Assessing Drug-Excipient Interactions in the Formulation of Isoniazid Tablets

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A suitable and efficient high-performance liquid chromatography (HPLC) method was developed for the simultaneous determination of tuberculostatic isoniazid and its related impurities, isonicotinic acid and isonicotinamide, in oral solid dosage forms. We studied the influence on chromatographic separation of mobile phase parameters, such as pH, ion pairing and ionic strength, as well as column bonded phase and brands. The use of buffer solution pH 6.8:acetonitrile 96:4 (v/v) at 0.8 mL min⁻¹, C18 column (250 × 4.6 mm, 5 µm), allowed proper separation of these ionizable analytes without the need of ion pairing agents. The limit of quantification of the method (0.1 µg mL⁻¹) was suitable for the analysis of impurities. A drug-excipient compatibility study was carried out with the analytical method proposed in this work and addressed frequent excipients used in isoniazid coated tablets. The dye FD&C blue 2 lake promoted the highest degradation of the tuberculostatic agent.

Keywords: tuberculosis, isoniazid, HPLC, drug-excipient compatibility

Introduction

Tuberculosis (TB) is a contagious disease and until nowadays can be lethal in many cases. When individuals present an active TB infection, transmission of this pathogen can occur either through the air during coughs and sneezes, or through their saliva. Different strains of mycobacteria can cause TB, particularly Mycobacterium tuberculosis, which was first isolated and reproduced in animals by Robert Koch in 1882. At that time, the disease was propagated over Europe and America, killing one out of seven individuals. Robert Koch’s research opened the way for the diagnosis and cure of TB.¹

According to the World Health Organization (WHO), approximately nine million people become sick with TB every year in the world, and 1.4 million die from the disease. Emergence of multi-drug resistant (MDR) strains of M. tuberculosis and co-infection with acquired immune deficiency syndrome (AIDS) harden the treatment of TB. The estimated number of people falling ill with TB is declining very slowly and the world is on track to achieve the Millennium Development Goal to reverse the spread of TB by 2015.²

The discovery of tuberculostatic activity of streptomycin in 1944 was the beginning of a new era for TB treatment. Isoniazid (INH) was introduced in the treatment in 1952, followed by ethambutol, rifampicin (RIF) and pyrazinamide (PZA), which are the most efficient drugs for TB.³

Mohan et al.⁴ evaluated the high-performance liquid chromatography (HPLC) method described in the United States Pharmacopeia (USP)⁵ for quantitative determination of RIF, INH and PZA in fixed dose combination formulations, studying its ability to resolve major degradation products of RIF in the presence of INH. They observed that the requirements of theoretical plates listed in this method were met for RIF, but were not achieved for INH and PZA, even when columns of different suppliers were tested. Moreover, the resolving power of the method

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was dependent upon the column brand, thus requiring the need to fix all the specifications of the column.

The selection of the optimum column is one of the most critical parameters of HPLC method development. This choice is usually based on the analyte solubility and chemical behavior. INH and its related impurities are soluble in polar solvents, therefore normal-phase separation could be considered. However, reversed-phase chromatography is more frequently used for routine analyses due to its better reproducibility and easier application.

This work aimed to develop a method for the determination of INH and its degradation products isonicotinamide (INAAM) and isonicotinic acid (INA), a potentially neurotoxic substance, in solid dosage forms. Additionally, a drug-excipient compatibility study was carried out in order to access the stability behavior of INH in the presence of tablet excipients.

Experimental

Chemicals and reagents

INH (purity > 99%) was obtained from Taizhou Jiangbei (Zhejiang, China). INA (purity > 98%), ethyl acetate, dichloromethane, anhydrous sodium phosphate, monobasic sodium phosphate, sodium 1-heptanesulfonate (HS), tetrabutylammonium hydroxide (TBA), phosphoric acid and sodium hydroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dichloromethane GC grade, ethyl acetate PA, triethylamine (TEA), methanol (MeOH) and acetonitrile (ACN) HPLC grade were obtained from Tedia (Ohio, OH, USA). Raney nickel was purchased from Fluka (Buchs, Switzerland).

