Original article

Determination of isoniazid acetylation patterns in tuberculosis patients receiving DOT therapy under the Revised National tuberculosis Control Program (RNTCP) in India

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ARTICLE INFO

ABSTRACT

Isoniazid is the most commonly used drug for treatment of tuberculosis, and is administered individually or in combination with other drugs as standard first line therapy. Offsetting its efficacy, severe adverse effects, especially peripheral neuropathy and hepatotoxicity, are associated with isoniazid therapy, limiting its use in tuberculosis. Isoniazid is acetylated *in vivo* producing hydrazine and acetyl hydrazine, which are responsible for hepatotoxicity. Marked pharmacogenetic differences in acetylation have been reported among different population across the globe. This study evaluates isoniazid acetylation patterns in tuberculosis patients receiving DOT therapy under the Revised National Tuberculosis Control Program (RNTCP) in a specialized tuberculosis hospital in north India. Of 351 patients from whom samples were taken for biochemical analysis of adverse events, 36 were assessed for acetylation patterns. Blood samples were taken 1 h after administration of a 600 mg dose of isoniazid, and plasma concentrations of isoniazid were determined using a validated HPLC method. Of these 36 patients, 20 (55.56%) were slow acetylators and 16 (44.44%) were fast acetylators. Our results are consistent with those of an earlier study conducted in a different region of India. Most biochemical changes produced during long-term isoniazid therapy resolve after therapy is terminated.

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1. Introduction

Tuberculosis (TB) is an infectious disease normally caused by *Mycobacterium tuberculosis* (*MTB*), and is a major global health problem in growing countries (Tripathi, 2013). It is a major killer of females, rivaling maternal mortality, and is a prime cause of death in people with human immunodeficiency virus (HIV). One-third of the world's population is infected with the bacteria that cause TB, but only a small proportion of people will develop active TB. In most infected people, the tubercular bacilli are dormant and produce no symptoms. Such infections are called latent (Broekmans et al., 2005). In more than 85% of tuberculosis cases, the lungs are the affected organ. This form of TB is called pulmonary tuberculosis, but *MTB* can attack any part of the body, including the skin, brain, kidney, spine, and genital organs. People living with or coming into contact with a patient with undiagnosed or untreated infectious tuberculosis (in particular, smear positive TB) are at risk of infection. Hence, it is very important to diagnose new patients early in the course of the disease and begin treatment (Managing the Revised National Tuberculosis Control Programme in your area, 2005). If not treated appropriately, TB can be lethal.

For the treatment of TB, standard therapies are preferred. These usually combine isoniazid (INH) with rifampicin, pyrazinamide, or ethambutol (Hall et al., 2009), but INH can also be used alone for prophylaxis (Sia and Wieland, 2011). Offsetting its beneficial effects, INH can cause some severe adverse effects including

peripheral neuropathy and hepatotoxicity (Boelsterli and Lee, 2014; Carlson et al., 1956; Nolan et al., 1999; Metushi et al., 2011).

INH is a low-molecular weight, water-soluble compound that can be rapidly absorbed from the gastrointestinal tract (GIT) (Metushi et al., 2014). Its pharmacokinetic properties are affected by various patient-specific factors, including genetics, age, comorbidities, and coadministered food or drugs (Mach et al., 2016; Lin et al., 2010; Requena-Méndez et al., 2014; Seng et al., 2015; Ramachandran et al., 2013; Saktiawati et al., 2016; Wiltshire et al., 2014).

The main pathways of INH metabolism (Fig. 1) include: (1) Acetylation by N-acetyltransferases to form acetyl-INH (AcINH), and (2) Hydrolysis by amidases, producing isonicotinic acid (INA) and Hydrazine (Hz). AcINH can also be hydrolyzed to form isonicotinic acid (INA) and acetylhydrazine (AcHz). In addition, Hz can be acetylated, yielding AcHz and diacetylhydrazine (DiAcHz) (Preziosi, 2007). Hz and AcHz are thought to be further oxidized to reactive metabolites that underlie INH hepatotoxicity (Delaney and Timbrell, 1995; Lauterburg et al., 1985; Mitchell et al., 1976; Nelson et al., 1976; Timbrell et al., 1980). These further oxidation reactions are thought to be mediated by microsomal P450s, especially CYP2E (Delaney and Timbrell, 1995; Sarich et al., 1999).

