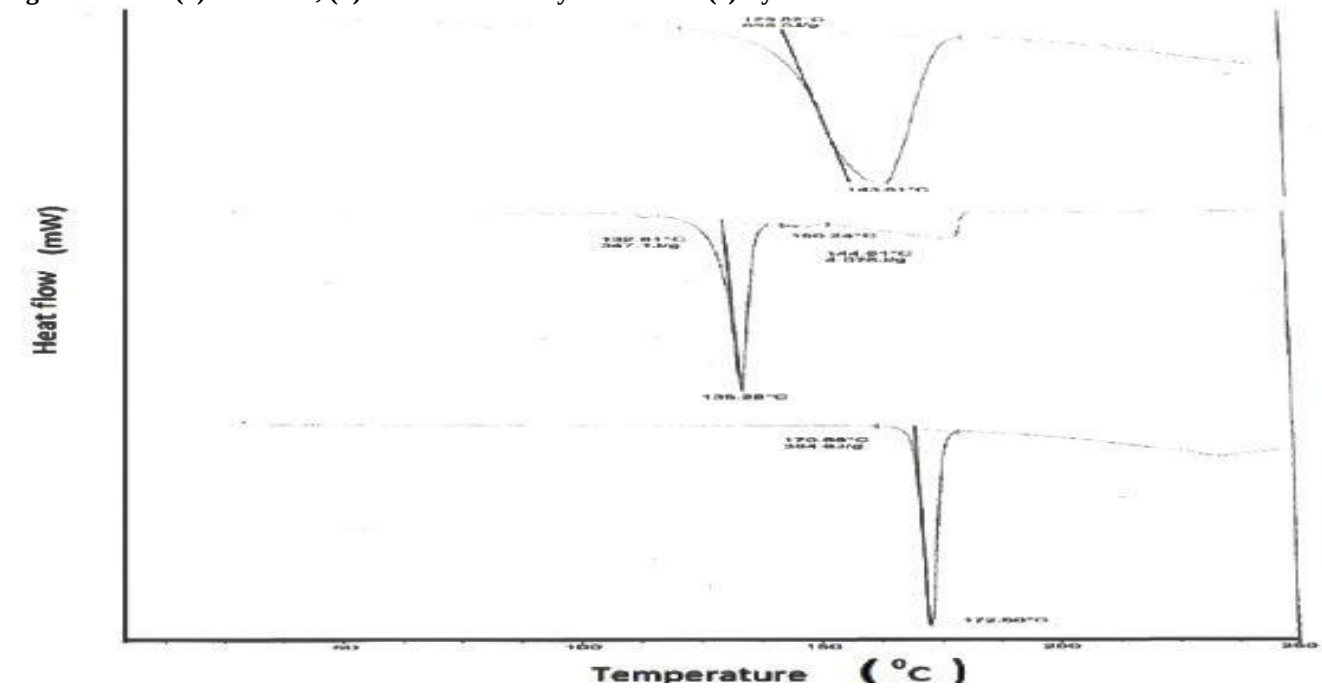


Fig 15. SEM photographs of (a) Isoniazid, (b) Para amino salicylic acid and (c)crystal form 1

Fig 16. XRD photographs of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-5)crystal form 1

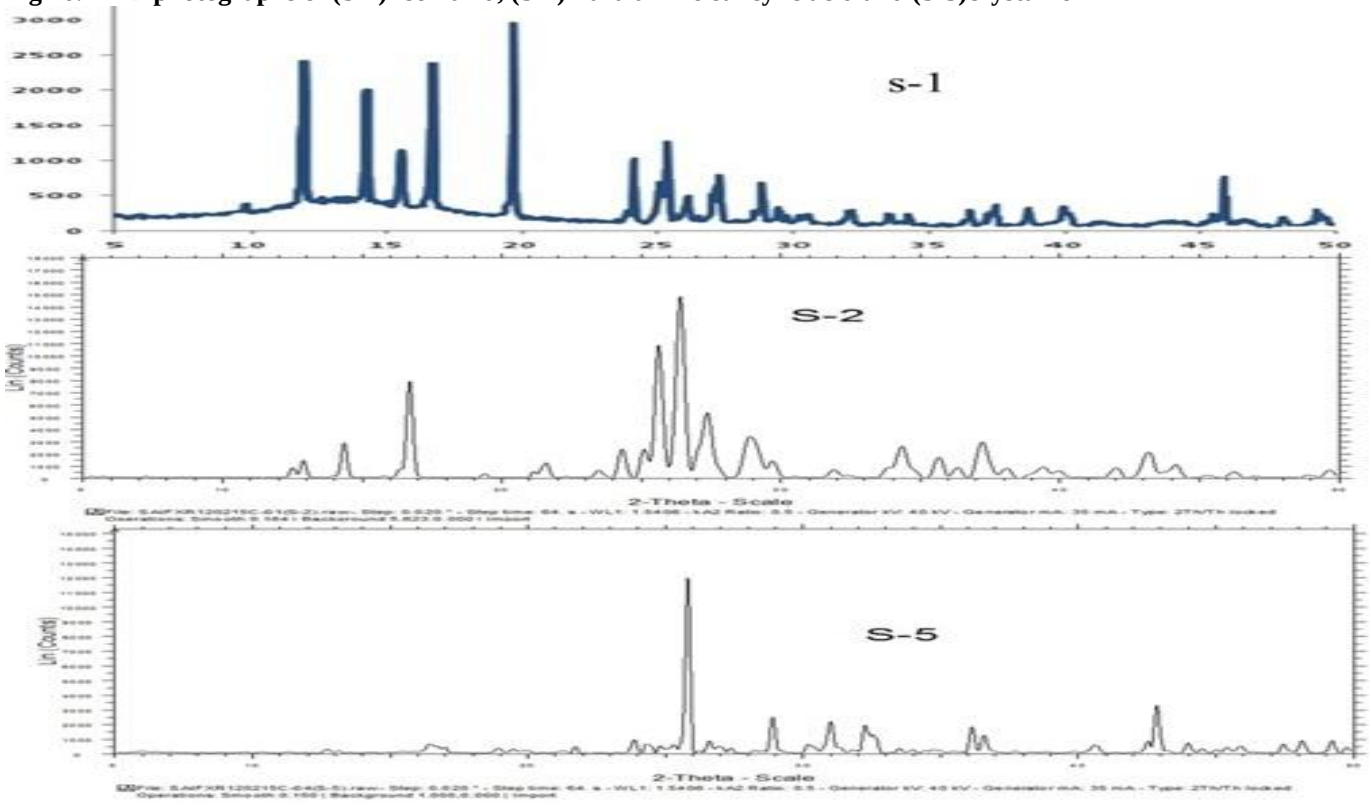


Fig 17. FTIR photographs of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-4)Crystal form II

