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Determination of Isoniazid and Pyridoxine in Plasma sample of Tuberculosis patients by micellar liquid chromatography

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Abstract:	It is no doubt Isoniazid is a powerful tuberculosis drug, but it might give rise to Vitamin B6 (Pyridoxine) deficiency. In this case, a usual treatment is the combined administration of Isoniazid and Pyridoxine. An easy-to-conduct procedure based on Micellar Liquid Chromatography has been developed to quantify Isoniazid and Pyridoxine in plasma from Tuberculosis patients. The sample was diluted in mobile phase, filtered and directly injected, thus avoiding extraction or purification steps. Both drugs were adequately resolved from the matrix and endogenous compounds using a mobile phase made up of 0.15 M sodium dodecyl sulfate - 8%(v/v) 1-butanol - 0.01 M phosphate buffer at pH 3, running at 1 mL/min through a C18 column at 25ºC, in less than 8 min. Detection was carried out by UV-Absorbance at 265 nm. This innovative analytical method was validated following the Food and Drug Administration Bioanalytical Method Validation Guidance for Industry (2018) with satisfactory results. The response of the drugs was linear in the range 0.1 to 15 mg/L, with $r^2 > 0.9990$ for both compounds. Trueness and precision were 89.2-102.3% and <8.5%, respectively. Limits of detection was 0.03 mg/L and no significant carry-over effect was noticed. Finally, the analytical method was used to determine both compounds in plasma samples of Tuberculosis patients and for stability studies.

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Editor
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Dear Sir,

We enclose hereby the revision version of the manuscript number nº MICROC-D-20-00305.R1 entitled, **Determination of Isoniazid and Pyridoxine in Plasma sample of Tuberculosis patients by micellar liquid chromatography,** to be considered for publication in **Microchemical Journal.** The paper has been modified according to the comments from the reviewers and the changes have been highlighted in green. A point-per-point answers to each reviewer's comment has been uploaded in a separate file.

Isoniazid is one of the first-line antituberculosis drugs. Although it is very effective, it can bring about polyneuropathy and hepatotoxicity, related to its conjugation to Pyridoxine, which induces Vitamin B6-defficiency. A solution to this problem is the administration of Pyridoxine supplements to patients following an Isoniazid based therapy. Therefore, the monitoring of the plasmatic concentration of the two drugs is useful for a better follow-up of the therapy and perform dose-adjustment to prevent side effects. Therefore, clinicians require analytical tools to determine isoniazid and pyridoxine in plasma of tuberculosis patients.

The paper describes the development of a procedure based on direct injection - micellar liquid chromatography to determine isoniazid and pyridoxine in plasma. The main advantage is the simplification and shortening of the sample processing, which is limited to a dilution, filtration and direct injection, without requiring clean-up or purification steps. This reduces the participation of operators and the associated sources of variance, and augments the sample throughput. What's more, the use of hazardous chemicals was minimized for the entire analysis. The method was successfully validated according to the guideline of the Food and Drug Administration, thus confirming the high quality of the results.

The analytical and practical performances of the method make it an excellent

choice for the analysis of these drugs in plasma with clinical purposes, as it results relatively inexpensive, sustainable, safe, useful for routine analysis, easy-to-conduct, and available for all laboratories. These characteristics fit the current trend in Analytical Chemistry.

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We look forward your answer.

With regards,

Juan Peris

RESPONSE TO REVIEWERS

The paper has been modified according to the valuable comments of the reviewers. Changes from the first revision (R1) to the second (current) revision (R2) have been highlighted in green (Modifications from the original version to the R1 are kept in yellow). A point-per-point response to each reviewer's comment is below provided:

Reviewer 2:

The acronym MEKC is spelled MECK on p.8; this can be corrected in the proof stage.

- ***Response***: This has been corrected

Reviewer 3:

I agree with the changes proposed by the authors. I recommend the publication, after adjusting the number of significant figures in tables 2 and 3.

- ***Response***: The number of significant figures has been adjusted

HIGHLIGHTS

> We have determined ioniazid and pyridoxine in plasma by micellar liquid chromatography

> Sample treatment was expedited to a dilution and direct injection

> Method was validated by the guidelines of FDA 2018

> It was eco-friendly, rapid, economic and with high sample throughput

> It was applied to stability studies and incurred samples

Determination of Isoniazid and Pyridoxine in Plasma sample of Tuberculosis patients by micellar liquid chromatography

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Abstract.

