British Journal of Pharmaceutical Research
4(14): 1781-1801, 2014

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Segregated Delivery of Rifampicin and Isoniazid from Fixed Dose Combination Bilayer Tablets for the Treatment of Tuberculosis

A. M. Silva[1], B. Abrahim-Vieira[1], F. A. do Carmo[1], L. H. do Amaral[1], L. C. Silva[1], C. S. Escudini[1], M. A. Lopes[2], V. P. Sousa[1], H. C. Castro[3], F. Veiga[2], C. R. Rodrigues[1], A. J. Ribeiro[2] and L. M. Cabral[1*]

[1]Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro 21941-902, Rio de Janeiro, Brazil.
[2]Centre for Pharmaceutical Studies, Faculty of Pharmacy, University of Coimbra, Azinhaga de Coimbra, 3000-548 Coimbra, Portugal.
[3]Institute of Biology, Federal University of Fluminense, Niterói 24210-130, Rio de Janeiro, Brazil.

Authors' contributions

This work was carried out in collaboration between all authors. Authors AJR, VPS, HCC, FV, CRR and LMC designed the study, authors AMS, BAV, FAC, LHA, LCS, CSE and MAL performed the experiments and statistical analysis, authors AMS and BAV wrote the protocol, authors FAC and LCS wrote the first draft of the manuscript. Authors AMS and BAV managed the analyses of the study. All authors read and approved the final manuscript.

Original Research Article

Received 20th May 2014
Accepted 4th July 2014
Published 19th July 2014

ABSTRACT

Aims: Develop an anti-tuberculosis (TB) Fixed Dose Combination (FDC) tablet containing an immediate release layer (IRL) composed of both rifampicin (RIF) and pyrazinamide (PYR) and a retarded release layer (RRL) comprised of isoniazid (INH) which would allow segregated delivery of RIF and INH.
Study Design: Trials were conducted on the pre-formulations and formulations to assess the compatibility of excipients and obtain a modified release profile, for an IRL consisting of both RIF and PYR and a RRL containing INH.
Place and Duration of Study: This study was performed at the Laboratory of Pharmaceutical Industrial Technology, Drug and Pharmaceutical Department, Faculty of

**Corresponding author: Email: lmcabral@pharma.ufrj.br;*

Pharmacy, between March 2008 and April 2010.
Methodology: Preformulation studies were performed on RIF and PYR, alone and in combination with excipients. The pharmacopeic attributes of the distinct layers and the FDC tablets were evaluated for hardness, friability and disintegration time. The FDC bilayer tablets were analyzed for their drug content and cumulative dissolution of the drug.
Results: Fourier transform infrared, x-ray diffraction and differential scanning calorimetry results revealed the presence of amorphous and crystalline RIF forms and no potentially relevant incompatibilities were identified in the kneaded system containing RIF, PYR and excipients. In vitro drug release from the FDC tablets revealed that the intragranular addition of croscarmellose sodium into the IRL rendered a cumulative dissolution of RIF and PYR within the limits of simulated gastric fluid. And, for RRL, the most effective retardant matrix excipient was found to be hydroxypropyl methylcellulose.
Conclusion: Segregated delivery of RIF and INH from bilayer tablets is an option for the development of immediate release FDC tablets and the retarded release of INH, this strategy proved suitable for preventing contact of these two drugs under acidic conditions. This finding suggested that RIF had a high in vivo bioavailability which qualifies this FDC for future bioavailability studies.

Keywords: Tuberculostatic drugs; bilayer tablet; drug interactions; fixed dose combination; segregated release.

1. INTRODUCTION

Tuberculosis (TB) is caused by species of the *Mycobacterium tuberculosis* complex, which is one of the world's most debilitating and deadly pathogens [1-3] and remains an infectious disease threat with an enormous impact on global public health [4, 5]. It is estimated that 9 million new TB cases occur per year and, in 2012, 6.1 million cases of TB were reported by the National Tuberculosis Control Programme (NTP) and to the World Health Organization (WHO) [6].

A combination of first-line anti-TB drugs can dramatically reduce mortality rates. Currently, the regimen recommended by World Health Organization (WHO) for the treatment of new cases of drug-susceptible TB is highly effective and combines three or four different first-line drugs: rifampicin (RIF), isoniazid (INH), ethambutol (ETB) and pyrazinamide (PYR). The treatment regimen lasts for 6 months, the initial treatment lasts two-months and involves the simultaneous administration of these three drugs, RIF, INH and PYR, followed by administration of RIF and INH for four months [2,3,7]. Anti-TB drugs can be administered as single-drug formulations or as fixed dose combination (FDC) formulations, combining two or more drugs in fixed proportions within the same formulation. Considering the severity and the spread of disease, WHO implemented the use of FDC as part of the control strategy to ensure that TB was adequately treated [4,8,9].

Strict adherence to TB treatment is important to assure successful recovery. This reduces the risk of drug resistance and also helps reduce the possibility of further TB transmission [9, 10]. FDC improves medication compliance since it combines two or more necessary drugs into a single dosage form, assuring that the correct drug dosages are delivered [4,11,12]. The combination of drugs also improves the therapeutic effect, safety and patient compliance compared to separate administration of the drugs [12,13]. However, concerns exist regarding the quality of FDC tablets, and reservations concerning the therapeutic

effectiveness of combined dosing strategies [11,14], namely the increased risk of interactions amongst simultaneously administered drugs which could compromise their bioavailability. Furthermore, RIF-containing formulations have demonstrated varied bioavailability, which could result from alterations in crystalline forms [15,16], drug-drug interactions, drug-excipient interactions and decomposition of drugs in formulations [16]. For example RIF in the FDC exhibits polymorphism and alterations in its crystalline form when INH and PYR are present and/or during the pharmaceutical development of tablets. These factors are assumed responsible for the variable bioavailability of RIF and contribute to its instability in the acidic environment of stomach [11,15-23].

The susceptibility of the drugs to decomposition within the FDC formulation occurs in this order: RIF > INH > PYR [16]. Moreover, the degradation of these drugs during the gastrointestinal passage may also be a concern since *in situ* gastric studies have revealed that INH has a negative effect on RIF's stability. Therefore, the incompatibility of these two anti-TB drugs should be taken into account while designing a delivery system.

Although there are some tuberculostatic FDC tablets available in the global market that employ innovative technology, i.e. the core is composed of INH and coated with special polymers for site-specific release, avoiding contact with RIF, which is known to adverse interact with INH. Therefore, incorporation of RIF into the outer layer helps avoid the unfavorable interactions, however these types of adjustments can lead to higher production costs for formulations [24].

Hydroxy propyl methylcellulose (HPMC) based monolithic hydrophilic matrix tablets that contain PYR, RIF and INH could provide sufficient initial release and extend release for up to 24 h for two of the drugs. It has been proposed that a segregated delivery system in which RIF is released in the stomach and INH is released in the intestine could be developed [17,18,25]. Thereby, a bilayer tablet which is produced with a reduced number of unit processes and using low-cost manufacturing process may provide a new approach for developing effective tuberculostatic FDC tablets.