INH can also be conjugated with numerous endogenous metabolites (Khan et al., 2016; Li et al., 2011), including ketone acids, vitamin B6 (pyridoxal and pyridoxal 5-phosphate), and NAD$^+$. In this manner, INH is able to disturb the homeostasis of endogenous metabolites such as vitamin B6, bile acids, cholesterol, and triglycerides (Cheng et al., 2013; Cilliers et al., 2010; Wason et al., 1981). The enzymes Catalase-peroxidase (KatG) of *MTB*, and human neutrophil myeloperoxidase can catalyze the formation of INH-NAD$^+$ adducts (Khan et al., 2016; Rozwarski et al., 1998). Conjugation of INH (INH) with ketone acids and vitamin B6 (pyridoxine) occurs at significant rates uncatalyzed.

Large differences exist between individuals in rates at which INH is acetylated. INH acetylation rate is inherited in simple Mendelian fashion. Individuals are slow, heterozygous rapid, or homozygous rapid acetylators. This patient phenotype forms the basis for determining a patient's level of INH-induced hepatotoxicity (Ellard GA, 1976). Herein, we evaluated INH acetylation patterns in tuberculosis patients receiving DOT therapy under the Revised National Tuberculosis Control Program (RNTCP) in a specialized government hospital in India.

2. Material and methods

2.1. Study design

This was an observational & experimental study aimed at assessing INH acetylation patterns in tuberculosis patients receiving DOT therapy.

2.2. Biochemical analysis

Blood samples were collected for biochemical analyses from TB patients who developed any untoward or adverse reaction. Samples for complete blood count (CBC) were collected in EDTA tubes, and serum samples for biochemistry were harvested from whole blood. Whole blood samples can only be stored at 2–8 °C for 24 h. Serum samples for biochemistry were harvested from whole blood after centrifugation, and supernatants obtained were used for biochemical analyses. This serum can be stored at −20 °C for several days. Biochemical analyses were performed using a fully automated instrument (Product no. Roche/Hitachi 902, Japan). Samples were visually analyzed for coagulum, hemolysis, or any type of lipemia, because hemolysis can artificially increase results in tests for AST, ALT, Potassium, and creatinine, and lipemia can interfere with spectroscopy.
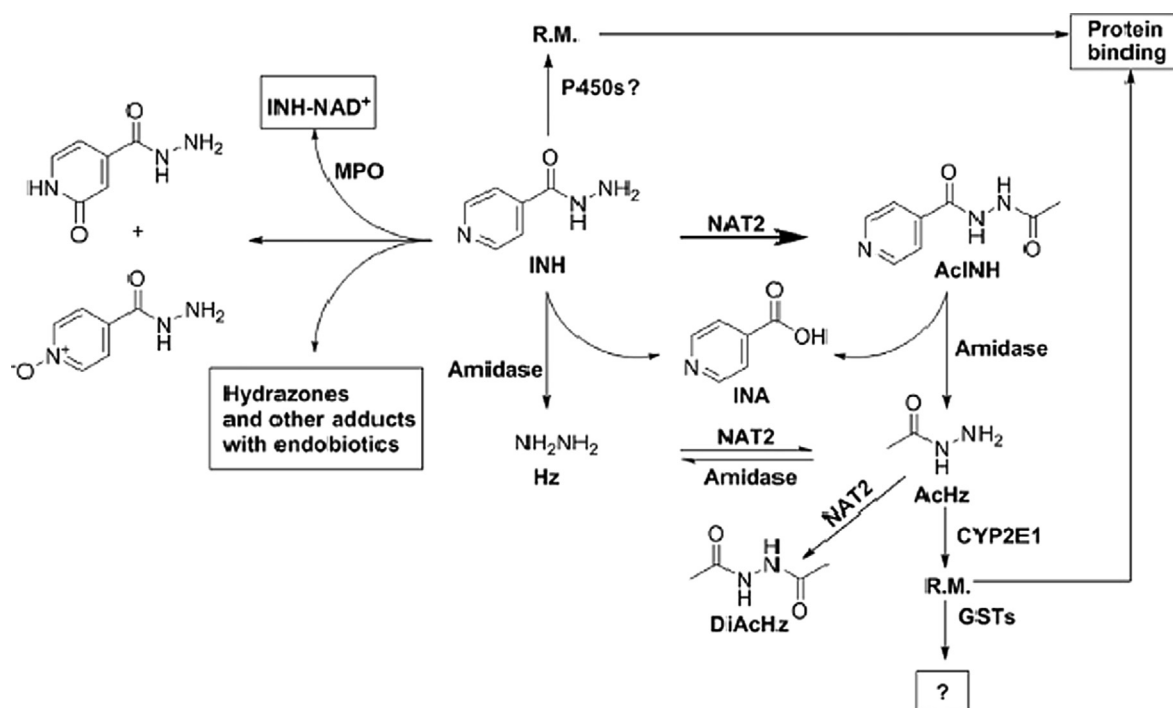

