ORIGINAL ARTICLE

Development and Validation of HPLC method for finding Isoniazid plasma levels in TB Patients with its Quantification in FDC Therapy

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ABSTRACT

Background: A fast, easy, subtle and inexpensive high performance liquid chromatographic method for the determination of plasma isoniazid levels in Pakistani Tuberculous patients was developed and validated as per guidelines of Food and Drug Administration.
Aim: To determine the quantity of INH as fixed dose combination therapy in pulmonary TB patients and HPLC method development using UV detection for determining plasma INH levels after 8 weeks of anti-tuberculous drugs treatment.
Methods: The instrument was of Shimadzu (HPLC) chromatographic system; a UV detector adjusted at 238 nm to detect INH.
Results: There was a combination of mobile phases A and B. There was a mixture of disodium hydrogen phosphate buffer 0.01M and acetonitrile in the following ratio of **95:5** and **50:50** (v/v) respectively. They were pumped at flow rate of 1ml/min. The limits of detection and quantification for isoniazid were 0.3 and 1.0 µg/mL respectively. The amount of INH after quantification of FDC prescribed to patients was 73.91 mg/tab. The method can be fruitfully practical for pharmacokinetic or bioequivalence studies of analyte.
Conclusion: High selectivity for the INH was observed as no interfering peaks from other drugs present in FDC therapy were pragmatic at the retention time in any sovereign blank plasma extracts gauged for the drug.
Keywords: Therapeutic drug monitoring; isoniazid; fixed dose combination; high performance liquid chromatography.

INTRODUCTION

Mycobacterium tuberculosis (MTB) is the most common causative agent for pulmonary tuberculosis in humans for thousands of years. This disease is curable but urgent and prolonged treatment for 6-24 months is required. It involves the organs like lungs, spines and central nervous system (1). Almost 10 million people approximately had TB and 1.5 million died from it. Pakistan is ranked 6th among countries for highest number of multidrug-resistant (MDR) TB[2].

It is more common in persons who live in closed, congested places like prisons, previously treated TB patients, people with HIV and other co-morbidities. Treatment for MDR-TB needs those drug regimens that are prolonged (18-24 months), more efficacious and less toxic. Globally, treatment success is only 50%[3]. Nowadays, drug resistant strains of MTB have evolved that lead to MDR-TB cases. In Pakistan, there are lot of reasons like poverty, less awareness about illness, false faiths about drugs being prescribed in TB clinics and parallel treatment systems causing treatment failure[4].

Anti-tuberculous drugs of first line defence includes pyrazinamide, isoniazid, ethambutol and rifampicin[5]. These drugs are used in fixed_dose combination as therapy during tuberculosis treatment nowadays[6]. Isoniazid (INH) is the backbone drug among first line anti-mycobacterial drugs. It inhibits the synthesis of mycolic acids and is metabolized by *N*-acetyltransferase-2 (NAT2) through acetylation in liver. Isoniazid shows good and rapid absorbtion from the gastrointestinal tract. Highest plasma drug levels after 2hrs of ingestion of 300 mg of isoniazid were 3–8 µg/mL[7,8].

Therapeutic drug monitoring (TDM) creates an option to overcome the problem of therapeutic failure by optimization of a dose that maximizes therapeutic benefit with limited toxicity[9]. Need of hour is specific and sensitive analytical method that can measure the drug in biological fluids as low anti-tuberculous drugs concentrations result in poor clinical outcomes with treatment failure. Therefore, Method development and validation is essential for finding plasma isoniazid levels in Pakistani Tuberculous patients. A try was done to confirm the exactness, correctness, sturdiness and other parameters, of developed and validated HPLC method as per FDA guidelines[10].
Objectives: The aims were to determine the quantity of INH as fixed dose combination therapy in pulmonary TB patients and HPLC method development and validation for determining plasma INH levels after 8 weeks of anti-tuberculous drugs treatment using UV detection.

MATERIALS AND METHODS

The study was held from January-December 2017 in Department of Bioequivalence Study Center, University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. Fixed dose combination (FDC) protocol followed in Gulab Devi Hospital, Lahore was adopted.

Received on 17-04-2019
Accepted on 03-08-2019

Chemicals and Reagents: Fixed dose combination anti-tuberculous drugs batch no:A606195, Isoniazid (purity 98.00%, w/w) was purchased from Merck Ltd., Acetonitrile was purchased from Merck Ltd, Methanol (HPLC grade) was purchased from Merck Ltd.,Orthophosphoric acid, Merck, Germany, Disodium hydrogen phosphate (Na_2HPO_4), Allied Signal, Germany, Double distilled water prepared in quality operation laboratory (QOL) of UVAS, Lahore. All other chemicals and reagents were of analytical grade.