However, designing formulations and developing formulation processes for FDC tablets can be challenging and requires chemical compatibility studies for the drugs and excipients used [26]. Variations in formulation processing have been shown to significantly influence drug release, namely the intra or extra granular addition of RIF into tablets, can significantly impact release profiles of both RIF and INH from hydroxy propyl methylcellulose formulations [27].

The goal of this study was to investigate the feasibility of formulating an anti-TB FDC tablet containing an immediate release layer (IRL) composed of both RIF and PYR and a retarded release layer (RRL) composed soley of INH, which would allow the segregated delivery of RIF and INH (Fig. 1). Furthermore, to ensure that the FDC is of good quality, preformulation studies were performed on RIF and PYR, alone and also in combination with the excipients. FDC tablets were submitted to physical tests and the *in vitro* release of drugs was assessed using a cumulative dissolution test. The results obtained were compared to similar, immediate release FDC tablets currently on the market.

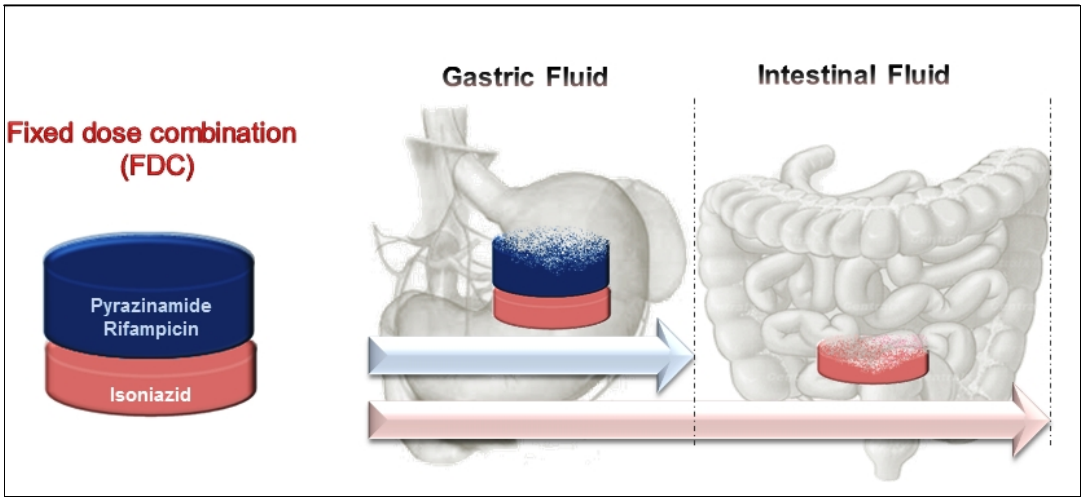

Fig. 1. Graphical abstract depicting the FDC strategy utilized in this study

2. MATERIALS AND METHODS

2.1 Materials

Pyrazinamide (PYR), rifampicin (RIF) and isoniazid (INH) were supplied by Calyx Chemicals (Mumbai, India), Shenyang Meiyao Chemical Co. Ltd. (Shenyang, China) and G. Amphray Laboratories (Bhiwandi, India), respectively. All primary and secondary standards were obtained from the United States Pharmacopeia (USP). The commercially available FDC tablets were obtained from local chemists and contained 150 mg RIF, 75 mg INH and 400 mg of PYR. Sodium docusate (SD) was purchased from Aldrich Chemical Company (USA); sodium metabisulfite (SMB), shellac (gum lac) (GL) and sodium lauryl sulfate (SLS) were purchased from Vetec (Rio de Janeiro, Brazil); cellulose acetate phthalate (CAP) from Fluka Analytical (USA); microcrystalline cellulose 102 (MCC-102) from Blanver (São Paulo, Brazil); croscarmellose sodium (CS) from FMC Biopolymer (USA); colloidal silicon dioxide (Aerosil®200) and Eudragit® L100 and S100 from Degussa Rohm Pharma Polymers (Darmstadt, Germany); magnesium stearate (MS) from AMC-Brazil (São Paulo, Brazil); hydroxypropyl methylcellulose (HPMC) from Shin-Etsu Chemical Co. Ltd (Tokyo, Japan); polyvinylpyrrolidone (PVP) K30 from Boai New Kaiyuan Pharmaceutical Com. Ltda (Henan, China); Viscogel® B8 from Bentec (Livorno, Italy). All solutions were prepared using MilliQ purified water and all other reagents utilized were of analytical grade.

2.2 Methods

2.2.1 Drugs characterization

PYR, RIF and INH were characterized using powder X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC).

Powder X-ray diffraction patterns were obtained on a Rigaku Miniflex diffractometer (Rigaku, Tokyo, Japan) equipped with a Cu-Kα radiation source (λ = 0.1542 nm) operating at 40 kV and 30 mA at room temperature. The diffraction angle (2θ) of the patterns was recorded

between 2º and 60º employing a scan rate of 0.05º.s^{-1}. FTIR spectra were collected an IR Pre utilizing a stige-21 Shimadzu (Shimadzu, Kyoto, Japan) spectrometer, 1% KBr pellets with a wavenumber between 4000 and 400 cm^{-1}. DSC analysis were performed between 35 to 500ºC with a Shimadzu DSC-60 thermal analyzer (Shimadzu, Kyoto, Japan) using partially closed aluminum pans, nitrogen flow of 50 mL.min^{-1} and a heating rate of 15ºC.min^{-1}. Approximately 3 mg of sample was used for DSC analysis, and the instrument was calibrated using indium and zinc standards [23-28].

2.2.2 Compatibility studies

Compatibility studies were performed to assess the physical mixture of RIF and PYR in a kneaded system (PVP 10% w/v in ethanol), these systems were also assessed when combined with the pharmaceutical excipients. The specific amounts and the methods used in preparation of the samples to be analyzed by XRD, FTIR and DSC are displayed in (Table 1). Prior to the analysis, samples were dried at 40ºC for 1 h and after packaging in amber glass bottles. Next, they were submitted to an accelerated stability test in a climatic chamber 420CLD-150 (Nova Ética, São Paulo, Brazil) at 40ºC±2ºC/75% RH ±5% RH for 90 days.

Table 1. Qualitative and quantitative composition of samples (A-E) used in the compatibility/stability studies as well as their preparation methods

Samples		A	B	C	D	E
Drug (mg)	RIF[a]	10.0	10.0	150.0	150.0	150.0
	PYR[b]	10.0	10.0	400.0	400.0	400.0
Excipients(mg)	SMB[c]	-	-	-	55.0	55.0
	SD[d]	-	-	55.0	-	55.0
Preparation method		Kneaded system	Physical mixture	Kneaded system	Kneaded system	Kneaded system

[a] rifampicin; [b] pyrazinamide; [c] sodium metabisulfite; [d] sodium docusate

2.2.3 Tablet formulation

2.2.3.1 Immediate release layer (IRL)

RIF and PYR were included in the IRL by varying the amount of microcrystalline cellulose (MCC-102) and croscarmellose sodium (CS) included in either the internal or external phases, as described in (Table 2). The internal and external phases contained 3.6 and 7.2 mg of SMB and MS, respectively. The FDC tablets were produced using the wet granulation method. The granulation agent was an ethanolic solution of 2.0% PVP K30 (w/w) and 0.36% sodium docusate (w/w). After drying in the stove (MARCONI MA035/5, São Paulo, Brazil) at 40ºC for 1 hour, granules were obtained with a moisture content of less than 3.0%. The granules were passed through a 10-mesh sieve and mixed with the external phase in a conical mixer (Wan of Brazil, São Paulo, Brazil). For evaluation of the IRL, all combinations to be tested were compacted into tablets weighing about 720 mg containing 150 mg RIF and 400 mg PYR in an eccentric tablet press (FABBE, São Paulo, Brazil) using 10 mm diameter biconcave punches.