Isonicotinamide synthesis

A mixture of 100 mg of INH and 1 g of Raney nickel in 10 mL ethanol was refluxed during 8 h. The reaction was followed by silica gel 60 F 254 thin layer chromatography (Merck, Darmstadt, Germany) using ethyl acetate:MeOH (4:1, v/v) as eluent. INAAM was extracted with dichloromethane and ethyl acetate and vacuum dried at 40°C. Identity was confirmed by mass spectrometry and nuclear magnetic resonance. A chromatographic purity of 97.9% was obtained by HPLC, according to the method described below.

Sample preparation

Solutions of INH (200 µg mL⁻¹), INAAM (20 µg mL⁻¹) and INA (20 µg mL⁻¹) were prepared in purified water (Milli-Q system, Millipore, Billerica, MA, USA) unless otherwise indicated, filtered through 0.45 µm regenerated cellulose units (25 mm, Macherey-Nagel, Düren, Germany) and kept at −20 °C before HPLC analysis.

Chromatographic measurements

Sample analyses were performed using HPLC Shimadzu LC-20 Prominance (Kyoto, Japan) equipped with photodiode array detector (PDA) at 200-400 nm. Separations were achieved at room temperature, 20 µL volume of injection, 0.5 to 1.5 mL min⁻¹ and quantification at 254 nm. The following chromatographic columns were tested: Symmetry Shield C18 250 × 4.6 mm, 5 µm; Spherisorb ODS2 C18 250 × 4.6 mm, 5 µm; and Symmetry C8 250 × 4.6 mm, 5 µm by Waters (Milford, MA, USA); BDS Hypersil C18 250 × 4.6 mm, 5 µm by Thermo Electron Corporation (San Jose, CA, USA); Ace 5 C18 250 × 4.6 mm, 5 µm by Ace (Aberdeen, United Kingdom); Zorbax 300SB-CN 150 × 4.6 mm, 3.5 µm by Agilent Technologies (Palo Alto, CA, USA); Nucleosil 100-5 C6H5 150 × 4.6 mm, 5 µm by Macherey-Nagel (Düren, Germany).

Ten mobile phase compositions were tested for HPLC-PDA aiming retention and resolution improvements: (i) water (pH adjusted to 2.5 or 6.8, with phosphoric acid or sodium hydroxide at 10%); ACN (96:4); (ii) 0.01 mol L⁻¹ phosphoric acid (PBS) pH 6.8:ACN (96:4); (iii) water:HS 0.1%:ACN (60:30:10) with and without 0.1% TEA supplementation, pH 2.5; (iv) TBA 0.025 mol L⁻¹:ACN:MeOH (96:2:2), pH 2.5; (v) TBA (0.025, 0.012, or 0.005 mol L⁻¹):ACN:MeOH (96:2:2), pH 6.8; and (vi) TBA 0.005 mol L⁻¹ supplemented with 0.020 mol L⁻¹ PBS:ACN:MeOH (96:2:2), pH 6.8. Solutions were filtered through 0.45 µm nylon membrane (47 mm, Millipore, Bedford, MA, USA) prior to analyses. Proportions were expressed in volumetric basis. Selectivity was studied in samples containing INH and impurities and it was expressed by the resolution values.

Experiments of liquid chromatography-mass spectrometry (LC-MS) were carried out on a Waters EM-03/ZQ Mass Spectrometer (Milford, MA, USA), equipped with electrospray ionization (ESI) source, and interfaced to a Shimadzu LC-10VP Class-VP HPLC (Kyoto, Japan). The chromatographic method was developed using a Symmetry Shield C18 column 250 × 4.6 mm, 5 µm at room temperature. Mobile phase consisted of water:ACN (96:4) at 0.8 mL min⁻¹ (the flow rate was split 1:3 prior to getting into the MS). All MS data were collected in positive ion mode with scan of m/z 40-300. The optimized parameter settings for ESI were: capillary voltage, 3 kV; cone voltage, 50 V; extractor, 1 V; source temperature, 0 °C; desolvation...
Different C18 columns, Symmetry Shield and BDS Hypersil (data not shown). Peak shape was improved by using an aqueous diluent (Figure 1b). We attribute the peak splitting observed in MeOH to a partial INH distribution into the two different solvents, namely MeOH as the diluent, and buffer-ACN as mobile phase. Therefore, a portion of INH eluted within the diluent solvent, while the remaining eluted with the mobile phase in a different retention time.