It is no doubt Isoniazid is a powerful tuberculosis drug, but it might give rise to Vitamin B6 (Pyridoxine) deficiency. In this case, a usual treatment is the combined administration of Isoniazid and Pyridoxine. An easy-to-conduct procedure based on Micellar Liquid Chromatography has been developed to quantify Isoniazid and Pyridoxine in plasma from Tuberculosis patients. The sample was diluted in mobile phase, filtered and directly injected, thus avoiding extraction or purification steps. Both drugs were adequately resolved from the matrix and endogenous compounds using a mobile phase made up of 0.15 M sodium dodecyl sulfate - 8%(v/v) 1-butanol - 0.01 M phosphate buffer at pH 3, running at 1 mL/min through a C18 column at 25ºC, in less than 8 min. Detection was carried out by UV-Absorbance at 265 nm. This innovative analytical method was validated following the Food and Drug Administration Bioanalytical Method Validation Guidance for Industry (2018) with satisfactory results. The response of the drugs was linear in the range 0.1 to 15 mg/L, with r^2 > 0.9990 for both compounds. Trueness and precision were 89.2-102.3% and <8.5%, respectively. Limits of detection was 0.03 mg/L and no significant carry-over effect was noticed. Finally, the analytical method was used to determine both compounds in plasma samples of Tuberculosis patients and for stability studies.

Keywords: Antituberculosis; Drug; Micellar; Optimization; Patients; Plasma.

1. Introduction

Isoniazid (INH; Figure 1A) is highly prescribed against tuberculosis (as part of combination therapy) or latent tuberculosis infection [1]. INH is also named as isonicotinyl hydrazine or isonicotinic acid hydrazide and acts by the inhibition of the biosynthesis of mycolic acid [2]. Although it is strongly effective, it exhibits a highly relevant adverse drug effect: its ingestion may bring about polyneuropathy (PNP) at treatment dose [3]. This is related to the conjugation of INH with vitamin B6 (pyridoxal and pyridoxal 5-phosphate), which leads to the depletion of pyridoxal 5-phosphate, inducing B6 vitamin deficiency on plasma levels [4]. Pyridoxine (PYR; Figure 1A) deficiency has been identified as a cause for peripheral neuropathy [5].

As risk factors for developing neuropathy induced by isoniazid treatment, we can stress out renal failure, chronic hepatic failure, alcoholism, HIV infection old age, pregnancy, slow acetylator status and malnourishment [6]. Patients taking medications which antagonize B6 effects, such as penicillamine, hydralazine, cycloserine, and retroviral drugs are also at high risk of developing neuropathy caused by INH therapy [7]. However, INH preventive treatment is increasingly recommended for TB patients, particularly those whit HIV infection [8]. This distinction is relevant, given that new cases of TB mainly arise in Asia and Africa in association with HIV [9]. Vitamin B6, mainly as its biologically active coenzyme form pyridoxal 5'-phosphate, is involved in a wide range of biochemical reactions, among then we can emphasize the synthesis of neurotransmitters [10]. Because of structural similarity, pyridoxine (B6 vitamin) is the only antidote for INH toxicity [11]. Acute toxicity of INH gives rise to seizures and neuropathy, while chronic toxicity results in hepatotoxicity [12]. Otherwise, Pyridoxine does not interfere with the pharmacokinetics of isoniazid [13]. Consequently, INH and B6 vitamin are often administered together, and then their simultaneous determination is of the utmost importance to monitor the progress of the therapy and avoid side effects by dose adjustment. It is well-known that HPLC is the gold standard technique for this sort of analysis [14].

Literature reveals only few HPLC-methods have been developed to quantify simultaneously Isoniazid and Pyridoxine in pharmaceuticals and biological samples. As the analytes are quite polar, they are barely retained in reverse phase columns. To obtain useful retention times, different strategies are proposed, like the use of mobile phases with a high content in water [13,15], very low flow rates [16] or HILIC columns [17,18]. Detectors used are

Absorbance [13,15,16,18,19] or mass spectrometry [17]. Besides, the injected solution has to be strongly purified to avoid column clogging and interferences from the matrix peaks, and then long and cumbersome sample treatments are required, what increases the sources of variance, because of the complexity of biological samples. However, these techniques require sophisticated and delicate instrumentation, trained laboratory staff, toxic waste handling and are relatively expensive, considering the necessary reagents and maintenance. An interesting alternative to shorten and reduce reagent consumption is the use of micellar electro kinetic chromatography (MEKC), which has been employed to simultaneously quantify these two drugs, by optimizing the experimental conditions using a factorial design [20].

We have previously demonstrated Micellar liquid chromatography (MLC), using sodium dodecyl sulfate as an anionic surfactant, is a useful strategy to determine drugs in biological fluids [21], including isoniazid in urine [22], providing useful advantages over hydroorganic-RP-HPLC. The main one is than plasma can be directly injected, after dilution in a micellar solution and filtration, what strongly reduces the sample processing. Otherwise, micellar mobile phases contain far less proportion of organic solvent (<15% face to up to 100%) and uses biodegradable reagents, and then are less toxic and flammable. and providing quite stable and reproducible responses. Therefore, MLC leads to highly-throughput, eco-friendly, safe, reliable and cost-effective methods. MLC is a better alternative to determine basic polar drugs. Indeed, the SDS-monomers are adsorbed on the stationary phase with the sulfate group oriented outwards, thus generating a negative layer, which interacts by electrostatics with the cationic solutes and increases retention. What's more, chromatographic responses are quite stable and reproducible [23].