Table 2. Qualitative and quantitative composition of immediate release layer (IRL) formulations produced by wet granulation. The granulation solution contained 20 mg polyvinylpyrrolidone (PVP- K30) and 3.6 mg sodium docusate dissolved in ethanol

Composition	Immediate release layer (IRL)		
	1	2	3
Internal phase (mg)			
RIF[a]	150.0	150.0	150.0
PYR[b]	400.0	400.0	400.0
MCC-102[c]	114.0	117.6	-
CS[d]	21.6	-	7.2
External phase (mg)			
MCC-102[c]	-	-	117.6
CS[d]	-	14.4	7.2
Aerosil®	-	3.6	3.6

[a] Rifampicin ; [b] Pyrazinamide; [c] Microcrystalline cellulose; [d] Croscarmellose sodium. Internal and external phases contained 3.6 and 7.2 mg of sodium metabisulfite and magnesium stearate, respectively. The total weight of each FDC was 720.0 mg

2.2.3.2 Retardant release layer (RRL)

INH was included in the RRL in combination with different excipients (CAP, Eudragit® L100, Eudragit® S100, Viscogel® B8, GL and HPMC) which act as retardant matrix agents. The effect of formulation parameters on tablets physical resistance were studied including: the preparation method (direct compression or wet granulation), the matrix composition and its mass ratio with INH. Microcrystalline cellulose (MCC-102) and magnesium stearate (MS) were added as the filler and lubricant, respectively. All FDC tablets were submitted to direct compression except those containing CAP and GL, which were prepared using the wet granulation method. The wet granulation solutions used to prepare CAP 1% w/v and GL 2% w/w, were ethanol-acetone (1:1) (v/v) and ethanol-water (1:1) (v/v), respectively. Granules were dried and calibrated using the same procedure described for preparation of IRL. The final mixtures were compressed into tablets using an eccentric tablet press equipped with 10 mm diameter biconcave punches, the tablets weighed about 260 mg and contained 75 mg of INH.

2.2.4 Preparation of FDC tablets

Distinct FDC bilayer tablets were developed containing combinations of IRL with (RIF and PYR) and RRL combined with (INH). Both layers were compressed to obtain bilayer tablets using an eccentric tablet press that was equipped with a 10 mm diameter biconcave punch. The IRL (720 mg) was initially pre-compressed to a low hardness state and then the RRL (260 mg) was compressed over it until the desired hardness was achieved to produce a bilayer tablet. Thus, the total mass of the bilayer FDC tablet was 980 mg. A control tablet was prepared without HPMC (HPMC was replaced by MCC) using the same process.

2.2.5 Evaluation of tablets

2.2.5.1 Physical tests

The FDC tablets were subjected to different physical characterization studies. Tablet hardness studies were performed using an automatic hardness tester Nova Ética 298 ATTS

(Nova Ética, São Paulo, Brazil) with included a sample of ten tablets. The minimum hardness acceptance criterion adopted for this study was 30 N. The tablet's friability was determined using a Sotax F1 friabilator (SOTAX, Allschwil, Switzerland). Tablet disintegration was determined for the IRL and RRL layers and the FDC tablets separately using the Nova Ética 301-AC disintegration tester (Nova Ética, São Paulo, Brazil). IRL disintegration was performed in simulated gastric fluid (SGF) without pepsin at 37±0.5ºC for 30 minutes. The SGF was prepared by dissolving 6.0 g of sodium chloride into 21.0 mL of a hydrochloric acid: water mixture and quiescing to a total volume of 100 ml. This solution has a pH of about 1.2. Disintegration of the RRL and FDC tablets was measured in SGF at 37±0.5ºC for 30 minutes and then in simulated enteric fluid (SEF) at 37±0.5ºC for 45 minutes. The SEF was prepared by dissolving 0.85 g of sodium phosphate dibasic anhydrous into 600 mL of water and adjusting the pH to 6.8 using phosphoric acid [28].

2.2.5.2 Analysis of drug content

The FDC tablets were analyzed to determine RIF, PYR and INH content using a gradient HPLC method. Analysis was performed according to United States Pharmacopeia XXXIV (USP 34) using a YL9100 HPLC (Young Lin Clarity, Anyang, Korea) equipped with a YL9110 pump, PDA detector YL9160 and auto sampler YL9150. Separation was performed on a C8 column, 250 x 4.6 mm, 5 µm (Merck Hitachi Column, Dartford, UK). Several mobile phases were prepared: mobile phase A contained phosphate buffer pH 6.8 and acetonitrile in a ratio of (96:4) (v/v), whereas mobile phase B contained phosphate buffer pH 6.8 and acetonitrile in a ratio of (55:45) (v/v). Initially, mobile phase A was run through (5 min at 100%) followed by a 1 min linear gradient that transitioned to 100% mobile phase B, followed by 10 min flow of 100% mobile phase B. After this, a 1 min gradient was performed that ended at 100% mobile phase A with an additional 10 min of 100% mobile phase A..The flow rate was 1.5 $ml.min^{-1}$ at 20ºC. The detector was set at 238 nm and the injection volume used was 20 µl for all samples and standards. Standard solutions were prepared by dissolving the USP standards in 100 mL of phosphate buffer solution (0.01M, pH 6.8) containing 10 mL of methanol to final concentrations of 160, 80 and 430 µg/mL of RIF, INH and PYR, respectively. Prior to HPLC analysis, twenty of each FDC tablets were weighed and pulverized to powder. A small powdered sample (104.5 mg) was extracted using 10 mL of methanol in an ultrasound bath for 5 minutes and then the final volume (100 ml) was obtained using phosphate buffer solution (0.01M, pH 6.8). The solution was filtered through a 0.45 µm pore membrane [28].

2.2.5.3 Dissolution test

The dissolution tests were performed in a SR8-Plus Dissolution Tester (Hanson Research Corp., Chatsworth, CA, USA) using apparatus I for dissolution (baskets). According to USP 34, 900mL of SGF and SEF were used as dissolution medium. The stirring speed was set at 100 rpm for 30 minutes in the SGF medium and 45 minutes in the SEF medium, both at 37±0.5 ºC. The concentration of RIF released from the FDC tablets was determined by UV spectrophotometry (model number 2401PC, Shimadzu Corporation, Kyoto, Japan at a wavelength of 475 nm using the dissolution medium to set the zero point. The concentrations of INH and PYR in the FDC tablets were determined using HPLC as described hereafter. Release of each drug was calculated to determine their cumulative dissolution after 30 and 45 min, in SGF and SEF, respectively. Values were compared multiple-pair wise using a 95% confidence interval. For these ANOVA based methods, SPSS 8.0 for Windows (SPSS, Chicago, IL) was utilized [28].