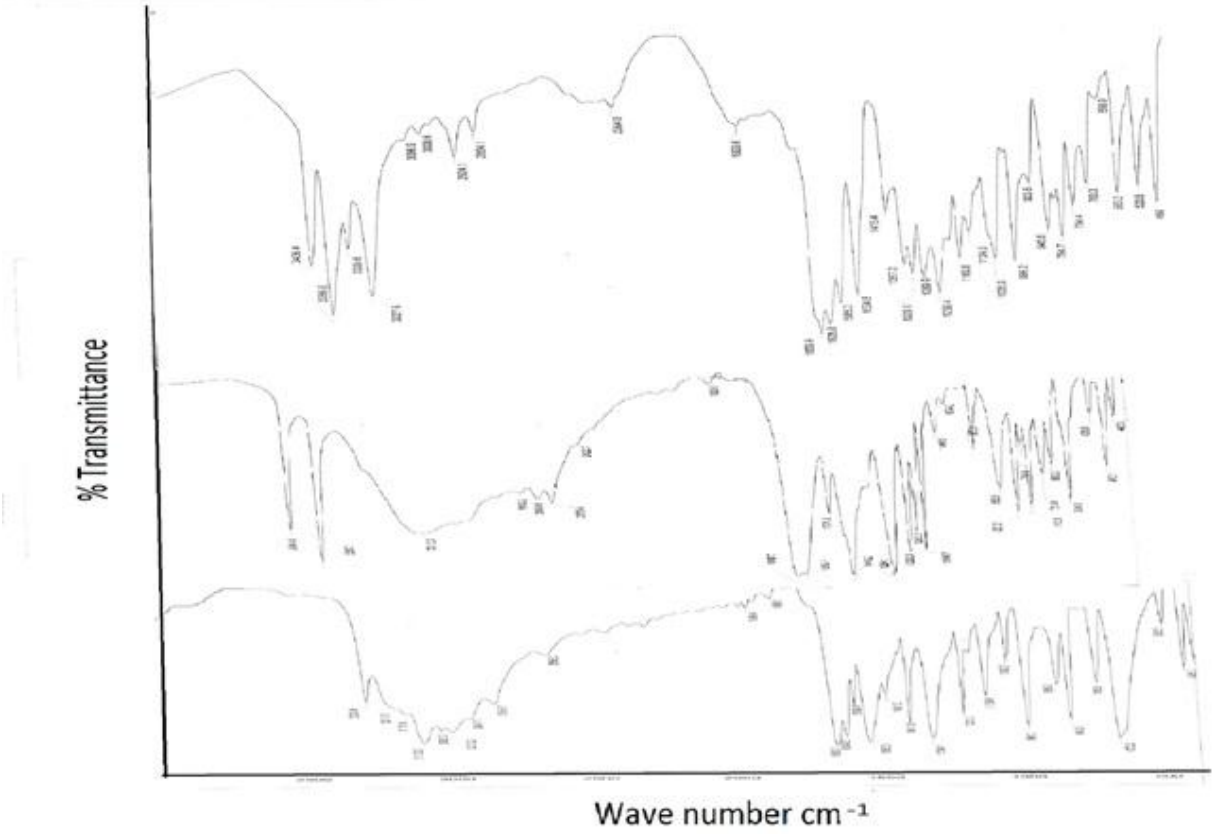


Fig 18. DSC of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-4)crystal form II

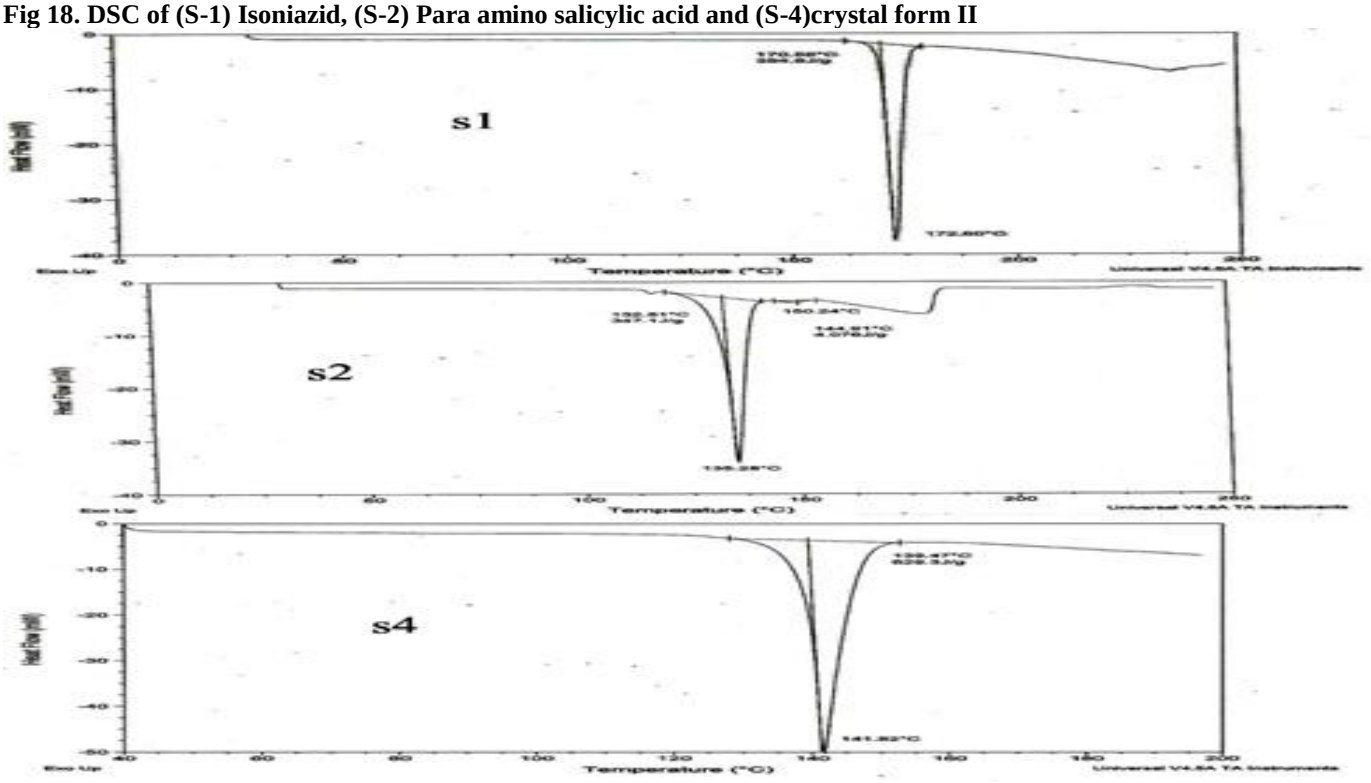
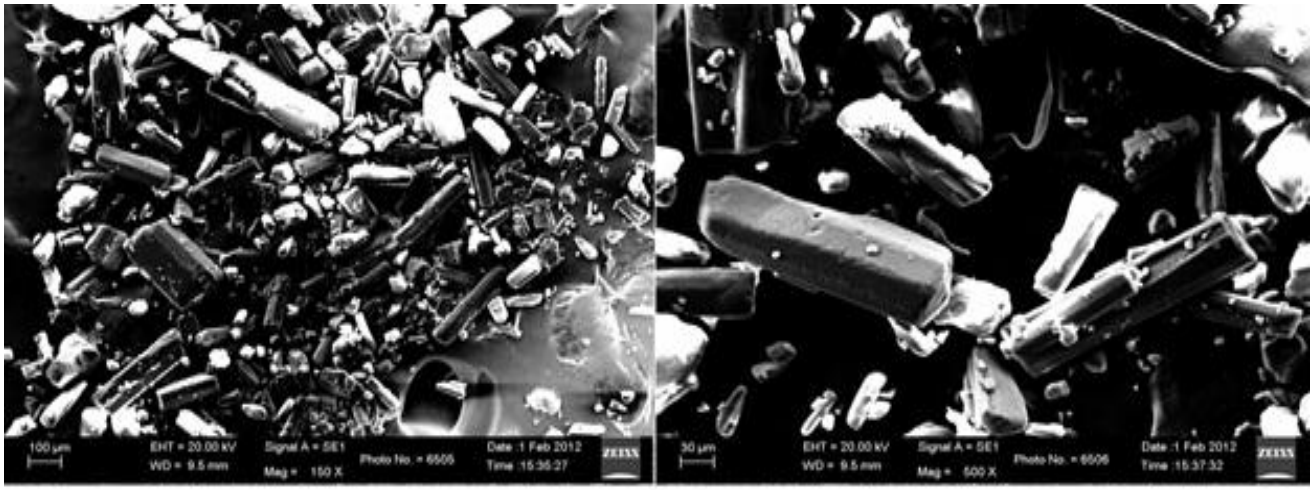
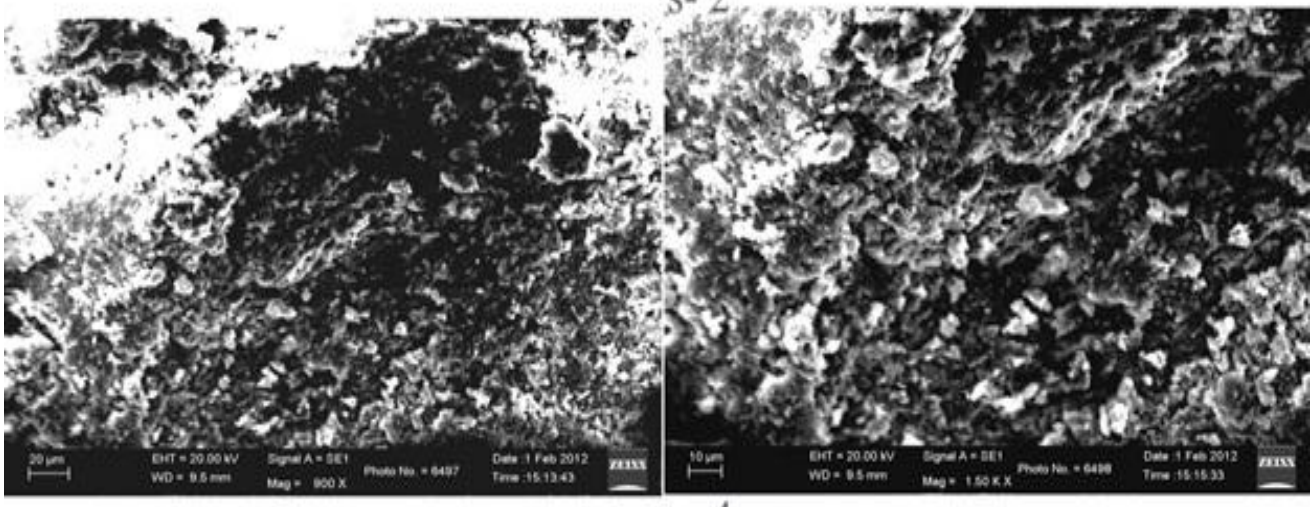