Under USP conditions, INH presented noteworthy small retention time (3.2 min, Figure 1b). This retention close to the dead time (1.6 min for this chromatogram) will compromise the separation of the related structure substances relevant for quality control, such as impurities of the active pharmaceutical ingredient and degradation products. This demonstrates the importance of developing an efficient method for the quantification of ionizable INH and related impurities.

Table 1 summarizes the results of retention factor for INH, INAA and INA, according to each experimental condition. It was studied changes in mobile phase composition, e.g., variation of organic solvent and water proportion, pH, and ionic strength as variable on INH, INAA and INA determination.

The effect of ionic suppression (Table 1, experiments 1-8) on the retention of compounds was verified on columns with different phase polarities. Mobile phases at pH values of 2.5 and 6.8 were tested based on the ionization behavior of the molecules. Other chromatographic parameters were initially set according to Bhutani et al.,9 who used water:ACN (96:4) instead of buffer:ACN (96:4), as adopted by USP methodology for mobile phase. The decrease in pH value was followed by an increase in INA retention, as well as a reduction in INH and INAA retention for C18 and C8 columns. Non-ionized forms of INH and INAA are predominant in neutral range pH, whereas the anion isonicotinate prevails for INA under this condition (Figure 2). The hydration of the anion decreased the column

**Results and Discussion**

**Method development**

Figure 1 shows the behavior of INH using the chromatographic conditions described in USP.1 Regarding the diluents adopted for sample preparation, Mohan et al.4 suggested using pure MeOH rather than buffer solution with zero or 4% of MeOH (for sample and standard, respectively) as adopted by USP methodology. The use of pure MeOH to dilute the samples resulted in INH peak splitting (Figure 1a). This behavior was similar in two
interaction and the INA eluted in a very small retention time. When the acid pH was used, the neutral neat charge of the Zwitterion form for INA decreased the hydration influence, enhancing therefore the column interaction and the retention time. Separation was considered better for C18 phase because peaks were broader on C8 column analysis and this stationary phase was not included in the following studies.

Table 1 also reveals that nitrile and phenyl columns presented the same retention factor for INH and INAAM, regardless of mobile phase pH. Therefore, these columns were not included in the subsequent tests. Coelution in phenyl column suggested that the $\pi$-$\pi$ interactions between phenyl rings of stationary phase and pyridine moiety of either INH or INAAM prevailed and no separation was observed. The unresolved peaks of INH and INAAM in the nitrile column indicated that hydrogen bonding and dipolar interactions between the nucleophilic nitrogen of the molecules and the nitrile groups of the column made a small influence in the interaction. These findings allowed
An increase in INA retention at a smaller retention time when compared to the system without ion pairing, suggesting electrostatic repulsion with the pyridine charge of the analytes. Tests were performed at pH value of 6.8 to verify these hypotheses.

Table 1 shows that the use of neutral pH with cationic pairing increased retention factor for all the analytes in comparison with acidic condition, confirming the need for non-ionized pyridine ring to avoid repulsion with the quaternary ammonium pairing agent. The influence of the ion pairing concentration in the binding of the solute to the reversed-phase chromatographic matrix was evaluated by the variation of the TBA hydroxide concentration at pH value 6.8, when only INA is ionized. Unexpectedly, the retention factor for INA decreased when the concentration of the ion pairing agent was higher, while hardly any difference in retention time was observed for the neutral species INH and INAA. We believe that the ion mobility is faster in higher concentrations of the ion pairing agent, reducing the retention time for isonicotinate therefore. This assumption was proved when the mobile phase with the smallest concentration of TBA (0.005 mol L$^{-1}$) was supplemented with 0.020 mol L$^{-1}$ phosphate. This condition recovered the INA retention time that was obtained with the higher concentration (0.025 mol L$^{-1}$) of the quaternary ammonium. Though good retention was achieved for INA using cationic pairing at neutral pH value, resolution of the pair H/I was still unsatisfactory. Nevertheless, the findings for different ion concentration were the grounds for the following test of the influence of ionic strength in Table 1, experiment 16) and C18 brands (Table 1, experiments 17-19) on separation, without ion pairing.