3. RESULTS AND DISCUSSION

3.1 Preformulation Studies

3.1.1 Solid-state characterization of RIF, PYR and INH

The XRD pattern, depicted in (Fig.2), revealed that RIF exists in two polymorphic forms as was previously reported [15, 23]. The RIF sample exhibited the characteristic peaks typical of form II at 9.93 and 11.1° (2θ angles) [23]. The presence of form I and amorphous forms could also be observed in the peaks of form I (13.65 and 14.35° 2θ) and also by the reduced intensity of peaks and the amorphous forms were evident since they led to the appearance of a halo at the baseline.

The XRD pattern of PYR showed its well-defined crystalline structure, with peaks appearing at 7.90, 15.40 and 17.70º (2θ angles) which are characteristic of form α [29-30]. Due to its crystalline nature, the XRD results of INH showed multiple peaks at 15.6º, 16.8º, 19.6º and 25.2º (2θ angles) [31-33].

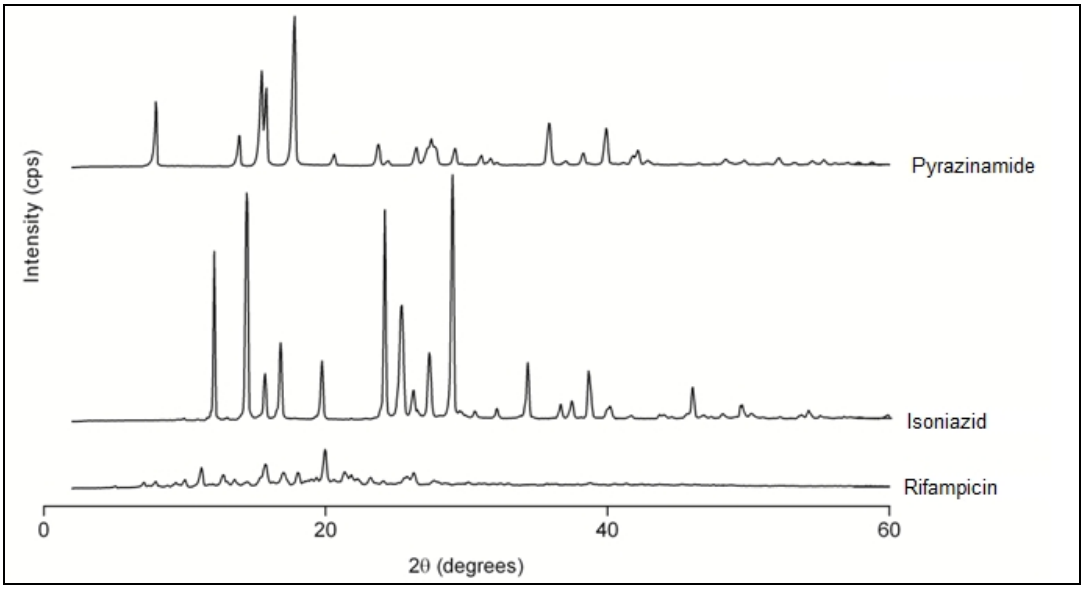

Fig. 2. XRD patterns of PYR (pyrazinamide), INH (isoniazid) and RIF (rifampicin)

The DSC curves are represented in (Fig. 3). The RIF curve exhibited the typical sequence of events seen for form II, with the melting endotherm at 185.6ºC, recrystallization to form I (exotherm, range 197-203ºC) and finally form I decomposition in the range of 255-266ºC [23]. The PYR sample appeared as an endothermic process, while the characteristic melting temperature ranged between 188-191ºC, with a melting peak at 189.1ºC. Whereas, INH had an endothermic event, characteristic of melting at 170ºC with endothermic decomposition occurring in the range of 237 to 264ºC. Around 150ºC, there was an additional weak endothermic peak which could be associated with a polymorphic phase transformation, from form α to form γ and an enantiotropic relationship between the phases involved [29,30]. The

last peak, which appeared within the range of 209 to 237ºC was due to the final thermal decomposition of PYR [29].

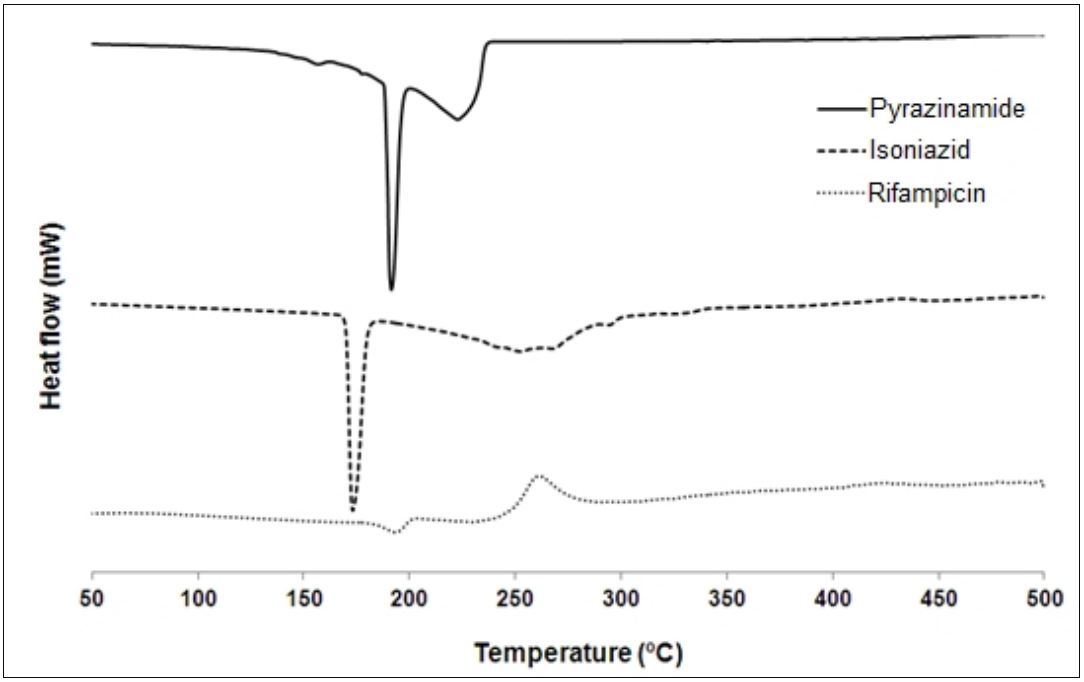

Fig. 3. The DSC curves of PYR (pyrazinamide), INH (isoniazid) and RIF (rifampicin)

The FTIR spectra are depicted in (Fig. 4). It was found that RIF exhibited a broadened band due to OH at 3446 cm^{-1} which was characteristic of RIF form II and amorphous forms. The double peak was due to furanone and acetyl (C=O) and was observed at 1716 and 1734 cm^{-1} which indicated the presence of form II [15,23]. The characteristic PYR bands were observed at 3412 cm^{-1} due to the (NH_2 asymmetric stretch), 3280 and 3153 cm^{-1} (NH_2 symmetric stretching), 1710 cm^{-1} (C=O stretching, amide), 1608 cm^{-1} (NH_2 in plane bending, amide), 1577 and 1523 cm^{-1} (ring stretching) and 1377 cm^{-1} (C-N stretching, amide). These peaks also indicated the presence of the α form [29,34,35]. The FTIR spectra of INH showed characteristic bands at 3304 cm^{-1} (NH_2 asymmetric stretching), 3217 cm^{-1} (NH stretching), and 3147 cm^{-1} (NH_2 symmetric stretching), 3111 cm^{-1} (CH stretching), 1664 cm^{-1} (C=O stretching), 1636 cm^{-1} (hydrazide/ring group CN asymmetric stretching), 1602 and 1557 cm^{-1} (ring CN symmetric stretching/H-N-N bending) [32,33].