Fig 19. SEM photographs of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-4)crystal form II



S- 1

S- 2



S- 4

Fig. 20. XRD photographs of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-4)crystal form II

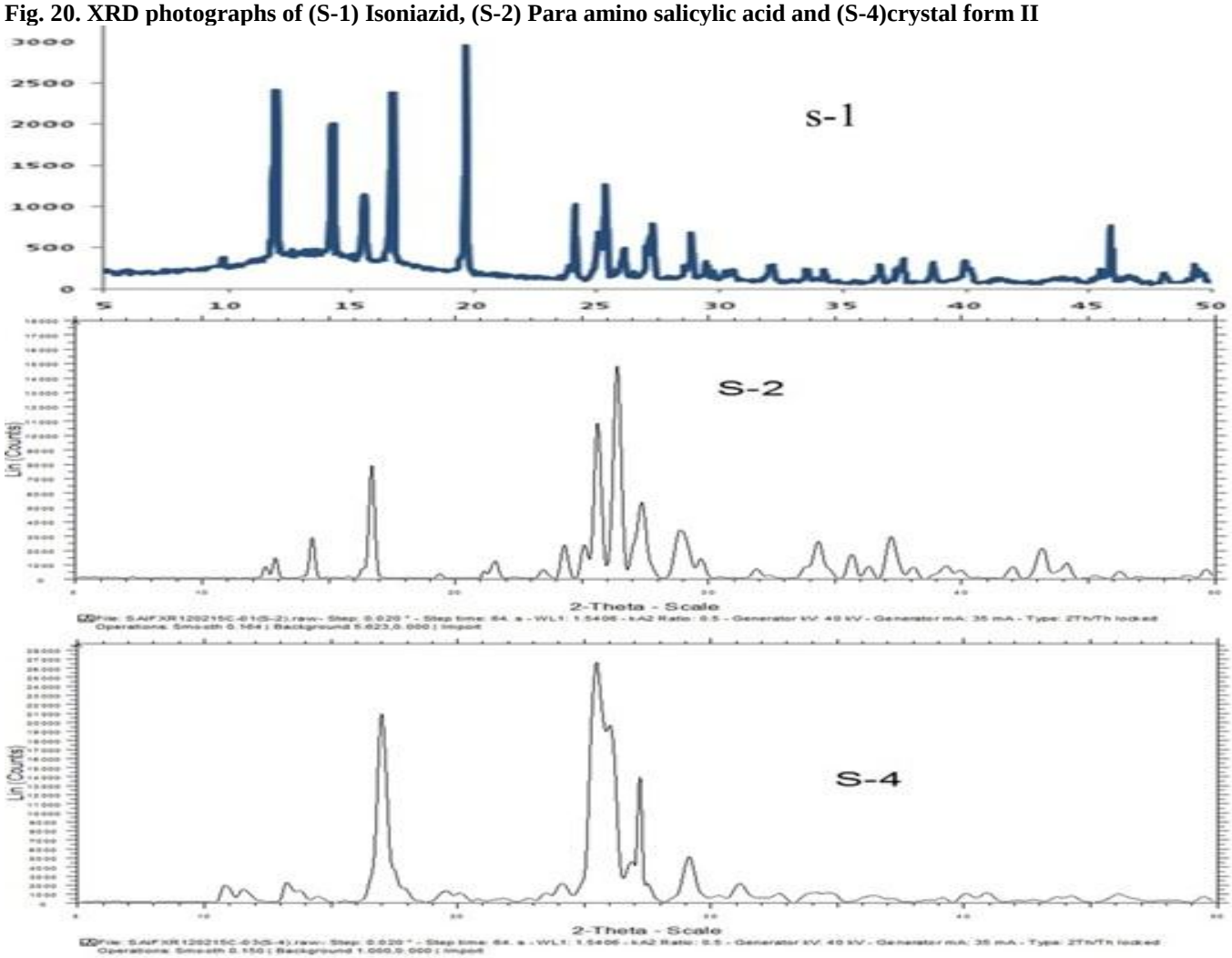


Fig 21. FTIR photographs of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-6) crystal form III