The objective of the study is to develop a rapid, simple, reliable and economic analytical alternative to quantify of Isoniazid and Pyridoxine (B6 vitamin) in plasma and urine, that could be used to treatment monitoring on medical centers, especially on tuberculosis patients. The method must be able to quantify the drugs reliably in clinical concentrations and be useful for routine analysis in a clinical laboratory. To test the analytical performance, the procedure must be validated by Food and Drug Administration Bioanalytical Method Validation Guidance for Industry (2018) [24]. Finally, its reliability should be demonstrated by analyzing plasma samples from patients with tuberculosis after a treatment based on these drugs.

2. Experimental

2.1 Reagents, Chemicals and Apparatus

Solid standards of Isoniazid (purity >99.9%) and Pyridoxine (>99.9%) were supplied by Sigma (St. Louis, MO, USA). SDS (>99%) was from Merck (Darmstadt, Germany). Sodium dihydrogen phosphate (>99%), sodium acetate (>99.0%), HCl (nearly 37%) and NaOH (>98%) were bought from Panreac (Barcelona, Spain). Solvents (HPLC grade) methanol, 1-butanol and 1-pentanol were from J.T. Baker (Holland). Ultrapure water was in-lab produced from deionized water, provided as running water by the University, employing an ultrapure water generator device Simplicity UV (Millipore S.A.S., Molsheim, France). This ultrapure water was used to prepare the aqueous solutions and mobile phases.

Ultrasonic bath was Ultrasons-H (Selecta, Abrera, Spain) and the pHmeter was a Crison GLP 22 (Barcelona) potentiometer equipped with a combined Ag/AgCl/glass electrode. The analytical balance used was a Mettler-Toledo AX105 Delta-Range (Greifensee, Switzerland).

2.2 Preparation of solutions

Micellar solutions were prepared by weighing the adequate amount of SDS and buffer salt, and solving it in ultrapure water using a magnetic stirrer. Afterwards, the pH was adjusted by adding drops of 0.01 M HCl or NaOH solutions whilst monitoring the pH, until the desired value is reached, and the solution was introduced in a volumetric flask. Then, organic solvent was added to reach the sought proportion, and ultrapure water was added up to the mark. Finally, the solution was ultrasonicated to achieve solubilization, and filtered through 0.45-μm-Nylon membranes (Micron Separations, Westboro, MA, USA) located in a funnel, with the aid of a vacuum pump, and stored in an amber bottle.

Isoniazid and Pyridoxine stock solutions (100 mg/L) were prepared dissolving the proper mass in ultrapure water, and the mixture was ultrasonicated for 2 min. Fresh solutions were prepared weekly. Working solutions were prepared by successive dilutions in water, and renewed each month. All these solutions were stored in amber vials in a fridge at +4ºC.

2.3 Chromatographic conditions

The chromatograph used was an Agilent Technologies Series 1100 (Palo Alto, CA, USA)

equipped with a quaternary pump, a degasser, an autosampler, a temperature controller for the column module and a diode array UV-Visible absorbance detector (DAD). A Kromasil 5 C18 column with a 5 μm particle size and 150 mm (4.6mm i.d.) (Scharlau, Barcelona) was used. HP ChemStation Rev. B.01.01. was used to control the instrumentation, as well as to acquire and process the signals.

The determination of Isoniazid and Pyridoxine was performed using a mobile phase made up of 0.15 M SDS - 8%(v/v) 1-butanol, 0.01 M phosphate buffer at pH 3 running under isocratic mode at a constant flow of 1.0 mL/min at a constant temperature of 25ºC. Under these conditions, pump pressure was nearly 150 bars Injection volume and absorbance wavelength were set to 20 μL and 265 nm, respectively. All injected solutions were filtered through a 0.45-μm-Nylon membranes, using a hand-pushed 3-mL plastic syringe.

The dead time ($t_0 \approx 1.0$ min) and the retention time (t_R, min) were directly taken from the chromatogram, while retention factor (*k*), efficiency (*N*, number of theoretical plates), asymmetry factor (*T*), peak width at half-height ($w_{0.5}$, *min*) and resolution (*Rs*) was calculated as in [25]. Working and cleaning instructions about the chromatographic instrumentation when dealing with micellar solutions is described in [26].

2.4 Sample collection and treatment

Incurred plasma samples were obtained from tuberculosis patients following isoniazid/vitamin B6 therapy, while blank samples were from healthy volunteers not taking any medication, from a local Hospital. Blood extraction was carried out by qualified medical staff. The investigation was approved by two local Ethics Committees, Hospital and the University Ethic Committee for Analysis of Research Projects. Written informed consent was obtained from all participants and all research was carried out in accordance with the 2013 Helsinki Declaration principles.