Therefore, in the present investigation, the characterization of XRD, DSC and FTIR indicated the presence of the stable PYR α polymorph. Four different PYR polymorphs (α, β, δ, ɣ) have already been detected [36,37], and at higher pressures ~ 4GPa it is possible to observe the transition from the ɣ to the β form. However, the same was not observed for forms α and δ when tested up to 14 GPa. A mixture of forms I, II and amorphous RIF forms was found, as previously reported, the predominant form found was the thermodynamically unstable form II. And further, form II of RIF had a lower intrinsic dissolution rate, at pH 2,0, compared to form I [15,23].

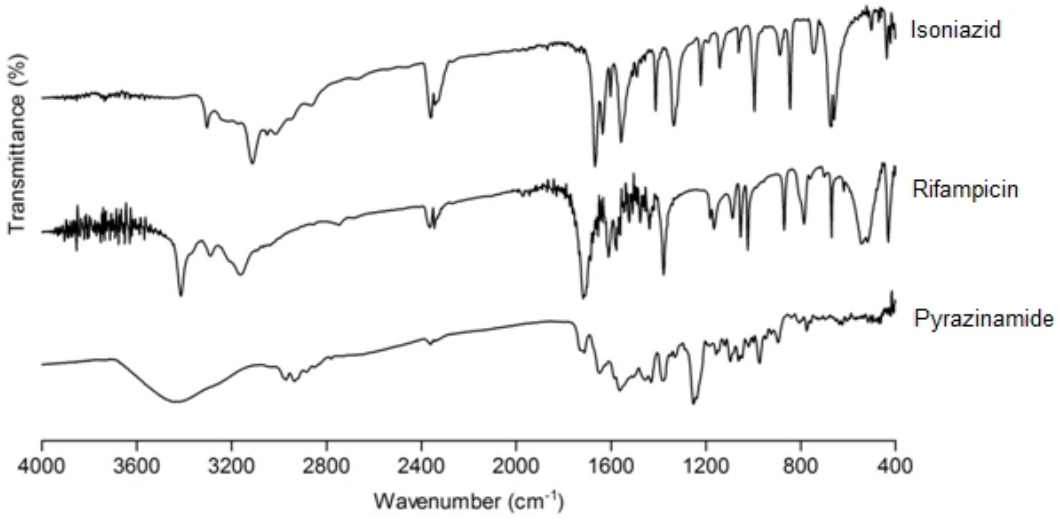

Fig. 4. The FTIR spectra of INH (isoniazid), RIF (rifampicin) and PYR (pyrazinamide)

3.1.2 Compatibility studies

Prior to the pharmaceutical development of the IRL, several trials were performed to assess interactions of RIF and PYR, with the excipients SMB and SD using the physical mixture or kneaded system preparation methods [16, 36]. Samples were analyzed by XRD, DSC and FTIR before and after the 90-day stability stress test. The results are shown in (Figs. 5, 6 and 7).

In general, the XRD pattern featured predominantly PYR signals, while RIF signals were not as pronounced, as shown in (Fig. 5). This could have been due to the greater crystalline purity of PYR compared to RIF, which exists in the sample as a mixture of crystalline and amorphous forms. Comparisons between the RIF and PYR kneaded system (sample A) and physical mixture (sample B) showed a decrease or disappearance of certain diffraction peaks associated with RIF (8.5º, 9.9º and 11.1º) after the kneading process. These changes can be attributed to the amorphization effect or RIF's decomposition which was probably due to the kneading process.

After the stability test, the RIF and PYR physical mixture showed no diffraction peaks that could be attributed to degradation phenomena and both drugs maintained their crystallinity. After 90 days the ethanolic kneaded RIF and PYR system, had a diffractogram (A-T_f) that had lower intensities or the disappearance of peaks which is closely related to RIF and also due to the amorphization effect. The addition of SD and/or SMB to RIF and PYR (samples C-E), did not significantly alter the XRD pattern, even after 90 days.

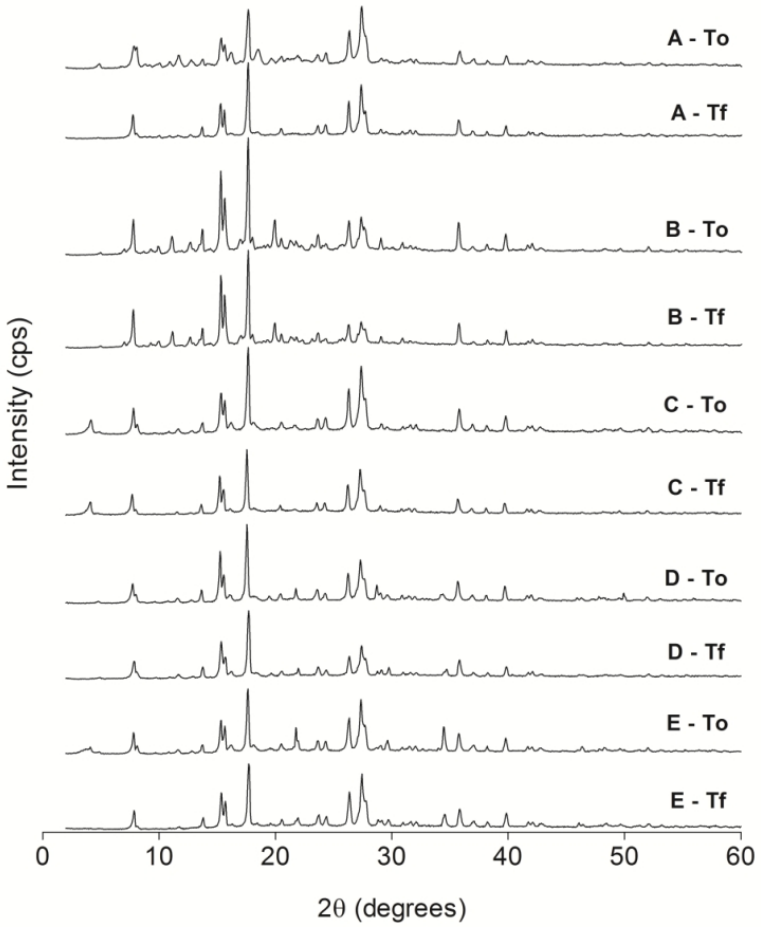

Fig. 5. Analysis of XRD patterns in the comparative study of the stability of the kneaded system of RIF-PYR (A); physical mixture of RIF-PYR (B); after ethanolic kneading of RIF-PYR-SD (C); after ethanolic kneading of RIF-PYR-SMB (D); after ethanolic kneading of RIF-PYR-SD-SMB (E). The initial analysis of T_o – through the analysis of T_f – in climatic a chamber at 40±2ºC / 75±5% RH after 90 days of stabililty testing

The FTIR spectra of the physical mixture (B) and the kneaded systems are shown in (A, C-E). As can be seen in (Fig. 6), there was no evidence of undesirable interactions nor degradation after 90 days.