Instrumentation: High performance liquid chromatograph (HPLC) Shimadzu chromatographic system, Japan equipped with a LC-20AT VP pump, an SIL-20AC HT auto-sampler, SPD-M20A, CTO 20 AC and CBM 20A controller unit, Vortex mixer, model no. M37610-33, Barstead International, USA., Column C18 (250 x 4.6 mm, particle size 5µm) Merck, Germany, Centrifugation machine, EBA 20, Germany, Analytical balance JJ224BC, Germany, pH meter Schott 850, Germany.

Analytical method: There were two mobile phases A and B. They were pumped at flow rate of 1ml/min at wavelength of 238nm.

Preparation of buffer for mobile phase: Fourteen hundred milligram (1.4g) of Na_2HPO_4 was dissolved in 1000ml of distilled water to make 0.01M solution. Orthophosphoric acid was employed for the pH adjustment of solution to 7.0 and buffer was passed through 0.45µm filter paper for filtration.

Calibration of standard curve: Isoniazid stock solution (1000µg/ml) was made in mobile phase. Spiking the blank plasma was used to make further standard solutions. Dilutions prepared were 1 µg/ml, 2 µg/ml, 4 µg/ml, 6 µg/ml, 8 µg/ml and 10 µg/ml. Quality control standard solutions were 3 µg/ml, 5 µg/ml, 9 µg/ml. Extractions of plasma samples were carried out by mixing 1000 µl plasma with 700 µl methanol in 2ml eppendroff tube. Samples were vortexed for 2 min by using vortex mixer then spinned at 10,000 rpm for 10 minutes. Supernatants were parted and filtered.

Chromatographic Parameters: The temperature was fixed at 30˚C throughout the procedure. Acetonitrile and 0.01 M disodium hydrogen phosphate were mixed in the ratio of 05:95 and 50:50 (v/v) respectively. The pH of the buffer was sustained at 7.0 ± 0.1. Before start of HPLC process, mobile phases were filtered and plasma samples were also passed through filter paper of 0.22 µm pore size for filtration process.Volume injected was 40µl.

HPLC Analysis: Drug levels versus area ratio calibration curve for isoniazid was made.

Method validation: Goal of validation method was to yield truthful, dependable and relentless results that can be applied. Method validation was confirmatory with standards of good laboratory practice (GLP). Validation showed that the method was correct as results produced were in lines with ICH guidelines (ICH Guidelines, 1996; FDA Guidance for Industry, 1998). Following certain parameters were checked for validation of mathod adopted.

Linearity: Linearity was used to access the expected range of the drug. For the evaluation of linearity, isoniazid standard solutions range from 1-10 µg/ml were used. The concentration versus area ratio calibration curve was constructed. In order to calculate concentrations and linearity least squared regression analysis was used.

Specificity: Specificity was done in order to find that the process was very accurate to be used under study. No interference in the retention time of isoniazid was found after injecting samples.

Recovery: Low, medium and high Quality control checks of different plasma concentration were used to examine the recovery. Analyzing the responses between extracted and solvent QC samples determined the percentage mean recoveries.

Precision and Accuracy: It is the nearness of replicate determinations of drug by an assay. Six replicate injections results of quality control checks were used to estimate the system correctness. Precision within day (intraday) was precision 1. Precision between days (inter-day) was precision 2. The percentage of drug recovered by the assay was accuracy and was done once.

Stability: Stability was judged by comparing the fresh samples results light explored, freeze and thaw at ambient, 24 hrs and 48 hrs room temperature sample results.

Limit of detection (LOD): Lowest level of drug in a blood sample is determined by LOD. It may quantify a false value. It is the bottommost concentration of drug with a signal-to-noise (S/N) ratio of at least 3. Samples having known concentrations of drug were used to measure the LOD. Setting the lowest concentration at which targeted drug quantity can be correctly identified.

Limit of quantification (LOQ): It is the minimum quantity of targeted analyte in a sample with appropriate precision and accuracy. To evaluate LOQ calculated sample signals of known low drug concentrations with blank samples were used.