Fig. 1. A schematic representation of isoniazid (INH) metabolism and the enzymes catalyzing steps in the metabolic pathways of INH. AcHz: acetylhydrazine; AcINH: acetylINH; DiAcHz: diacetylhydrazine; GST: glutathione S-transferases; Hz: Hydrazine; INA: isonicotinicacid; MPO: myeloperoxidase; NAT2: N-acetyltransferase 2; P450: cytochromeP450; RM: reactive metabolite.

2.3. Study population and dose administration

A total of 1011 patients were enrolled in this study, but only 351 experienced adverse events resulting in sample collection for biochemical and adverse event analyses. Of these 351 patients, 36 had matching DOT therapy start times and gave informed consent to take samples for INH analysis. Blood samples were taken 1 h after administration of a 600 mg dose of INH. This study was carried out over a period of 18 months.

2.4. Bioanalytical procedure and plasma sample analysis

An HPLC connected to a UV detector (Shimadzu, Japan) was used for sample analysis. The method described by Sadeg with some modifications was used to estimate isoniazid concentrations (Sadeg et al., 1996). The mobile phase, consisting of absolute water-acetonitrile-triethyleamine-acetic acid with the ratio 600:400:2:1 was freshly prepared each day and used to separate analytes using a C-18 column (5μ, 250 × 4.6 mm). The mobile phase flow rate was fixed at 1.3 ml/min, and the analyte retention time was 5.4 min, in a total run time of 13 min. Analytes were detected at 340 nm.

2.4.1. Preparation of stocks and working standard solutions

As the drug is soluble in water and sparingly soluble in methanol, drug stock solutions were prepared in methanol. Solutions were made by accurately weighing 25 mg of INH working standard, transferring it to a 25 ml volumetric flask, and dissolving it in methanol to obtain a concentration of 1000 μg/ml. Five hundred microliters of this solution were transferred to a 25 ml volumetric flask and diluted to 25 ml with water for a final concentration of 10 μg/ml. This solution was used as a stock from which working standards were prepared. Serial dilutions from this stock were prepared with water to achieve working standards spanning the concentration range 0.5–8 μg/ml (Table S1).