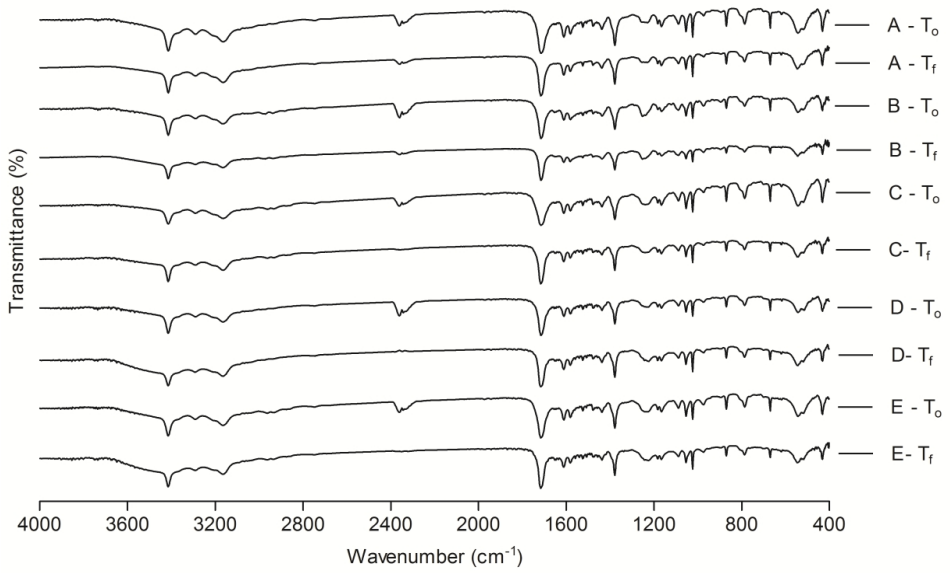

Fig. 6. The FTIR spectra generated during the comparative study examining the stability of the physical mixture obtained by ethanolic kneading of RIF-PYR (A); the physical mixture of RIF-PYR (B); ethanolic kneading of RIF-PYR-SD (C); ethanolic kneading of RIF-PYR-SMB (D); ethanolic kneading of RIF-PYR-SD-SMB (E). T_o – initial analysis and T_f – analysis in a climatic chamber at 40±2ºC / 75±5% RH after 90 days

The DSC curves can be seen in (Fig. 7), and showed that the first endothermic event occurred between 180 and 200 °C which corresponded to the RIF and PYR melting point and a second endothermic peak which was due to the thermal decomposition of PYR [29]. Thermograms of the RIF and the PYR kneaded system (A) and physical mixture (B) exhibited a small and broad exothermic peak after 200 ºC which corresponded to the decomposition of RIF. After the stability test, the physical mixture ($B–T_f$) showed no evidence of degradation, however the thermogram of the kneaded system ($A–T_f$) showed the appearance and shifting of some endothermic peaks and the disappearance of the exothermic peak between 200°C and 250°C. These spectral changes indicated degradation of RIF.

DSC curves obtained for RIF and PYR ($A–T_0$) and RIF, PYR and SD ($C–T_0$) exhibited broadening and shifting of the first endothermic event from 187.4ºC for sample A to 181.6ºC for sample B. And also after 90 days, visible signs of degradation were evident which indicated instability of the mixture.

The results revealed that no incompatibilities existed between RIF, PYR and SD. Also, the RIF, PYR and SMB mixture ($D–T_0$) did not exhibit undesirable interactions nor any degradation after 90 days. These two findings suggest a drug-excipient compatibility between RIF, PYR and SMB. Based on this analysis, SMB was found to be compatible with RIF and PYR, therefore it can be used in the FDC as an antioxidant. Although sample C has shown the potential for incompatibility between drugs and SD, when RIF and PYR were combined with both SD and SMB excipients together (sample E), no significant incompatibilities were found by DSC. Thus IRL was prepared using both excipients, SD in the granulation ethanolic solution and SMB in the internal phase. The contents of all drugs

were analyzed, and all the samples studied were within the limits specified by their respective pharmacopoeic monographs (data not shown).

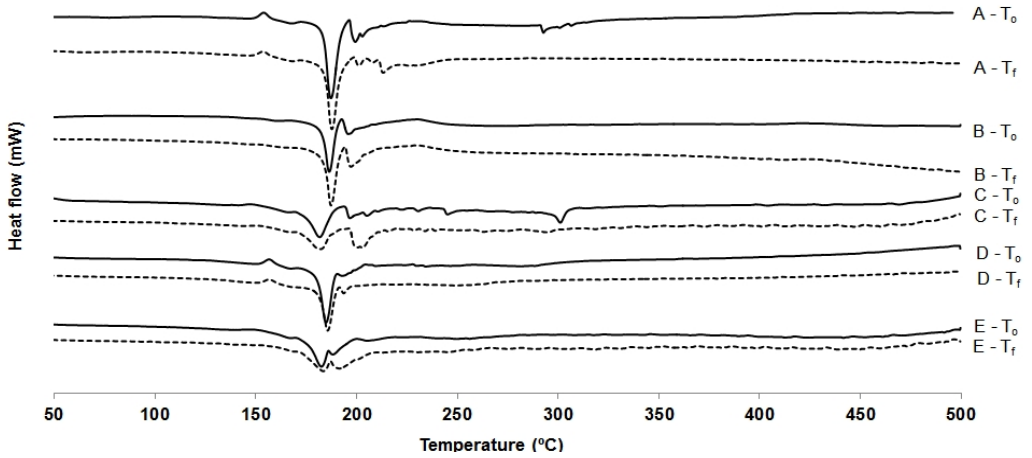

Fig. 7. The DSC curves obtained during the comparative study assessing stability of systems produced by ethanolic kneading of RIF-PYR (A); the physical mixture of RIF-PYR (B); ethanolic kneading of RIF-PYR-SD (C); ethanolic kneading of RIF-PYR-SMB (D); ethanolic kneading of RIF-PYR-SD-SMB (E). T_o – initial analysis and T_f – analysis in climatic chamber at 40±2ºC / 75±5% RH after 90 days

3.2 Tablet Evaluation

3.2.1 Immediate release layer of the tablet – RIF and PYR

The hardness and disintegration time of IRL in SGF were determined, the results obtained are shown in (Table 3).

When the FDC contained both microcrystalline cellulose and croscarmellose sodium (IRL-1 and IRL-2) much better disintegration results were obtained (less than 20 minutes), with similar hardness and friability. Thus, IRL-1 and IRL-2 were selected for the development of the FDC tablets.

3.2.2 Retardant release layer of the tablet – INH

All formulations exhibited satisfactory hardness and friability values according to USP 34. Despite the potential of Eudragit®L100, Eudragit®S100, Viscogel®B8 and GL as excipients for retardant release, their inclusion in the RRL did not increase disintegration time. Most of the RRL containing these excipients disintegrated in the SGF within a few seconds (data not shown). As can be seen in (Table 4), a longer disintegration time or even no disintegration could be obtained for an RRL that contained either CAP or HPMC as retardant matrix agents. The INH/HPMC ratios of at least 1.00/0.60 were effective in protecting the tablet's structure in SGF.