RESULTS

Validation of Analytical Method

Limit of Detection (LOD) and Limit of Quantification (LOQ): Samples analysis with known concentrations of drug was done to measure the LOD with setting at the minimum level on which the concentration of drug can reliably be calculated. The LOD for INH was 0.3ug/ml. Limit of quantification is used to calculate the lowest concentration of drug in a blood sample that can be quantitatively measured with appropriate precision and accuracy . The LOQ for isoniazid was 1.0 ug/ml.

Selectivity: Evaluation of method selectivity was done by spiking the drug in blank plasma and ensuring the retention time. The retention time of isoniazid in spiked plasma was 4.40 ± 0.1 minute.

Calibration Of Standard Curve Or Linearity: Calibration of standard curve was conducted to obtain isoniazid plasma concentrations linearity. In the mobile phase, Isoniazid (100µg/ml) stock solution was made. Further, standard solutions were arranged in blank plasma and run to obtain average regression equation.

$y = 44456x + 15809$

$R^2 = 0.9911$.....The value of R^2 is within the limit as described by ICH (1996). Results shown in table: 2 and figure:1 below.

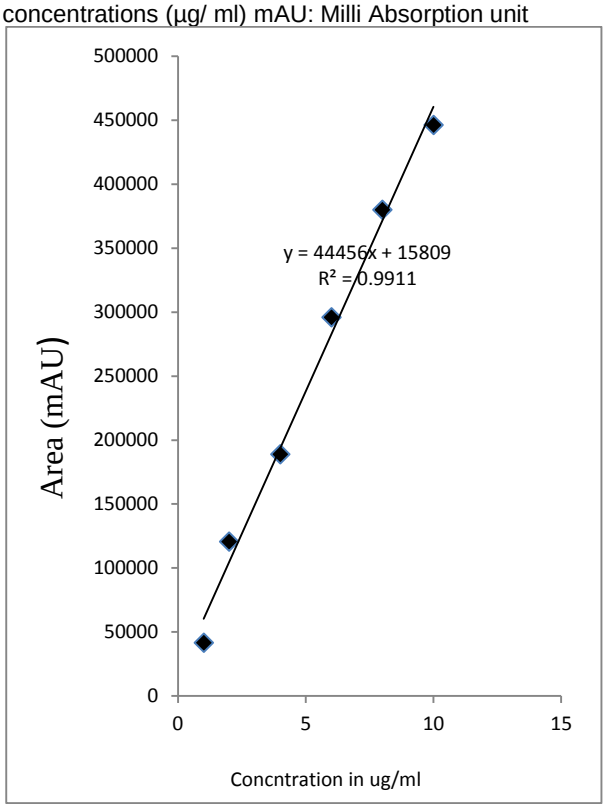
Table 1: Limit of detection and limit of quantification of isoniazid

Standard	**Isoniazid**
Linear regression equation	y = 44456x + 15809
R^2	R^2 = 0.9911
Linear range ug/ml	1-10.0
LOD ug/ml	0.3
LOQ ug/ml	1

Table2: Calibration of Isoniazid in plasma at different concentrations (ug/ml)

Concentration of Isoniazid (ug/ml)	**Area** (mAU)
1	41496
2	120645
4	188769
6	295982
8	379933
10	446161

Figure 1: Isoniazid calibration curve in plasma at different concentrations (µg/ ml) mAU: Milli Absorption unit



Chromatograms of Isoniazid in Solvent and spiked plasma are shown below. The retention time of Isoniazid was 4.4 ± 0.1 min**.**

Figure 1: INH in solvent

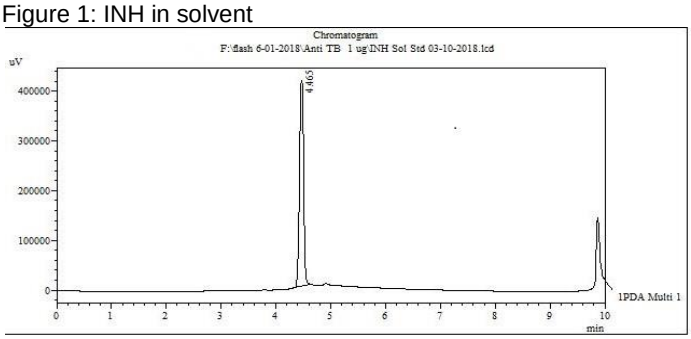
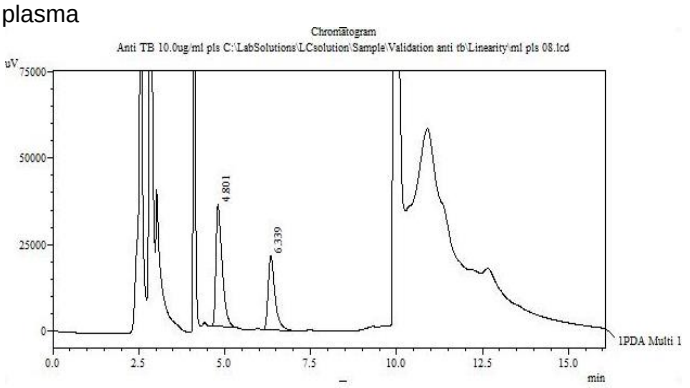