The use of buffer slightly changed retention time for INH and INAA, which are non-ionized in neutral range pH. For INA, higher retention factors were achieved with buffer in the mobile phase when compared to water (Table 1, experiment 1). Presumably, the presence of the sodium cation in the buffer acted as the counterion for the isonicotinate species, disturbing the solvation layer and approximating the analyte to the column alkyl chains. Therefore, a better interaction between INA and the column was observed.

Replacement of Hypersil column by either Spherisorb, Ace or Symmetry Shield phase increased H/I resolution (Figure 3). A better resolution was presented by the Ace column and could be related to its high carbon load (15.5%), in comparison with Hypersil (11.0%) and Spherisorb (11.5%) columns. Symmetry Shield, the C18 phase with the highest carbon load (17.0%) from those tested, presented lower resolution for the H/I pair, however. The worse separation when compared to Spherisorb and Ace columns can be attributed to the excessively high density
of bonded C18 phase, which makes the stationary phase very hydrophobic. This can affect separation on highly aqueous mobile phases because it is more difficult for water to wet the bonded phase. If the water-rich mobile phase does not easily adsorb or permeate into the stationary phase, a surface layer of structured water is formed and this limits access of the analyte to the surface.\textsuperscript{12} Even though, Symmetry Shield column presented suitable performance for the separation in terms of acceptable resolution and symmetric peak, confirming the importance of high carbon load for this analysis.

The comparison of results from Figure 3 and Table 1 allowed the selection of phosphate buffer (pH 6.8):ACN (96:4) as the mobile phase composition and either Ace or Symmetry Shield as the C18 phase, due to the promotion of a higher retention time for INA and the best peak shape for INH and INAAM.

Final experiments were executed increasing flow rate from 0.5 to 0.8 mL min\textsuperscript{-1} in order to optimize analysis time. Separation was completed in less than 10 min, while 15 min were needed for the lower flow rate. Chromatographic parameters were not changed after the increase in flow rate and were fit for purpose: INH theoretical plate number was 4100; IA/H and H/I resolution were 6.5 and 4.0, respectively; INH peak symmetry was 1.5. Method sensitivity was suitable for the analysis of impurities; limit of quantification was 0.1 µg mL\textsuperscript{-1}, corresponding to 0.05% of INH concentration in the sample.

Drug-excipient compatibility study

There is no universal method for drug-excipient compatibility studies and literature is poor of information on that. We could find only one article in literature addressed to INH adsorption by specific tablet excipients.\textsuperscript{13} The way drugs and excipients are combined in order to test their compatibility can strongly affect the results. In case of tablets, the best approach to predict the behavior of the product would be the direct compression of the drug and specific excipients, whose compatibility was being evaluated. This is not always feasible, however, because some combinations drug/excipient will not make tablets without the aid of other excipients. Moreover, this procedure would require large amounts of the substances in order to reach the minimum volume for the tablet press. Therefore, powder INH and excipients were mixed and stored in glass vials covered with filtration paper in order to maximize exposure to conditions of accelerated stability test, according to the Brazilian Health Surveillance Agency, 40 °C and 75% RH.\textsuperscript{14} The formulation of a coated tablet produced by Fiocruz was adopted as the model for the study. In this dosage form, the coating is designed to reduce moisture uptake and to provide the product with a homogeneous and reproducible color. Fixed drug-excipient proportion, such as 1:100, is reported in literature to prepare binary mixtures for this purpose.\textsuperscript{15} Since the excipient amounts in our formulation range from 0.1 to 87.5% related to the INH dosage (200 mg), we decided to use the actual proportions in order to simulate a real tablet. Differential scanning calorimetry (DSC) can be applied to a fast screening for incompatibility issues between drugs and excipients.\textsuperscript{16} However, as we intended to follow not only the stability of INH but also which degradation products were being produced, we used the HPLC method described in the first part of this article.

The drug-compatibility study (Figure 5) showed that, among excipients from the tablet core, only sodium lauryl sulfate and magnesium stearate promoted INH degradation to INA. This event was observed only after 180 days and

\textbf{Figure 3.} Role of C18 stationary phase on separation of isoniazid (INH)/isonicotinamide (INAAM) and INH peak shape. Mobile phase constituted of 4% acetonitrile (ACN) in buffer at pH 6.8, flow rate 0.5 mL min\textsuperscript{-1}. Error bars correspond to standard deviation (n = 3).