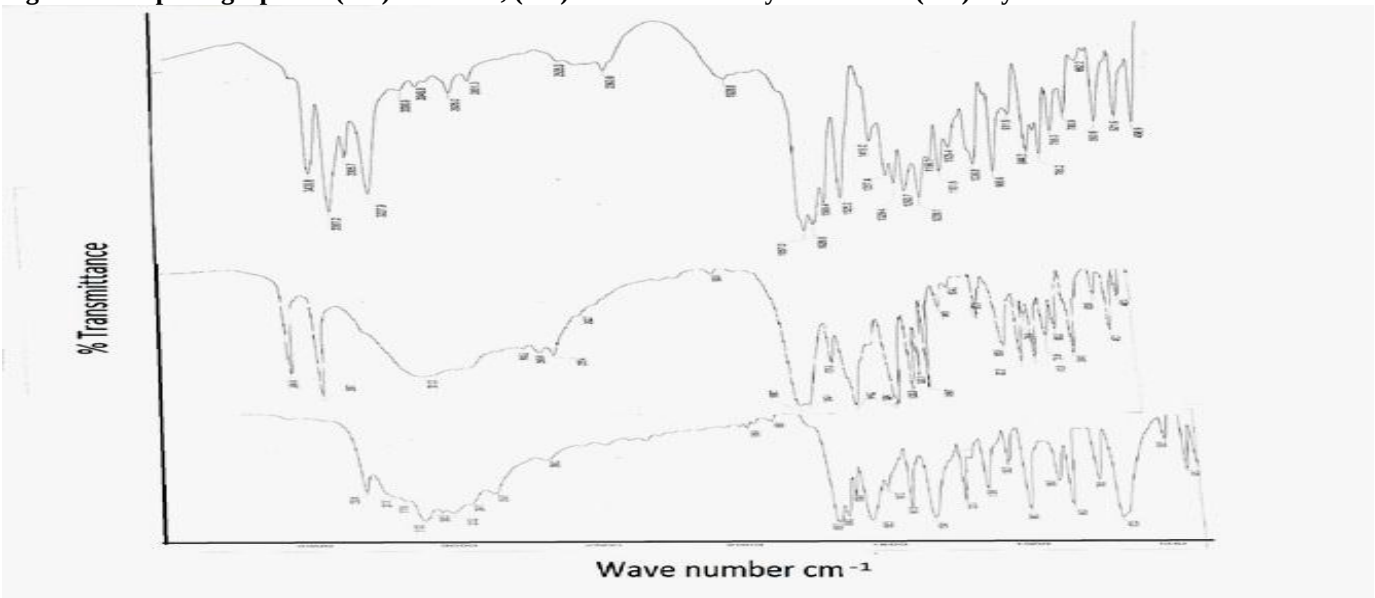


Fig 22. DSC of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-6) Crystal form III

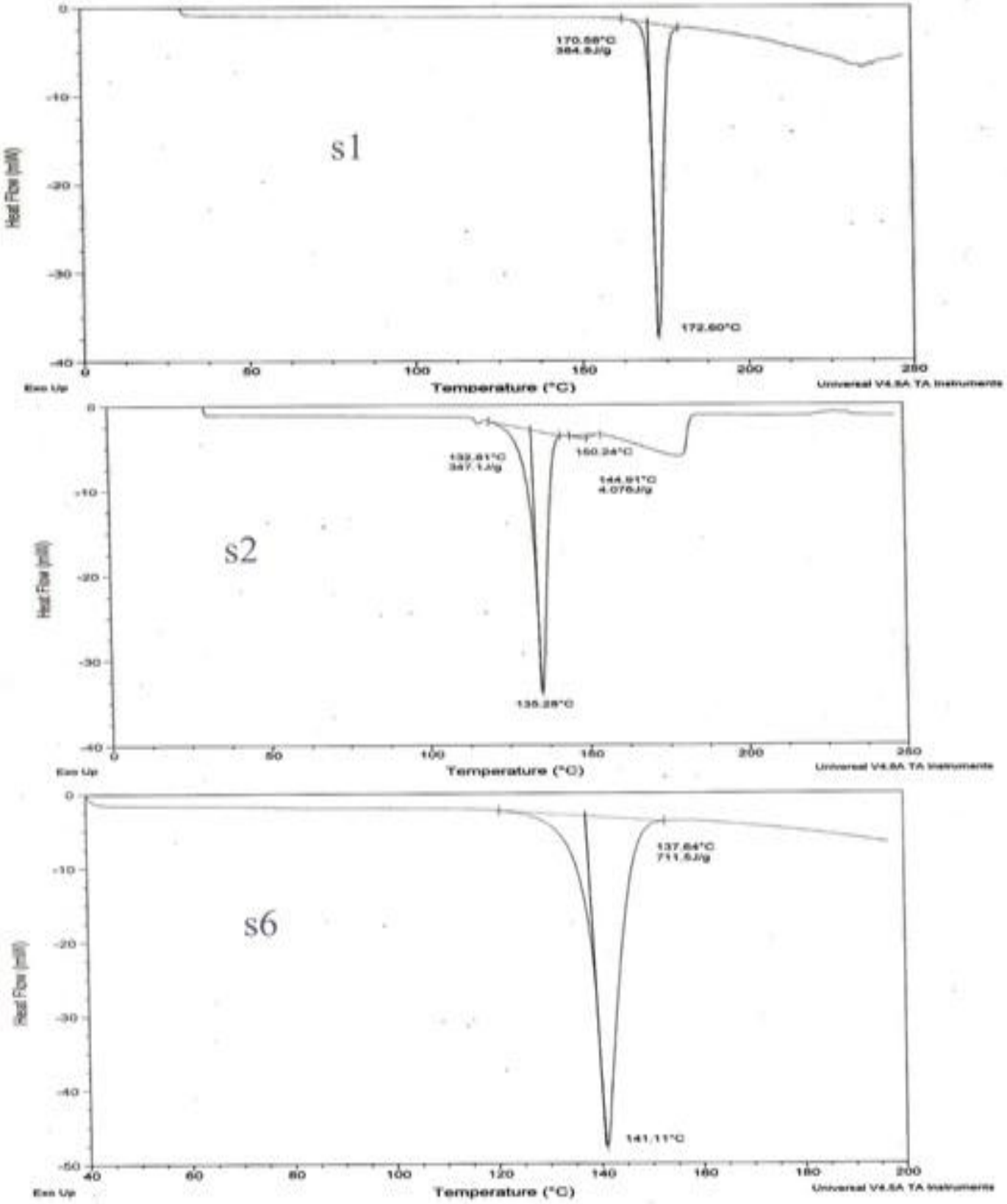
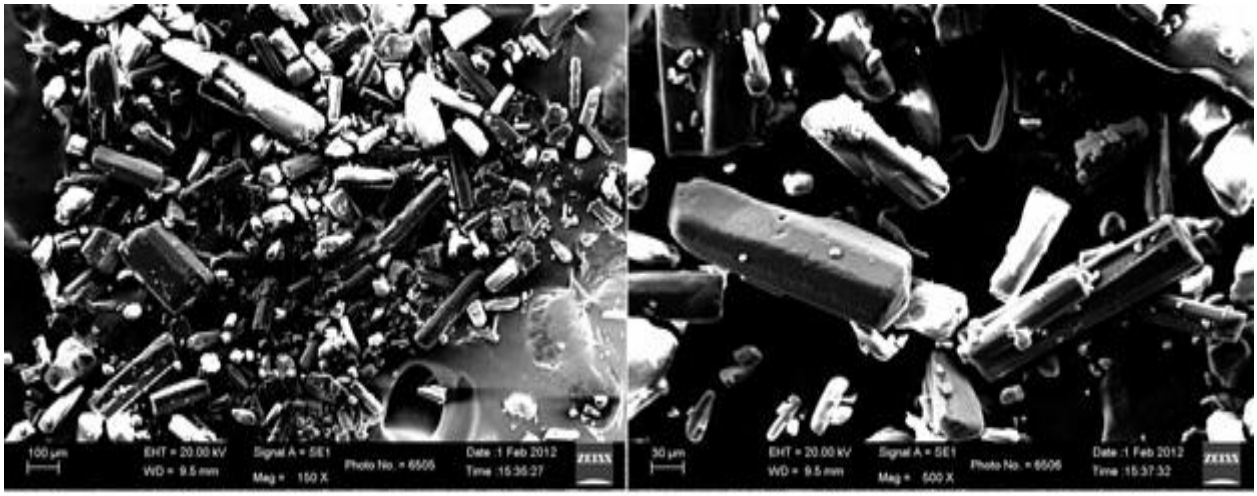
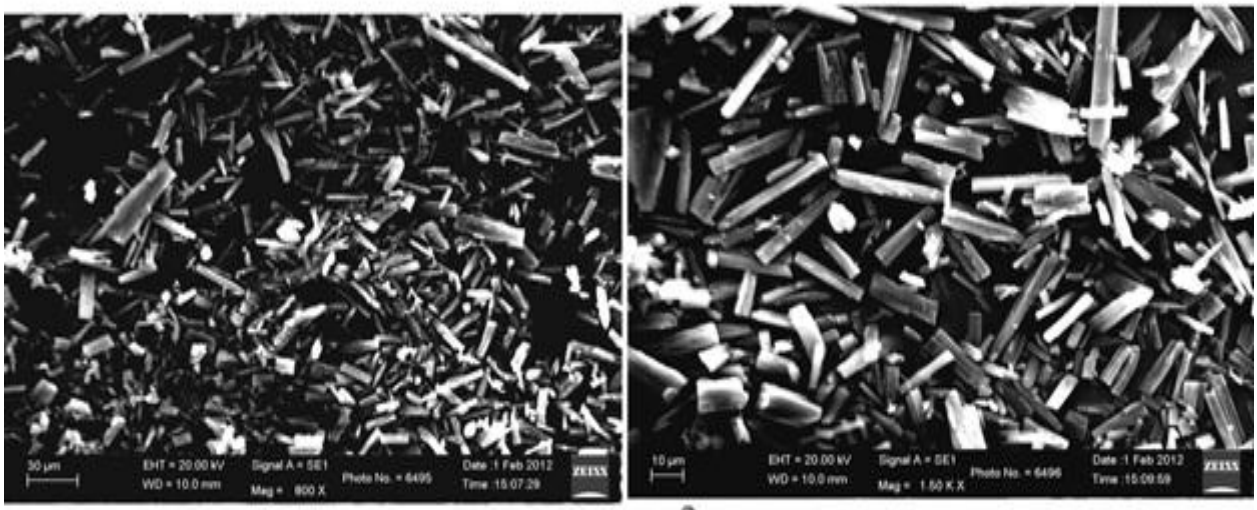