For confidentiality reasons, samples were sent unlabeled. Neither personal nor clinical information about the patients or the healthy volunteers was provided from the Hospital. The laboratory commits not to transfer any information to other institutions (except that indicated in the paper) and to destroy all the samples (except those from healthy volunteers, which may be stored for further studies) and all the chromatograms and the experimental data (except that here published) one year after the publication of the paper.

Blood samples were collected using a DB SST Tube (BD Vacutainer Systems, Plymouth, UK), and were centrifuged for 5 min at 756 Relative Centrifugal Force (RCF) or G-force at 4ºC, to obtain the non-cellular fraction. Plasma was immediately frozen and stored at −20°C. Plasma samples, either blank or patient samples, were treated likewise. They were thawed until complete melting the same day of the analysis. Afterwards, an aliquot was 1/5-dissolved in mobile phase, filtered and directly injected. The fortification was performed by adding the appropriate volume of a standard solution of the analytes, before the dilution [27].

3. Results and discussion

3.1 Optimization of chromatographic conditions

The mobile phase selection was based on the maximization of the resolution and peak shape, while minimizing the analysis time. INH is a quite polar compound (log P/ow = -0.7) and contain three ionizable nitrogen atoms: pyridine N (pKa = 1.8); hydrazine–NH (pKa = 3.6) and hydrazine NH_2 (pKa = 10.8) [22]. Pyridoxine is also polar (log Po/w = -1.1) and has an ionizable pyridine N with a pKa of 5.1 and an acidic OH with a pKa of 8.4 [28]. As both compounds are quite polar, we expect the retention strongly depends on pH [14]. In the development of the MLC method for the determination of Isoniazid and Pyridoxine, eight factors were identified that influence the separation analytes: SDS and modifier concentrations, concentration of buffering species, running buffer pH, column length, column temperature, sample injection volume and flow rate. The assays were performed by analyzing a working solution of 0.2 mg/L isoniazid and vitamin B6.

First of all, we studied the effect of the pH on the chromatographic response over the working range of the column: 3.0; 7.0 (phosphate buffer) and 5.0 (acetate buffer) [29]. For INH, the pyridine N and the hydrazine NH_2 are unionized and protonated, respectively, at the three tested pHs. At pH 3, the hydrazine–NH is partially protonated (80%), and then INH exhibits a formal charge of +1.8; while at pH 5 and 7, it is neutral, and INH has a formal charge of +1. For Pyridoxine, at the three pHs, the –OH is neutral. At pH 3 the pyridine-N is ionized and PYR has a formal charge of +1, at pH 5, it is partially protonated (56%) and PYR has a formal charge of +0.6; and at pH 7, it is neutral and PYR has a formal charge of +0.

Therefore, the pH of 3 favours the retention, thanks to electrostatic interaction, what was confirmed experimentally. The pH of 3 was selected due to was the most suitable pH in terms of higher retention time and better resolution. Second, molarity of the buffering agent was observed to have less influence on the separation and was therefore kept constant at 0.01 M. Third, the column length was studied making efficiency increase a 10% in a column of 25 cm compared to one of 15 cm, but retention time and analysis increased in a 35%, so a column of 15 cm was selected. Fourth, the influence of the temperature was checked in the range from 25 to 40ºC, result shows that this parameter does not improve the resolution or analysis time: 25ºC was selected for further studies. Fifth, the studies were focused in the injection volume, changing it between 0.5-30 μL, observing overloading at 25 μL: an injection volume of 20 μL was selected. Sixth, flow rate was experimented from 0.5 to 2 mL/min showing a decrease of analysis time, but decreasing the resolution dramatically, so 1 mL/min was selected. Finally, SDS and modifier concentrations have an important influence and were simultaneously optimized.

Considering the high retention of the compounds caused by their charge, the concentration of SDS was set to the maximal value, 0.15 M. Although pure micellar SDS mobile phases would be useful, it is better to introduce an organic solvent to decrease viscosity of the mobile phase (to reduce pump pressure), increase the interaction between mobile phase and stationary phase (to prevent stationary phase collapse) and enlarge the lifespan of the column. By the same reason, only the most hydrophobic alcohols were tested. Using 1-pentanol, Isoniazid and Pyridoxine elute too quickly and overlap with the protein band. As a retention time is a priority parameter, 1-butanol was selected as the modifier for the analysis running, and was set to the maximum value, 8%. Under these conditions, both compounds were eluted well-resolved, far from the expected elution interval time of protein band for plasma [30], in a low analysis time (Pyridoxine, 3.8 min and Isonazid, 5.0 min) and with a symmetrical shape.

Finally, the detection conditions were examined. Both compounds were determined by the optimal conditions, and their UV-Absorbance spectrum was in-time measured at the retention time. We appraise 265 nm provides a good signal-to-noise for both drugs, and then was taken as optimal value.