Figure 3: Chromatogram of INH (1.0µg/ml) in spiked plasma



Recovery: Quality controls samples at three differnt concentrations ranges i.e low, medium and high were employed to access recovery of drug. It was found quite similar and reliable, independent of the concentration range. Recovery of drug on average was more than 95% . As given in table 4.

Precision and accuracy: Quality control samples were used to calculate the interday and intraday precision of analyte samples. Results obtained after the injections of six quality control checks were summarized in table 3.

The above mentioned procedure was used to make all calibration standards; calibration curves were drawn from the cohesive peak areas.

Stability: Under the studied concentrations, in plasma significant degradation of INH in samples was noted. The freezer and room temperature stability of plasma samples was checked by keeping them frozen at -20℃ and 25℃ temperature respectively. The peak area ratio of the solutions of standard isoniazid was monitored inorder to evaluate stability of standard and sample solutions. Results regarding stability of samples were summarized in table: 5.

Table 3: Interday and intraday accuracy and precision of isoniazid

Nominal concentration ug/ml	**Intra Day (n=6)**				**Intra Day (n=6)**			
	Value found	**SD**	**Accuracy %**	**Precision %**	**Value found**	**SD**	**Accuracy %**	**Precision %**
3	3.16	0.035	101.35	101.3	3.02	0.04	100.80	100.8
	5.39	0.07	104.03	104	4.73	0.04	94.55	94.55
9	9.37	0.14	102.93	102.9	7.96	0.093	88.41	88.41

Table 4: Recovery of INH at quality check points

	Conc. Added (ug/ml)	**Conc.Cal.Pls** (ug/ml)	**Conc. Cal. Sol.** (ug/ml)	**Recovery** (%age)	**Conc. Added** (ug/ml)	**Conc. Cal. Pls** (ug/ml)	**Conc.Cal. Sol.** (ug/ml)	**Recovery** (%age)	**Conc. Added** (ug/ml)	**Conc. Cal. Pls** (ug/ml)	**Conc.Cal. Sol.** (ug/ml)	**Recovery** (%age)	**Mean Recovery** (%age)
	3	2.85	3.29	86.74	5	5.03	5.19	96.95	9	8.99	9.29	96.78	
	3	2.81	3.06	92.00	5	5.14	5.17	99.32	9	9.04	9.24	97.86	
	3	2.96	3.07	96.32	5	5.07	5.07	99.88	9	8.91	9.24	96.48	
	3	2.81	3.05	92.09	5	4.95	5.09	97.32	9	8.96	9.25	96.87	
	3	2.94	3.08	95.44	5	4.92	5.18	94.98	9	9.00	9.21	97.73	
	3	2.87	3.08	93.21	5	5.05	5.09	99.33	9	9.03	9.22	97.89	
Mean		3.16		92.63		5.40		97.96		9.46		97.27	95.95%
±SD		0.04				0.10				0.12			
RSD/CV		1.34				1.86				1.25			

Table 5: Stability study of Isoniazid in ambient and freezer conditions

	Ambient		**Freezer 1**		**Freezer 2**		**Ambient**		**Freezer 1**		**Freezer 2**	
	Conc. Added (ug/ml)	Conc. Cal. (ug/ml)	Conc. Added (ug/ml)	Conc. Cal. (ug/ml)	Conc. Added (ug/ml)	Conc. Cal. (ug/ml)	Conc. Added (ug/ml)	Conc. Cal. (ug/ml)	Conc. Added (ug/ml)	Conc. Cal. (ug/ml)	Conc. Added (ug/ml)	Conc. Cal. (ug/ml)
	3	3.23	3	3.01	3	2.61	9	9.37	9	9.30	9	7.97
	3	3.12	3	3.00	3	2.62	9	9.47	9	9.34	9	8.03
	3	3.11	3	3.08	3	2.49	9	9.32	9	9.29	9	8.14
Mean		3.15		3.03		2.57		9.39		9.31		8.05
SD		0.07		0.04		0.07		0.08		0.03		0.09
RSD		2.11		1.42		2.80		0.84		0.27		1.07