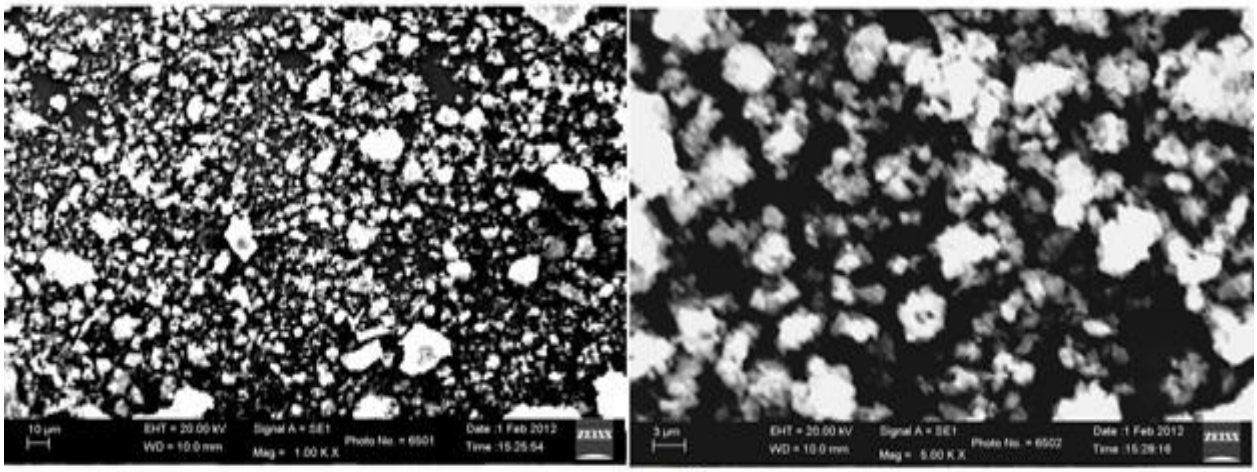
Fig 23. SEM photographs of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-6) Crystal form III



s- 1



s- 2



s- 6

Fig 24. XRD photographs of (S-1) Isoniazid, (S-2) Para amino salicylic acid and (S-6)crystal form III