Standard solutions of both compounds were analyzed by sextuplicate to make a system suitability testing. The experimental values for Pyridoxine/Isoniazid and the

acceptance criteria [25,29] are in Table 1. Instrumentation and experimental conditions were valid for analysis, as the responses comply with the acceptance criteria. Like in MEKC, the increase of SDS concentration leads to lower retention times for these two drugs, even though the role of the surfactant is rather different. MLC offered similar retention times than MEKC, but with less symmetry (MEKC nearly 0.9) and slightly less variability in peak area (MEKC nearly 2.5%) [20].

Table 1. Results of the system suitability test (SD, standard deviation; RSD, relative standard deviation)

Parameter	Pyridoxine	Isoniazid	Acceptance criteria
t_R (min)±SD (RSD, %)	3.81±0.07 (1.8)	5.00±0.09 (1.8)	RSD ≤2%
RSD of peak area	1.9	2.0	≤2%
RSD of width at half-height	1.5	1.7	≤2%
retention factor	2.8	4.0	2.0-20
Efficiency	3342	3054	≥2000
Asymmetry	1.1	1.2	0.8-1.6
Resolution	2.1		≥2.0

t_0 = 1.0 min

3.2 Method validation

This method for the determination of isoniazid and pyridoxine in plasma of tuberculosis patients was validated according to the FDA Bioanalytical Method Validation Guidance for Industry (2018) [24], and assisted by other documents about validation [25,31]. All the parameters were determined in matrix, otherwise specified.

3.2.1 Calibration range and linearity

Blank plasma samples were spiked at nine concentration levels of Isoniazid and Pyridoxine in the range 0.1 - 15 mg/L, and analyzed by triplicate. The zero calibrator was also analyzed. The highest and lowest variance were compared by an Snedecor F's test with a

significant level of 0.05, and were found equal, thus proving the homoscedasticity of the model. The slope, y-intercept and determination coefficient were obtained by plotting the average areas of the chromatographic peak versus the concentration by least-square linear regression. The curves were:

Isoniazid: $A = (94 \pm 3)$ [Isoniazid] - (1.4 ± 0.9), $r^2 = 0.99992$
Pyridoxine: $A = (71 \pm 3)$ [Pyridoxine] - (0.9 ± 0.7) $r^2 = 0.9990$

In both cases, no significant trend was noticed by plotting the residuals *v.s.* concentration. Linearity was adequate ($r^2 > 0.990$) and there was any outlier (standardized residuals were <3). The y-intercept confidence interval included 0, pointing out to the absence of systematic error. All the calibrators were <15% of the nominal value.

Lower limit of detection (LOD) of quantification (LOQ) were calculated as indicated in [32], and were 0.04 mg/L and 0.11 mg/L, for both drugs. Response for LOQ was more than 5 times that of the zero calibrator.

3.2.2 Selectivity

Ten blank plasma were analyzed, before and after being fortified at 1 mg/L of Isoniazid and Pyridoxine (Figure 2A and 2B, respectively). The protein band was from the dead time to nearly 2.5 min, and the baseline was quite stable and straight. We did not notice any peak at or close to the window time of the analytes. Otherwise, the peaks of the two antituberculosis drugs can be observed as being sufficiently separated from each other, avoiding overlapping. Chromatograms from fortified blanks were compared to that obtained by standard solutions (1/5 diluted) by overlaying, and peaks exhibited similar shape and area, thus indicating the absence of coeluting compounds. Therefore, the method is free of interferences, and then the absence or presence of the drugs in the sample can be inferred from the chromatogram, and the entire peak area can be effectively assigned to the corresponding drug.

3.2.3 Carry-Over Effect

A plasma sample spiked with Isoniazid and Pyridoxine at 15 mg/L was injected, and immediately afterwards, a blank plasma sample was analyzed. In this last one, no peak

appeared in the chromatogram at the window time of any analyte. Thus, the carry-over was considered negligible at the working concentrations.

3.2.4 Precision and trueness

These parameters were calculated at four levels (0.1; 0.5; 5 and 15 mg/L) using spiked plasma samples. A sample containing 20 mg/L was analyzed as the same way, by introducing and extra-dilution 1/10 in mobile phase to evaluate dilution integrity.

Within-run values were determined by the successive analysis of 6 replicates in the same sequence run and prepared the same day. The trueness was the quotient between the found average concentration divided by the nominal concentration (recovery) and the precision was the relative standard deviation (RSD) of the six found concentration (repeatability). The same approach was carried out five different days over a 3-month period for between-run calculations. Trueness was the grand mean of the 30 found concentrations by the nominal value, and the precision (intermediate precision) was the relative standard deviation of the found concentrations. The results are shown in Table 2. The data show good trueness (89.2-102.3%), adequate precision (<8.5%) for both analytes, which are useful for routine analyses. Besides, the dilution of the sample does not affect the analytical performance, and then samples with a concentration over 15 mg/L can be analyzed. These results were accomplished due to the direct injection and the lack of complex purification steps.

Table 2. Within-run and between-run accuracy and precision ([a]n=6; [b]n = 5). Acceptance criteria: trueness 80-120% for LLOQ and 85-115 for higher values; precision, for LLOQ <20% and for higher values, <15%.