Table 3. Results of physical tests of the immediate release layer (IRL) containing RIF (150 mg) and PYR (400 mg) in the internal phase

Composition	Immediate release layer (IRL)		
	1	2	3
Internal Phase (mg)			
MCC-102[a]	114.0	117.6	-
CS[b]	21.6	-	7.2
External Phase (mg)			
MCC-102[a]	-	-	117.6
CS[b]	-	14.4	7.2
Aerosil®[c]	-	3.6	3.6
Physical tests			
Hardness (N)[d]	96.04±2.10	95.06±1.30	98.98±1.70
Friability (%)[e]	0.32±0.01	0.23±0.01	0.24±0.01
Disintegration time in SGF[f] (min)	19.0	12.0	22.0

[a] Microcrystalline cellulose; [b] Croscarmellose sodium; [c] colloidal silicon dioxide; [d] Results are expressed as mean ± standard deviation for n = 10; [e] Results are expressed as mean ± standard deviation for n = 20; [f] Simulated gastric fluid. The internal and external phases contained 3.6 and 7.2 mg of sodium metabisulfite and magnesium stearate, respectively

The FDC made using CAP as the retardant excipient (INH/CAP ratio of 1.00/0.50) was also effective in protecting the formulations. However, the FDC made with a higher concentration of CAP disintegrated in SGF, likely due to increased friability (1%). Hence, during the development stage, the FDCs that yielded the best results in physical tests, contained either CAP or HPMC as matrix agents.

In FDC control studies, the percentages of actives was selected according to USP 34 and became an acceptable option for the production of the generic FDC ILRs of these three drugs.

3.2.3 FDC tablets containing RIF, PYR and INH

The hardness, friability and disintegration results for the FDC tablets are shown in (Table 5). All formulations presented results that fell within specifications of the USP 34. As expected, control FDC, made without the retardant matrix agent, exhibited complete disintegration of its two layers in less than 30 minutes in SGF. The drug content assay of FDC tablets was performed using HPLC allowing an additional 10 minutes of running time between injections. The FDC formulations made using CAP, had low INH and RIF content (48.2 and 65.7%, respectively), therefore, were discarded from the dissolution studies.

The content analysis results of the active PYR indicated that all of the FDC were within the range specified by USP 34 (90.0 to 110.0%). Next, the FDC tablets were evaluated in SGF and SEF dissolution media because the FDC tablets developed in this study had a retardant release matrix layer for INH. The mechanism involved with drug release has not been discussed here since it is beyond the scope of the present study.

Table 4. Results of the physical tests of the retardant release layer (RRL) formulations

Retarded release layer (RRL)		Physical tests			
Matrix agent	**INH : matrix ratio (w/w)**	**Hardness (N)[a]**	**Friability (%)[b]**	**Disintegration (min)**	
				SGF[c]	**SEF[d]**
Cellulose acetate phthalate (CAP)[e]	1.00 : 0.25	57.82±1.70	0.41±0.01	23.0	20.0
	1.00 : 0.50	59.78±1.70	0.62±0.01	ND	24.0
	1.00 : 1.00	58.80±1.30	1.01±0.03	27	22.0
Hydroxypropyl methylcellulose (HPMC)[f]	1.00 : 0.20	61.74±1.70	0.81±0.01	0.5	NP
	1.00 : 0.30	60.76±1.70	0.67±0.01	0.6	NP
	1.00 : 0.40	58.80±1.30	0.78±0.01	1.0	NP
	1.00 : 0.50	58.80±1.10	0.50±0.01	7.0	NP
	1.00: 0.60	59.78±0.80	0.62±0.01	ND	2.0
	1.00 : 0.70	57.82±1.60	0.39±0.01	ND	3.0
	1.00 : 0.80	60.76±1.10	0.32±0.01	ND	5.0
	1.00 : 1.00	56.84±1.70	1.12±0.02	ND	17.0
Control	1.00 : 0.00	58.80±0.80	0.23±0.00	0.5	0.5

[ND] Not disintegrated; [NP] Not performed;; [a] Results are expressed as mean ± standard deviation for n = 10; [b] Results are expressed as mean ± standard deviation for n = 20; [c] Simulated Gastric Fluid; [d] Simulated Enteric Fluid; [e] Formulation prepared by wet granulation with agglutinant solution comprised of 1% cellulose acetate phthalate; [f] Formulation prepared by direct compression and contained 2.6 mg of magnesium stearate

Quantification of PYR and INH in the dissolution test was performed by HPLC, while RIF was quantified using UV spectrophotometry. The retention times obtained were: (INH 4.7 min, PYR 6.03 min), the theoretical plate values were: (INH 10028; PYR 12401), and the tailing factor values were: (INH 1.2; PYR 1.2). These values were all within the limits required by USP 34, thus demonstrating that the quantification methods are effective and accurate for used in the dissolution test of FDC tablets.

Table 5. Results of physical tests and drug content of the FDC tablets containing rifampicin (RIF), pyrazinamide, (PYR), and isoniazid (INH). The disintegration test of FDC tablets was conducted in simulated gastric fluid (SGF) for 30 minutes and then in simulated enteric fluid (SEF) for 45 minutes

Formulation		Physical tests				Drug content (%)		
IRL[a]	**RRL[b]**	**Hardness (N)[c]**	**Friability (%)[d]**	**Disintegration (min.)**		**INH**	**PYR**	**RIF**
				SGF	**SEF**			
Intragranular croscarmellose sodium	Control	98.98±1.7	1.12±0.01	2.0	NP	97.2	95.7	94.7
	CAP 2,00	108.78±2.1	0.69±0.01	No	27.0	75.0	95.1	73.2
	HPMC 1,25	96.04±1.9	1.32±0.01	No	17.0	102.0	96.7	94.6
	HPMC 1,00	96.04±1.4	1.22±0.01	No	28.0	95.3	95.3	82.3
Extragranular croscarmellose sodium	HPMC 1,25	96.04±0.7	1.24±0.00	No	23.0	99.5	95.3	93.1
	HPMC 1,00	98.98±1.2	1.07±0.01	No	29.0	100.4	94.3	93.8
Market formulation		NP	NP	NP	NP	NP	NP	NP

[NP] Not performed.; [a] Immediate release layer description made according to disintegrant modality addition; [b] Retarded release layer description according to the INH/matrix ratio (w/w), except control formulation. [c] Results are expressed as mean ± standard deviation for n = 10; [d] Results are expressed as mean ± standard deviation for n = 20

Theoretically, INH and PYR do not have issues with bioavailability since they are class I drugs (high solubility and high permeability) of the Biopharmaceutical Classification System (BCS). RIF was the only hydrophobic drug used in FDC tablets belonging to class II (low solubility and high permeability) of the BCS. The cumulative dissolution results are shown in (Table 6). Compared to the market formulation, the FDC control formulation had similar percentages of drug release and were in accordance with the acceptance criteria recommended in USP 34. Therefore, this represents an acceptable option for the development of immediate release FDC tablets.