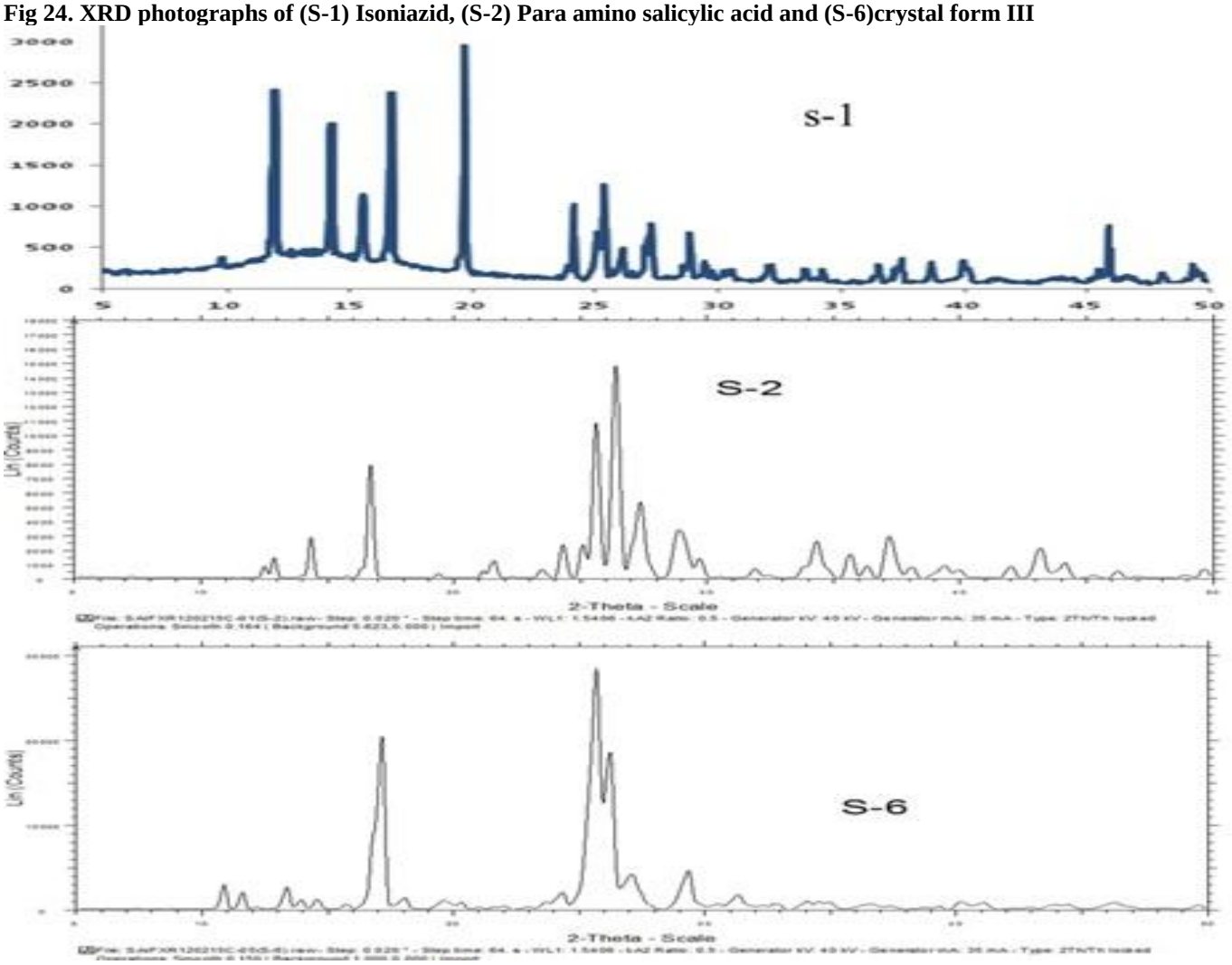


Figure 25. Solubility profile of INH and prepared co-crystals

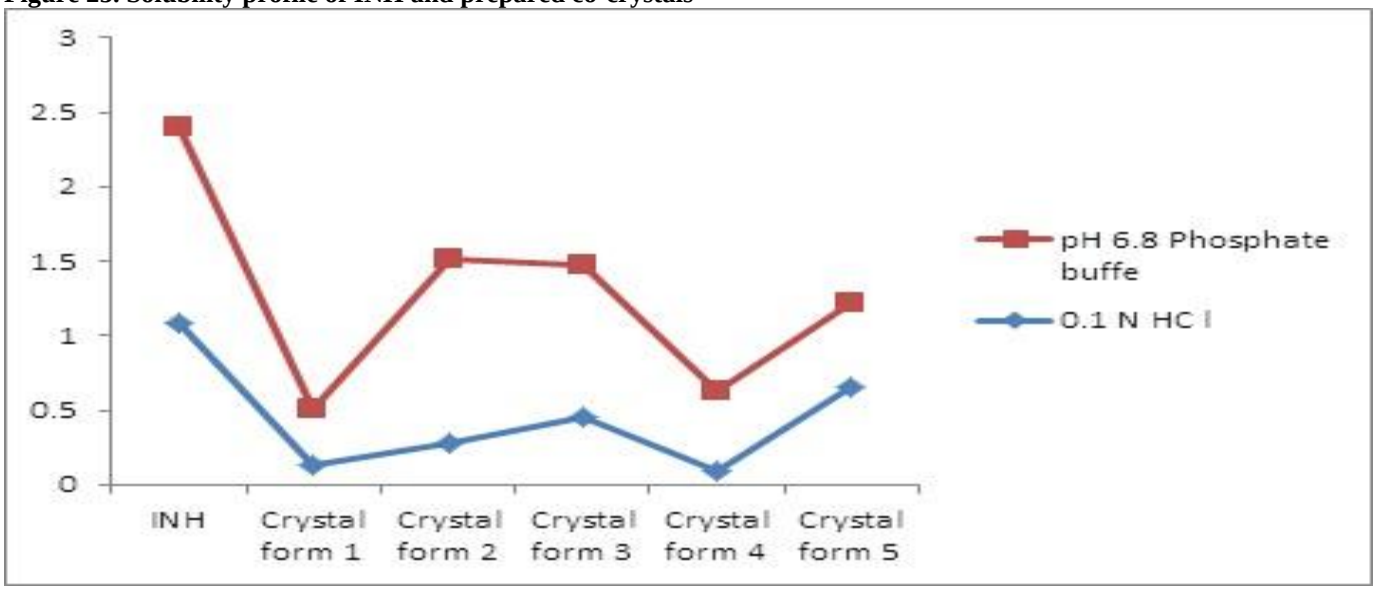


Figure 26. Dissolution profile of pure drug and prepared co-crystal forms in 0.1N HCl

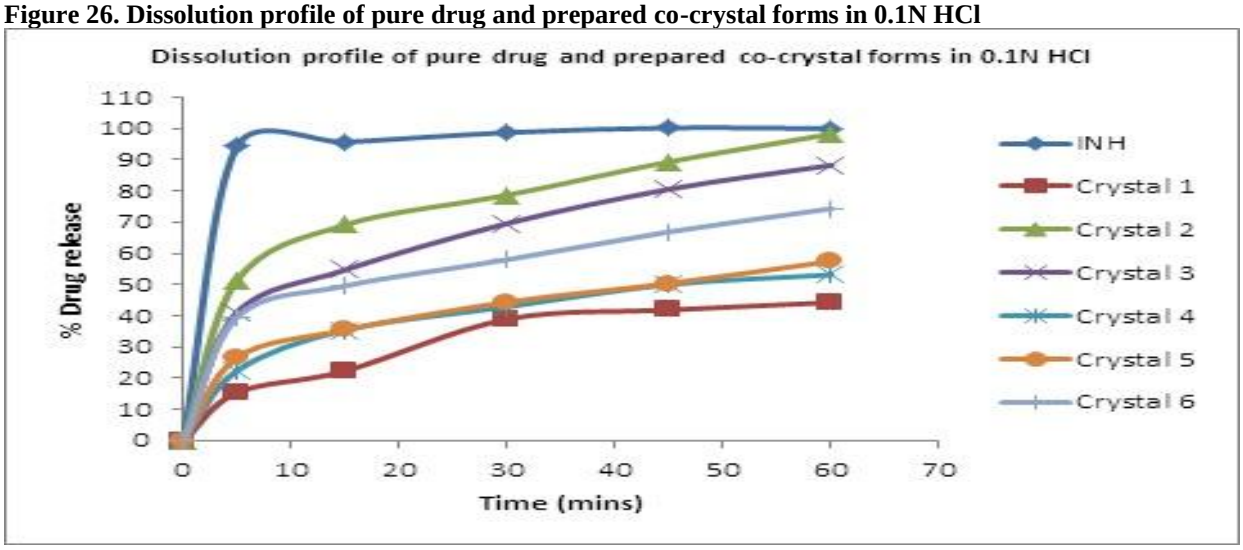


Figure 27. Dissolution profile of pure drug and prepared co-crystal forms in pH 6.8 phosphate buffer