Isoniazid				
	Within-run[a]		Between-run[b]	
Concentration	Precision	Trueness	Precision	Trueness
0.1	6.5	90.5	7.6	91.8
0.5	5.4	94.5	6.8	96.5
5	3.2	99.4	4.3	100.7
10	1.6	101.6	3.0	100.9
20 (1/10 dil.)	2.8	98.7	3.4	99.7
Pyridoxine				
Concentration	Precision	Trueness	Precision	Trueness
0.1	8.2	89.2	8.5	90.8
0.5	5.7	92.2	7.0	93.6
5	1.9	102.3	2.5	101.0
10	2.1	98.7	2.7	97.5
20 (1/10 dil.)	3.4	97.0	4.3	98.3

3.2.5 Stability

Stability can be defined as the resistance of a compound to chemical change throughout time, under different external factors and chemical environments. In biological samples, it should be studied to recommend storage conditions and period. As reported in the literature, Isoniazid major products of decomposition are Isonicotinic acid, Isonicotinamide, Hydrazine, Isonicotinic Acid N´(pyridyl-4-carbonyl)-hydrazide, Isonicotinic acid ethylidene-hydrazide, Isonicotinic acid pyridine-4-methylene-hydrazide [33]. In our case, it was investigated in bench-top processed sample; freeze-thaw; long-term and stock solution.

Stability studies were conducted by subjecting the drugs to the investigated conditions, and monitoring the concentration of the analytes over a long period. Both drugs were considered stable until their concentration falls 15% in matrix and 5% in standard solutions, from their nominal value. The experiments were as follows:

- Working solution in water (0.2 mg/L): the solution was stored in a fridge (darkness at +4ºC), and each day, thawed, analyzed and restored. Under these conditions, both drugs were found stable up to 7 days, and then working solutions were renewed after this period.
- Bench-top processed sample: a blank plasma sample was spiked at 1 mg/L, treated, placed in the autosampler tray and analyzed by successive injections each 8 min for a half day. We did not notice any decrease in concentration for both drugs, and then an incurred sample can be processed and thereafter analyzed in sequences of up to 12 h.
- Freeze-and-thaw cycles: a blank plasma sample was fortified at 1 mg/L and stored at -20ºC in a freezer (the usual storage conditions in a Hospital). Each day, the entire sample was thawn, and aliquot taken and analyzed, and the rest of the sample reintroduced in the freezer. After 7 days, the decay reached the acceptance criteria for Isoniazid and was 12% for Pyridoxine. Therefore, the sample can be stored for repeated analysis up to one weeks.
- Long-term stability: a blank plasma sample was spiked at 1 mg/L, divided into 8 parts and kept at -20ºC. Thereafter, each two days, one of them was thawed, analyzed and discarded. The drugs remained quite stable for the duration of the assay (concentration diminishing of 10% for isoniazid and 5% for Pyridoxine), and then a plasma extract can be kept during two weeks until analysis.

3.3 Application of the method to patient´s plasma

The method was applied to the analysis of plasma samples from tuberculosis patients following a combined therapy of 300 mg Isoniazid and 50 mg Pyridoxine taken by oral route. Samples were extracted over a 24-h period after administration. All the collected samples were processed, placed in the autosampler tray and injected in the same sequence run. Quality control (QC), blanks and incurred sample reanalysis (ISR) solutions were distributed among the samples to monitor the analytical quality of the method. Results can be seen in Table 3. Chromatograms obtained from patient 1 is shown in Figure 2C.

In all chromatograms, we appraised Isoniazid and Pyridoxine could clearly be quantified without interferences. Reliability of the method was maintained throughout the entire run, as the results obtained by the quality solutions comply with the acceptance criteria (QC, <15% of nominal value; blank, <20% of LLOQ and ISR, <20% of the mean, respectively).

The procedure requires a low manpower and offered a high sample throughput, as the whole set was analyzed in a day by a single operator. General reagents, material and instrumentation were used. Volume of hazardous chemicals handled and wasted (only the 8% of 1-pentanol in the mobile phase) was rather limited, considering sample treatment and chromatographic analysis). The cost of the determination per sample was minimal. Therefore, the method exhibited strong practical performances and is useful for routine analysis in laboratories with a high workload, like a clinical one.

Table 3. Analysis of incurred samples

Injection number	Sample	Isoniazid	Pyridoxine
1	0.5	0.4	0.5
2	M1	0.4	0.1
3	M2	1.3	0.4
4	blank	n.d.	n.d.
5	M3	0.8	n.d.
6	M4	1.0	0.2
7	2.0	1.9	1.9
8	M5	2.4	0.3
9	M6	3.2	0.5
10	M4-bis	1.1	0.2
11	M7	0.2	n.d.
12	M8	0.9	n.d.
13	5.0	5.1	4.8
14	M9	1.6	0.4
15	M10	2.0	0.4
16	blank	n.d.	n.d.