\textbf{Figure 4.} Chromatogram obtained in optimized conditions for isoniazid (INH) and related impurities. C18 Symmetry Shield, buffer at pH 6.8: acetonitrile (ACN) 96:4; flow rate 0.8 mL min\textsuperscript{-1}.
was attributed to the moisture uptake of both mixtures, as they presented a granular appearance after the study. Moreover, a synergic effect of all the excipients from the tablet core on the formation of INA and INAAM was already observed in 30 days of experiment.

**Figure 5.** Drug-compatibility study at 40 °C and 75% of relative humidity. All the samples contained isoniazid (INH) and the excipient(s) described. Isonicotinic acid (INA) and isonicotinamide (INAAM) were not detected in the sample that contained INH only, as well as in the mixtures of INH with the following excipients: 102 microcrystalline cellulose, sodium starch glycolate, polyvinylpyrrolidone, colloidal silicon dioxide, Eudragit®, talc powder 325 mesh, and polyethylene glycol 6000.

Considering exposure to isolated excipients from the coating, degradation of INH was more significant for its mixture with the colorant FD&C blue 2 lake. The blue colorant tested in this work is a lake pigment produced by precipitating the dye with aluminium hydroxide. Traces of Al\(^{III}\) from the colorant binder possibly acted as Lewis acid and catalyzed the hydrolysis of INH. Although the colorant FD&C red 6 lake contains the same binder, it produced lesser extent of INH degradation. We attribute this difference to the complexation of Al\(^{III}\) with the red dye, Ponceau 4R (Figure 6), which prevented INH from the catalytic role of the metal. Al\(^{III}\) can form bidentate complexes with azo dyes through their azo and hydroxyl moieties.\(^{17}\) The indigo carmine dye of the blue colorant is not efficient to complex with Al\(^{III}\), once its oxygen atoms are involved in intramolecular hydrogen bonding with the amine protons. Moreover, Al\(^{III}\) has a strong tendency toward displacement of protons from hydroxyl groups, which are present only in the red dye.\(^{18}\) From the other coating ingredients, only titanium dioxide promoted some extent of INA formation detected after 180 days. This finding supports the role of metals in the catalysis of INH hydrolysis. The combination of all the excipients of the tablet formulation (core and coating) promoted the highest level of degradation, greater than 0.5% of INA formation detected in 180 days. This might be related to a synergic effect of moisture and metal catalysis.

**Figure 6.** Chemical structures of Ponceau 4R (CAS 2611-82-7, present in FD&C red 2 lake) and indigocarmine (CAS 860-22-0, present in FD&C blue 2 lake).

The LC-MS analysis of the mixtures containing the colorants showed trace amounts of a third degradation product with [MH]\(^+\) of \(m/z\) 243. The mass spectrum and the retention time are consistent with the INH dimer reported by Bhutani et al.,\(^9\) isonicotinic acid N’-(pyridyl-4-carbonyl)-hydrazide. In the positive mode of MS, protonation of the hydrazide moiety was probably followed by cleavage of the N–N bond. leading to loss of INAAM, [MH-122]\(^+\), and formation of an abundant fragment ion of \(m/z\) 121. This dimer was also reported by Kakemi et al.\(^{19}\) after exposure of INH to alkaline conditions. According to the authors, dimerization was prevented by the addition of the chelating agent EDTA. This finding suggests the role of metals in the reaction, which is in line with our results of dimer formation in the colorant-containing mixtures.

**Conclusions**

A stability-indicating HPLC method was developed for INH and related impurities analysis with limit of quantification of 0.1 µg mL\(^{-1}\). These compounds present ionizable moieties, which required a comprehensive understanding of their ionic behavior during the mobile phase selection. The interaction of ionic species with alkyl stationary phases was promoted by the use of counterions in
a buffered eluent and all the peaks were baseline resolved. The drug-excipient compatibility study revealed that tablet formulation components containing high moisture levels or metals in their composition promoted INH degradation in the accelerated stability conditions. Considering the individual effect of each excipient, the FD&C blue 2 lake colorant was responsible for the highest formation of the degradation products. This finding suggested that colorants containing aluminium hydroxide as the binder should be avoided during INH formulation, unless the pigment presents an efficient mechanism for Al$^{III}$ quelation, as observed for the FD&C red 6 lake colorant.

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