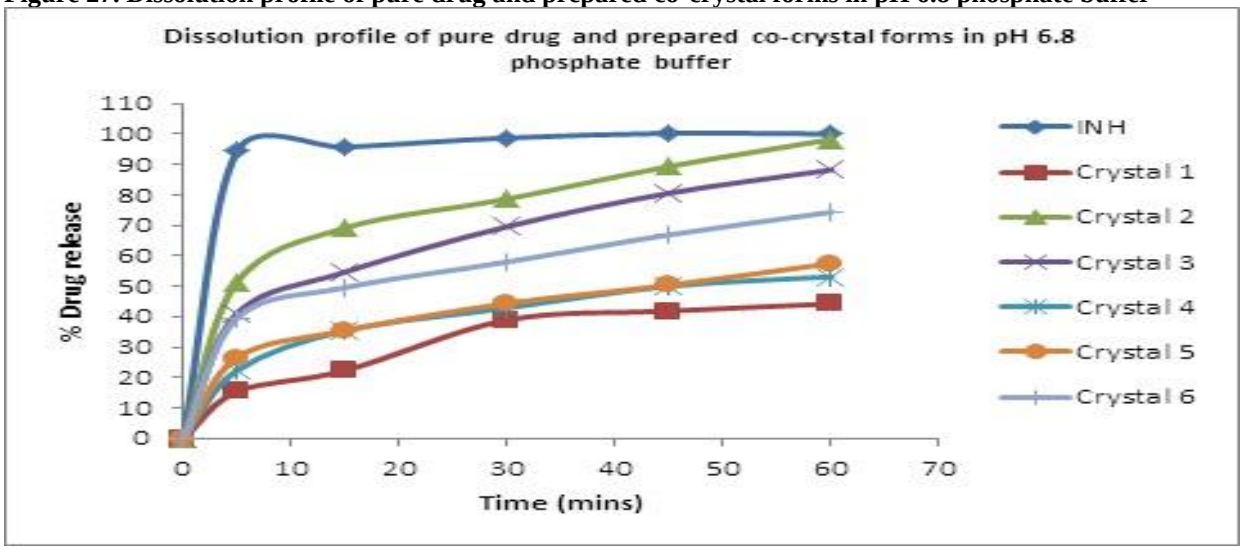


Figure 28. Microplate Alamar Blue assay of Isoniazid and its crystal forms

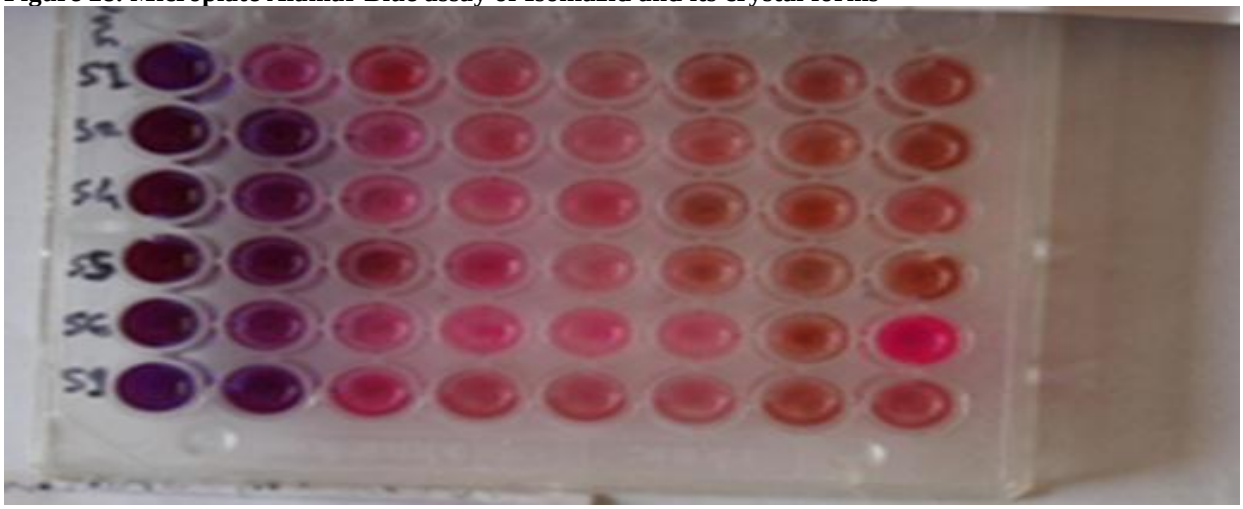


Table 1. Interpretation of IR spectra of INH

FTIR region cm^{-1}	Assignment
3303	N-H stretching
3055	C-H Asym. Stretching
1666.3	C=O stretching
1634.2	Ring Asym. Stretching
1602.4	Ring Asym. Stretching
1555.5	Pyridine nitrogen
1411.8	Ring sym. Stretching
1334.1	C-N stretching
1221.3	Ring C-C-H Asym. Bending
1138.9	N-X.stretching($X=NH_2$)
887.26	C-N-C bending
844.92	Ring C-C-H sym. bending

Table 2. FTIR interpretation of PAS

FTIR region cm^{-1}	**Asignment**
3494.4	NH_2 stretching vibration
3387.1	O-H stretching vibration
1650.7	C=O stretching vibration
1619.4	C=C stretching vibration

Table 3. Drug content of prepared crystal forms

	10 mg of crystal form contains (% Yield)	
	0.1N HCl	pH 6.8 phosphate buffer
Crystal form 1	83	94.8
Crystal form 2	74.8	91.6
Crystal form 3	96	88.4
Crystal form 4	100	91.6
Crystal form 5	90.3	96.4
Crystal form 6	88.9	96.4

Table 4. Micromeritic properties of isoniazid and prepared crystal forms

	INH	PAS	Crystal Form 1	Crystal form 2	Crystal form 3	Crystal form 4	Crystal form 5	Crystal form 6
Bulk density	0.64	0.22	0.4	0.6	2.0	0.61	0.39	0.39
Tapped density	0.84	0.36	0.5	0.85	2.54	0.81	0.57	0.53
Angle of repose	32.37	59.07	28.39	43.4	43.6	44.06	47.54	41.95
Carr's index	0.23	0.38	0.20	0.136	0.27	0.24	0.31	0.26
Hausner ratio	1.31	1.64	1.25	1.15	1.37	1.32	1.46	1.35
Compressibility %	23	38	20	13.6	27	24	31	26

Table 5. Dissolution data of INH and its crystal forms in 0.1 N HCl

Time (min)	INH	Crystal 1	Crystal 2	Crystal 3	Crystal 4	Crystal 5	Crystal 6
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	96.352	15.254	19.654	29.335	42.354	39.328	35.367
15	97.442	19.369	22.315	35.447	59.321	44.985	55.987
30	98.910	25.333	26.354	39.995	65.358	59.673	68.687
45	99.548	28.654	35.429	46.986	76.998	68.987	74.591
60	100.124	30.419	41.675	52.676	88.192	75.418	84.268

Table 6. Dissolution data of INH and its crystal forms in pH 6.8 phosphate buffer

Time (min)	INH	Crystal 1	Crystal 2	Crystal 3	Crystal 4	Crystal 5	Crystal 6
0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
5	94.325	15.398	51.351	40.894	22.398	26.584	39.584
15	95.659	22.337	69.147	54.684	35.421	35.448	49.584
30	98.699	38.925	78.653	69.587	42.873	44.398	57.999
45	100.215	41.875	89.337	80.558	49.998	50.379	66.897
60	99.985	44.266	98.219	88.216	53.14	57.495	74.329

Table 7. MABA results of isoniazid and prepared crystal forms

Sample	100	50	25	12.5	6.25	3.125	1.6	0.8	0.4	0.2
S1	S	R	R	R	R	R	R	R	R	R
S2	S	S	R	R	R	R	R	R	R	R
S4	S	S	R	R	R	R	R	R	R	R
S5	S	S	R	R	R	R	R	R	R	R
S6	S	S	R	R	R	R	R	R	R	R
S9	S	S	R	R	R	R	R	R	R	R

DISCUSSION AND CONCLUSION

Preformulation studies

From the preformulation studies the solid-state characterization of isoniazid by SEM, FTIR, DSC and XRD studies have clearly demonstrated that isoniazid is pure Crystalline phase. From the FTIR, SEM, DSC and XRD studies it can conclude that Para amino salicylic acid and methyl paraben samples are pure and it was selected for the further investigations.