4. Conclusions

Micellar liquid chromatography has been proved a useful technique to analyze human plasma to quantify Isoniazid and Pyridoxine. The main feature is the possibility of injecting a diluted sample directly into the column, avoiding *prior* extractions or purifications steps, and the increase of retention for polar and cationic compounds. The analytes were reasonably

resolved from the matrix within 8 min, using a mobile phase of 0.15 M SDS - 8%(v/v) Butanol - phosphate buffer at pH 3. Validation was performed according to the FDA Bioanalytical Method Validation Guidance for Industry (2018) with satisfactory results in terms of linearity, selectivity, precision, trueness, dilution integrity and carry-over effect. The sensitivity and calibration range were sufficient to detect the usual amount of Isoniazid and Pyridoxine in patients's plasma. Besides, it was applied for stability studies and analysis of incurred samples, with adequate results. In addition, this method involves at sustainable chemistry due to it is relatively inexpensive, easy-to-conduct, short-time, sustainable and with a high sample throughput.

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6. Conflicts of interest

The authors declare there are no conflicts of interest.

7. Author contributions

Conceptualization: S.C.B and J.E.R.; Data curation: J.P.V. and J.A.C.; Formal analysis: M.A.G.B. and J.A.C.; Funding acquisition and Project administration: S.C.B.; Investigation, M.A.G.B. and J.A.C.; Methodology, J.P.V. and S.C.B.; Resources, S.C.B. and J.E.R.; Software, J.A.C. and J.P.V.; Supervision, S.C.B. and J.E.R.; Validation: J.P.V. and S.C.B.; Visualization, J.A.C and S.C.B.: Roles/Writing - original draft: J.A.C. and J.P.V.; Writing - review & editing: J.P.V and S.C.B.

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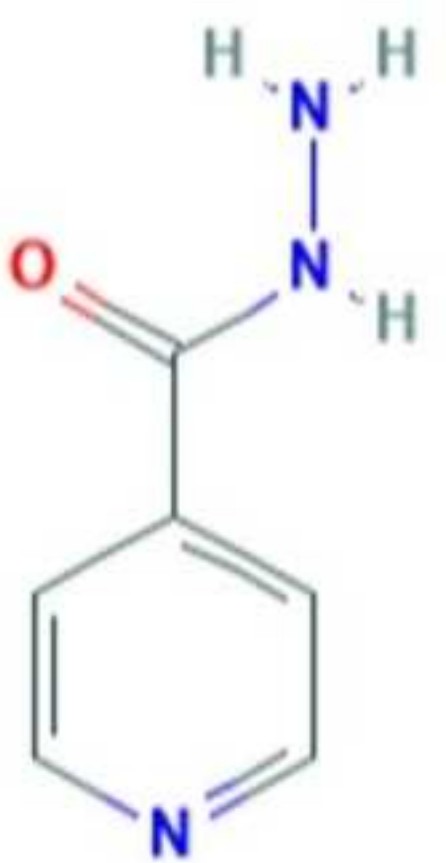
FIGURE CAPTIONS

Figure 1. Structure of Isoniazid and Pyridoxine

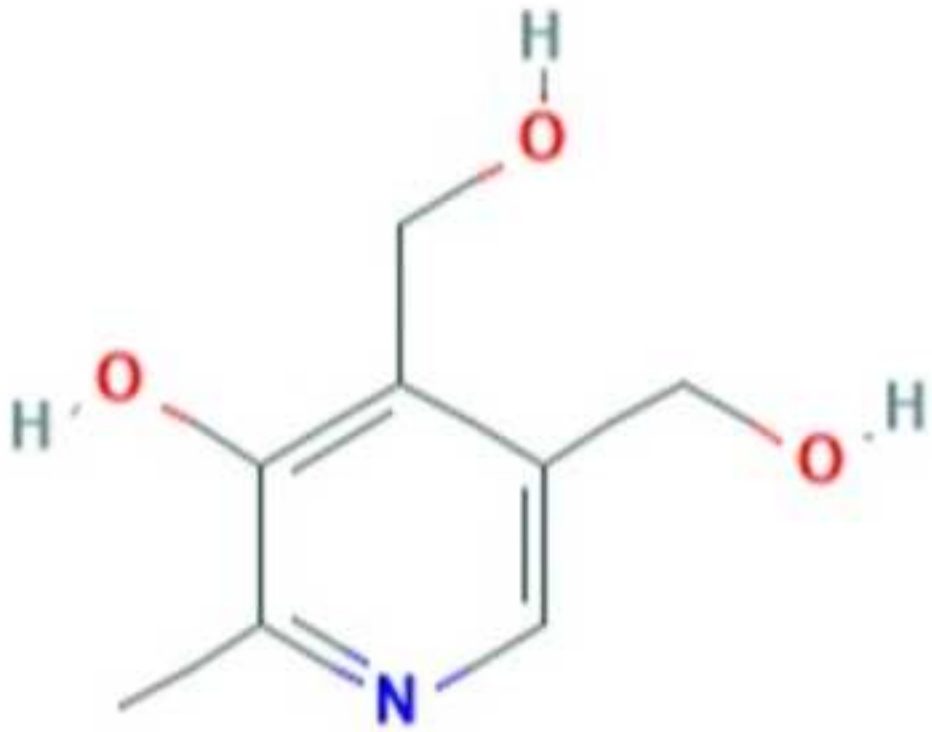
Figure 2. Chromatogram obtained by the analysis of a plasma sample: A) blank; fortified at 1 mg L^{-1} of both drugs, and C) from patient M1.