Among the FDC produced, the retardant action of HPMC was more effective when the ratio of INH/HPMC in the IRL was equal to 1.00. However, this formulation demonstrated lower performance in regards to PYR and RIF, compared to the formulation produced using an INH/HPMC ratio of 1.25, therefore it was discarded. INH and PYR showed the lower and higher cumulative dissolution efficiency, around 70 and 90%, respectively, when not considering the discarded formulation. In addition, an adequate total release of RIF and PYR, was observed, around 91 and 100% respectively, for the FDC tablet containing the IRL with the intragranularly-added disintegrant, croscarmellose sodium (CS); and the RRL prepared using an INH:matrix ratio of 1.25. This profile was better than the marketed FDC tablet.

Various factors may influence the release of INH from HPMC matrices: viz., polymer viscosity, polymer particle size, drug/polymer ratio, drug solubility, drug particle size, drug loading, compression force, tablet shape, formulation excipients, processing techniques, as well as the testing medium utilized [38]. INH is susceptible to hydrolysis and oxidation, despite this, it has been shown to be reliable for use in the FDC of tuberculostatic drugs [39]. DSC is a rapid method that can be used to explore the possibility of physicochemical interactions, between drugs and excipient(s). However, studies regarding the thermal behavior of INH by DSC are relatively few. As a matter of fact, the few available results revealed that no interactions occured between INH and microcrystalline cellulose [40] but INH interactions with HPMC have been reported [33].

Table 6. Cumulative dissolution of rifampicin (RIF), pyrazinamide, (PYR), and isoniazid (INH) from FDC tablets in simulated gastric fluid (SGF) after 30 minutes followed by 45 minutes in simulated enteric fluid (SEF). Marketed immediate release FDC tablets as well as control formulations without HPMC were submitted to the same dissolution experimental conditions

Formulation		Cumulative dissolution (%)				
IRL[a]	RRL[b]	SGF			SEF	Total
		INH	PYR	RIF	INH	INH
Intragranular croscarmellose sodium	Control	102.0±0.8	98.5±1.2	93.4±0.8	NP	NP
	CAP 2,00	48.2±0.3	96.7±1.1	65.7±0.8	NP	NP
	HPMC 1,25	32.5±0.6	100.1±0.9	91.3±0.8	37.2±0.8	69.7±1.1
	HPMC 1,00	30.2±1.0	93.7±0.4	89.7±1.4	37.6±1.2	67.8±0.7
Extragranular croscarmellose sodium	HPMC 1,25	31.6±0.5	94.4±0.6	90.4±0.8	35.9±0.9	67.5±0.4
	HPMC 1,00	33.7±1.2	89.5±0.8	86.0±1.4	37.9±0.3	71.6±0.8
Market formulation		106.9±0.7	104.0±1.8	108.1±1.2	NP	NP

[NP] Not performed; [a] Immediate release layer description prepared according to disintegrant modality addition; [b] Retarded release layer description prepared according to INH/matrix ratio (w/w), except control formulation

We strongly believe that the ability of HPMC to decrease INH release can be explained by the increased viscosity of the RRL as well as the formation of a gel layer with a longer diffusional path. This phenomenon resulted in the decreased effective diffusion of INH, therefore generating a slow-down effect of the INH release rate, which has been previously reported [41]. The ability of HPMC to affect INH release can be modulated by controlling the INH/matrix ratio towards one that allows better INH bioavailability. This phenomenon has been previously described for other HPMC-based modified release systems developed for INH [42].

The acceptable results of RIF in the *in vitro* dissolution test encourages additional *in vitro* and *in vivo* studies which are a prerogative for producing an FDC formulation with adequate bioavailability. The development of anti-TB FDC tablets which demonstrate high levels of RIF dissolution is important to ensure acceptable bioavailability in formulations. The release of total RIF under simulated gastric conditions, was around 90%, this result was expected since it is more highly solubile at a low pH, while conversely it solubility is reduced at higher pH [43]. However, the total release of RIF from the overall FDC bilayer tablets was lower than the quantified content value, therefore a possible polymorphic transition could not be excluded.

The polymorphic forms I and II of RIF have differing solubilities in aqueous media, however the RIF crystalline structure did not affect its cumulative dissolution from FDC bilayer tablets, as previously described [44]. The decomposition study of RIF in acidic conditions and in the presence of INH led to formation of a hydrazone between the RIF decomposition product. This interaction between 3-formylrifamycin and INH is a complex reaction process where, RIF becomes further degraded, while INH can be recovered [45]. This same study revealed that loss of RIF and INH upon their individual storage in 0.1M HCl at 37ºC, could be significantly reduced (almost 3X) when they are combined. Under acidic conditions, the bilayer tablet containing RIF and INH in different layers, which better correlates with conditions when they are stored individually. Moreover, recent *in vitro* degradation studies indicated that 15-25% of the RIF is degraded under acidic conditions when the ratio of RIF:INH is above 1:0.50. This tendency toward degradation is reduced to less than 10%, when the RIF:INH ratio is below 1:0.25 [43,45-46]. Taking into account the initial amount of RIF and INH present in the tablets obtained in this study, as well as the *in vitro* release data, we suggest that the potential interaction between the likely formed 3-formylrifamycin and INH was strongly minimized.

Previously published work [25] has demonstrated the preparation of a solid oral dosage with two tablets of RIF and a capsule of INH within a hard gelatin capsule which allowed segregated delivery of RIF and INH. However, this formulation strategy has produced a relatively low number of treatment strategies, only two anti-TB drugs were developed despite the numerous unit processes involved, thereby increasing the costs. Thereby, the segregated delivery of RIF and INH from the FDC bilayer tablets proposed in this work allows a higher cumulative dissolution of RIF and PYR and potentially improved INH bioavailability. Therefore the strategy demonstrated here can contribute to the development of a new and effective tuberculostatic FDC tablet. This development is mainly important for poor countries since this strategy reduces the number of unit processes and the cost of the manufacturing process. As shown for most of the high dosage tablets, the FDC tablets in this study could cause swallowing difficulties due to the total weight of the FDC tablet (980 mg).

4. CONCLUSION

The preformulation studies presented here allowed interactions between RIF, PYR and excipients to be assessed. There were no potentially relevant incompatibilities identified in the kneaded system containing RIF, PYR and excipients. The intragranular addition of croscarmellose sodium in IRL led to faster disintegration in the simulated gastric fluid as well as a higher cumulative dissolution of RIF and PYR. For RRL, the hydroxypropyl methylcellulose was found to be a more effective retardant matrix. Amongst the FDC tablets developed, the combination of the IRL with the disintegrant, where the croscarmellose sodium was added intragranularly and the RRL was prepared using an INH: matrix ratio of 1:25 which yielded better results, due to higher total release of PYR and RIF and retarded release of INH in simulated gastric fluid. These promising properties qualify this FDC for future bioavailability studies. The segregated delivery of RIF and INH could reduce degradation of RIF and consequently improve INH bioavailability. Finally, FDC tablets were formulated according to WHO recommendations, using doses of INH (75 mg), RIF (150 mg) and PYR (400 mg), which was also found to be a useful strategy for improving tuberculosis treatment and reducing the risk of prolonged monotherapy and the emergence of drug resistance.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=593&id=14&aid=5386