1. Crystal form 1: INH-PAS (1:1) co-crystal (solvent evaporation method)

Crystal form **1** (1:1 molar ratio, Co-crystal) was prepared by solvent evaporation- solution crystallization method and confirmed by comparison of the melting points with those of the starting materials. It characterized in terms of SEM, FTIR, DSC, and XRD subjected to Preliminary pharmaceutical characterization such as solubility, micromeritic studies, Invitro dissolution studies and Antitubercular activity.

The crystal structure determination of Crystal form 1 reveals that the two molecules (isoniazid and para amino salicylic acid) are associated via a carboxylic acid-pyridine hydrogen bond heterosynthon.
IR, DSC and SEM support the formation of co-crystal between isoniazid and para amino salicylic acid at 1:1 stoichiometric ratio. From the pharmaceutical characterization, Crystal form 1 shows 83% of practical yield. It was showing decreased solubility in 0.1N HCl but increased solubility in pH 6.8 phosphate buffers when compared to 0.1N HCl.

From micromeritic studies of Crystal form 1 show, decreased bulk (0.4) and tapped (0.5) densities, when compare with isoniazid bulk (0.64) and tapped (0.84) densities. Decrease of angle of repose value of Crystal form 1 (28.39) indicates good flow, this is supported by Carr's Index (0.20) and Hauser Ratio (1.25), than isoniazid, angle of repose (32.37), Carr's Index (0.23) and Hauser Ratio (1.31).

From Invitro dissolution studies it shows decrease in its dissolution profile, with 30.41% at the end of 60 min where pure drug has 100% drug release. But it has more Invitro antitubercular activity when compare with pure drug. However, the activity of Crystal form **I** is effective in antitubercular activity compared to that of the pure drug.

2. Crystal form II: INH-PAS (1:1) co-crystal (solvent drop method)

Crystal form II (1:1 molar ratio, Co-crystal) was prepared by solvent drop method and confirmed by comparison of the melting points with those of the starting materials. It characterized in terms of SEM, FTIR, DSC, and XRD subjected to Preliminary pharmaceutical characterization such as solubility, micromeritic studies, Invitro dissolution studies and Antitubercular activity.

The crystal structure determination of Crystal form II reveals that the two molecules (isoniazid and para amino salicylic acid) are associated via a carboxylic acid-pyridine hydrogen bond heterosynthon.
IR, DSC and SEM support the formation of co-crystal between isoniazid and para amino salicylic acid at 1:1 stoichiometric ratio. From the pharmaceutical characterization, Crystal form II shows 74.8% of practical yield. It was showing decreased solubility in 0.1N HCl but drastic increase in the solubility in pH 6.8 phosphate buffer when compared to 0.1N HCl.

From micromeritic studies of Crystal form II show, similar bulk (0.6) and tapped (0.85) densities, when compare with isoniazid bulk (0.64) and tapped (0.84) densities. Increase of angle of repose value of Crystal form II (43.4) indicates poor flow, this is supported by Carr's Index (0.136) and Hauser Ratio

(1.15), than isoniazid, angle of repose (32.37), Carr's Index (0.23) and Hauser Ratio (1.31).

From Invitro dissolution studies it shows decrease in its dissolution profile, with 41.67% at the end of 60 min where pure drug has 100% drug release. But it has more Invitro antitubercular activity when compare with pure drug. However, the activity of Crystal form II is effective in antitubercular activity compared to that of the pure drug.

3. Crystal form III: INH-PAS (1:1) co-crystal (co-grinding method)

Crystal form 1 (1:1 molar ratio, Co-crystal) was prepared by co-grinding method and confirmed by comparison of the melting points with those of the starting materials. It characterized in terms of SEM, FTIR, DSC, and XRD subjected to Preliminary pharmaceutical characterization such as solubility, micromeritic studies, Invitro dissolution studies and Antitubercular activity.

The crystal structure determination of Crystal form III reveals that the two molecules (isoniazid and para amino salicylic acid) are associated via a carboxylic acid-pyridine hydrogen bond heterosynthon.

IR, DSC and SEM support the formation of co-crystal between isoniazid and para amino salicylic acid at 1:1 stoichiometric ratio. From the pharmaceutical characterization, Crystal form III shows 96% of practical yield. It was showing decreased solubility in 0.1N HCl but drastic increase in the solubility in pH 6.8 phosphate buffer when compared to 0.1N HCl.

From micromeritic studies of Crystal form III show, increase bulk (2.0) and tapped (2.54) densities, when compare with isoniazid bulk (0.64) and tapped (0.84) densities. Increase of angle of repose value of Crystal form III (43.6) indicates poor flow, this is supported by Carr's Index (0.27) and Hauser Ratio (1.37), than isoniazid, angle of repose (32.37), Carr's Index (0.23) and Hauser Ratio (1.31).

From Invitro dissolution studies it shows decrease in its dissolution profile, with 52.67% at the end of 60 min where pure drug has 100% drug release. But it has more Invitro antitubercular activity when compare with pure drug. However, the activity of Crystal form III is effective in antitubercular activity compared to that of the pure drug.

The design of new multicomponent crystal phases of APIs with desired physicochemical properties by applying crystal engineering is an evolving novel concept. Capability to design new multicomponent crystal structures will depend mostly on supramolecular chemistry and on viewing a crystal structure with interactions of various types and strengths. Crystal engineering approach involves identification of interactions or supramolecular synthons that will covers an entire family of structures with the object of identifying a set of new crystal phases of API.

The development of new supramolecular complexes, co-crystal and polymorphs of drugs by crystal engineering is becoming progressively more important as an alternative to salt formation, mainly for neutral or weakly ionizable compounds. Even though lack of priority in marketed products and concerns about the safety and toxicity of co-crystal forming agents.

This concept was applied to the co-crystallization of isoniazid with para amino salicylic acid (PAS) by solvent evaporation, solvent drop and co-grinding assisted crystallization. The carboxylic acid-pyridine hydrogen bond has again been used to successfully create a new pharmaceutical co-crystal of isoniazid, Crystal form 1, 2, 3 with para amino salicylic acid (PAS). The supramolecular interaction of isoniazid (pyridine ring) with carboxylic acid of PAS resulted in genuine co-crystals. The crystallization of isoniazid with para amino salicylic acid (PAS) is in 1:1 ratio.

These prepared crystal forms I resolve the poor micromeritic problems of isoniazid and shows improved flow and compaction property than isoniazid. From the anti-tubercular test performed it has confirmed that the co-crystal forms of methyl paraben and INH (S9) had shown increased anti-tubercular activity compared to the pure drug shown increase in the anti-tubercular activity.

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