Table 6: Determining the quantity of INH in FDC

Sr.NO	**Std Area**	**Smp area**
1	415643	407556
2	403174	406036
3	419300	406520
Average	412706	406704

%age = Area of sample/Area of Standard * 100
= 412706/406704 *100
= 98.55%
Stated concentration of INH in mg/Tab = 75mg
Concentration in mg/Tab = 73.91 mg
Disintegration Time = 25 min
Quantification of INH in FDC:

Table 7: Dissolution results of INH

Wt. of tab (mg)	**%age**	**Conc. of INH** (mg)
1245	98.55	73.91
1237	97.92	73.44
1232	97.52	73.14
1240	98.15	73.61
1233	97.60	73.20
1230	97.36	73.10

DISCUSSION

Therapeutic drug monitoring of INH was planned to distinguish between MDR-TB cases or poor compliance. Therapeutic drug monitoring (TDM) for anti-tuberculous drugs is not usually performed in our setups. The anti-tuberculous health facilities work in colaboration with WHO. Treatment involves anti-tuberculous drugs in FDC as well as injectable form.

Causes of therapeutic failure are false faiths about drugs being prescribed in TB clinics and parallel treatment systems e.g traditional medicines and complimentary medicines drift patients away from taking treatment for 06 months add TB burden rather than eradicating it. More over a patient placed on 2nd line anti-mycobacterial drugs has to face severe drug reactions and to take medicine for long durations.

Method of enrollment was adopted in this research with some modifications[11]. Newly diagnosed Pakistani pulmonary TB patients admitted from April to August, 2017 were recruited to volunteer in current study. A written consent was taken from all the subjects. Identifiable codes were given to them for traceability.

Enrolled patients were given INH in a fixed dosage form according to their body weights. They recieved different number of tablets as per WHO guidelines. They were given drug dose at 5mg/kg (300mg/kg) body weight daily for 56 days (8weeks). Our work was in lines with previous researchers who prescribed same dose of INH 300 mg/kg to their patients in their stuies[8,11].

In present work plasma drug levels were determined by high-performance liquid chromatography (HPLC) using UV detector. Two mobile phases were used in our research.. Disodium hydrogen phosphate buffer 0.01M and acetonitrile were combined in proportions as 95:05 and 50:50 respectively. Wavelength used was 238nm for a detector and flowrate was 1ml/mint. In one reseach, mobile

phase was a combination of methanol, acetonitrile and H_2O in proportion of 5:30:65 with a wavelength was 242nm for a detector. In current study, injection volume was 40µl whereas in above mentioned study it was 20µl. In both studies column, C-18 was used[10].

During the method development of INH, many chemical buffers at various concentrations and altered pH ranges were used. Isoniazid is a basic drug due to its azide functional group. Therefore, acidic pH of mobile phase ionizes INH present in plasma causing reduced recovery. To extract the unionized drug, it is vital to alkalinize the pH. Our work was in line with the work done previously. In current study, pH was adjusted to 7.0 ± 0.1 with orthophosphoric acid as compared to other studies that used pH 2.7 ± 0.1 and 5.2 ± 0.1 with orthophosphoric acid respectively[10,12].

An easy and profound HPLC method is defined by an existing article with UV detection of target drug when given in FDC using easily available cheap laboratory reagents. Our work was in line with the work done for development and validation of HPLC method for INH plasma levels previously (13-17).

Our study had a number of limitations like financial constrains and less resources. Only single drug was evaluated in present study. Current project was a rare study in a sense that evaluated pharmacokinetics of an anti-tuberculous drug, INH, and quantification of FDC together. This study helped to evaluate reasons for increasingly reported MDR-TB cases in Pakistani population.

CONCLUSION

High selectivity for the INH was observed as no interfering peaks from other drugs present in FDC therapy were pragmatic at the retention time in any sovereign blank plasma extracts gauged for the drug.

Acknowledgement: I am thankful to Allah, who granted me with the courageousness, the direction and the perception to complete this research. Peace and blessings of Allah be upon our Holy prophet Muhammad (PBUH), his family and his companions. Foremost, I am great full to my teachers and family members.

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