Figure1

Click here to access/download;Figure;Figure 1.jpg



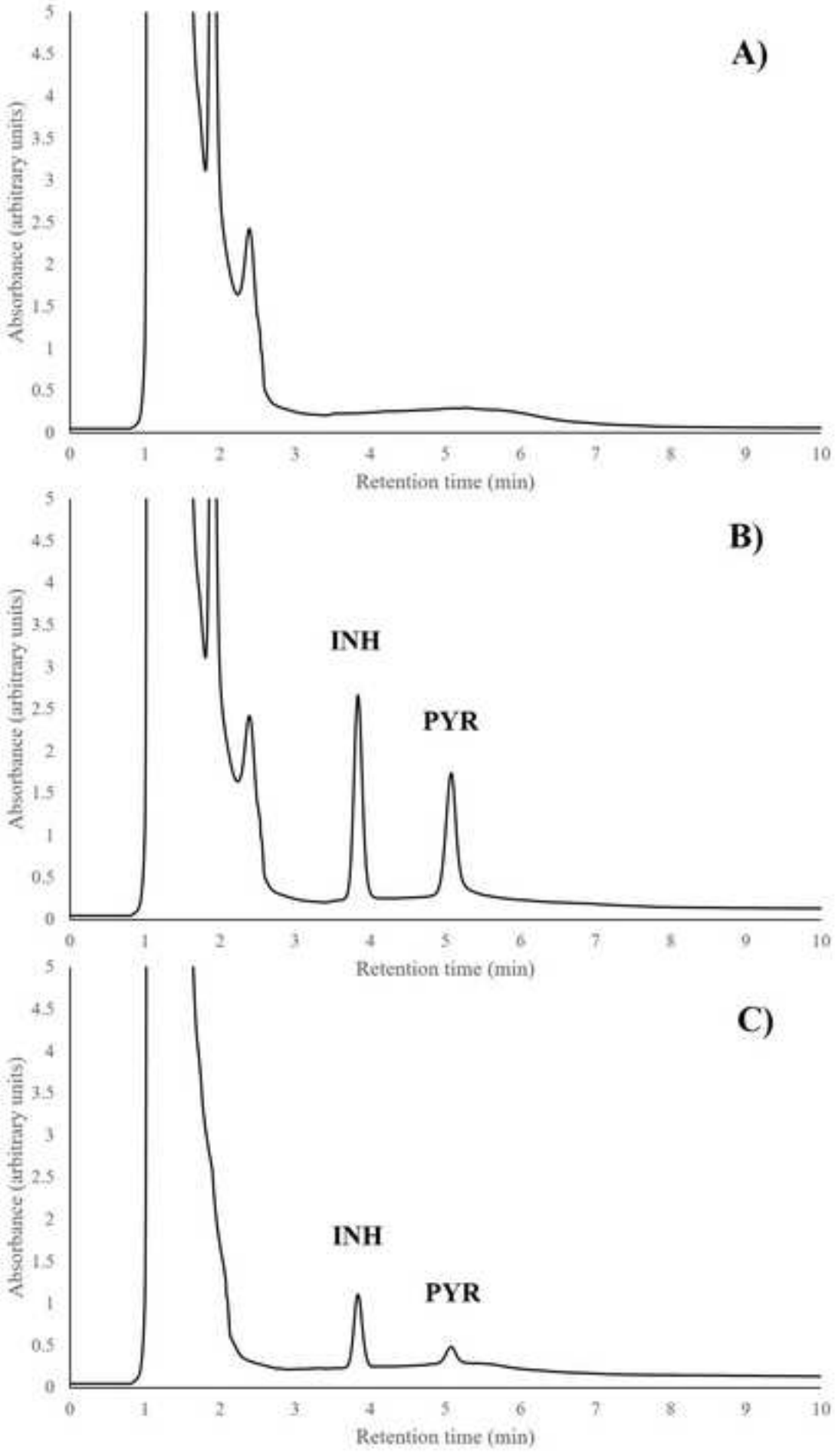
A)



B)

Figure2

Click here to access/download;Figure;Figure 2.jpg



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Software, J.A.C. and J.P.V.

Supervision, S.C.B. and J.E.R.

Validation: J.P.V. and S.C.B.

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Writing - review & editing: J.P.V and S.C.B.