2.4.2. Preparation of plasma calibration curve (CC) and quality control (QC) samples

Blank human plasma was spiked by transferring working standard solutions (0.5–8 μg/ml) into appropriate plasma volumes to achieve a calibration curve with a concentration range of 0.25–4 μg/ml. QC samples were also prepared by spiking plasma with working standard solutions (1, 3 and 6 μg/ml) to achieve low, middle, and high QC concentrations of 0.5, 1, and 3 μg/ml, respectively. All aqueous solutions were stored in a pharmaceutical refrigerator maintained at 4–6 °C. Spiked plasma and aqueous humor samples were stored in a freezer maintained at −20 ± 5 °C.

2.4.3. Sample preparation

Plasma samples (0.5 ml) were thawed at ambient temperature, vortex-mixed, and precipitated with 100 μl of 30% (w/v) trichloroacetic acid. They were then mixed on a vortex mixer and centrifuged for 5 min at 2000 rpm. Thirty microliters of 0.1% coupling reagent *trans*-cinnamaldehyde was added to 150 μl of the supernatant. After 10 min, 30 μl of 1 M KOH solution were added to obtain a pH range between 4 and 6. Finally, 20 μl of sample was injected into the HPLC for sample analysis.

2.4.4. Method validation

Our established method was partially validated prior to experimental sample analyses. Calibration curves were linear in the concentration range of 0.25–4 μg/ml. The correlation coefficient was found to be ≥ 0.998. Intra-day and inter-day precision and accuracy were evaluated using five replicates of all three QC samples, and analyte stability in plasma samples was determined according to guidelines for bioanalytical method validation.

2.5. Data analysis

In assigning phenotypes, a slow acetylator was defined as one whose plasma INH concentration was greater than the antimode, and a fast acetylator was one whose plasma INH concentration was less than antimode. Data are presented as incidence, means ± SD (90% C.I.) and odds ratios. Statistical analyses were performed using Statistical Analysis Software SPSS and ANOVA tests. P-values less than 0.05 were considered statistically significant.

2.6. Ethica approval

Ethical approval was obtained from the L.R.S. Institute of Tuberculosis and Respiratory Diseases, New Delhi, Human Research Ethics Committee, with reference AMS/EC/2008/12038. All patients gave informed consent for these studies.

3. Results

3.1. Demographic details of patients enrolled in DOTS

The demography of patients who experienced adverse events includes age, sex, weight in kg, height in cm, and BMI, and is presented as means ± SD for each center (Table 1). The mean ages for men vary from 28.45 (±14.873) to 34.00 (±16.405), whereas those for women vary from 23.00 (±11.205) to 39.14 (±25.354) years. BMIs (weights (kg)/heights squared (m^2)) are presented as means (±SD). Overall mean ages of men and women enrolled in the present study were 30.60 (±14.435) and 27.57 (±14.485), respectively. The average patient age was 29.30 (±14.514). Overall mean weight, height, and BMI of all patients in the present study were 45.30 kg (±10.550), 157.89 cm (±7.698), and 16.86 kg/m^2 (±4.160), respectively.

3.2. Biochemical changes and adverse events in patients undergoing DOTS

Biochemical changes during drug therapy are common because every drug is an active chemical entity which has pharmacological and toxic effects on the body that can lead to adverse drug effects. Blood samples were taken from patients before and after drug administration. Blood samples from patients who developed adverse events during combination anti-tuberculosis drug therapy were assessed for biochemical changes in liver and kidney functions, and CBC as per protocol.

Monitoring of liver function is very important in patients receiving DOT therapy, because components of DOT have been reported to cause hepatotoxicity. In this study, baseline ALT (SGOT) and AST (SGPT) levels were 23.77 ± 13.198, and 29.40 ± 13.752, respectively. After initiating standard DOT therapy, blood samples were taken from patients who developed any untoward reaction for relevant analyses. One blood sample was taken when an adverse event occurred, and a second blood sample was taken when the adverse event resolved or disappeared. In some patients, a third blood sample was taken later if assay results from the second sample were not normal even though symptoms had resolved.

Post-treatment liver transaminase values were high in patients who experienced adverse events. AST and ALT values (expressed as Mean ± SD) were within the normal range (i.e., 26.58 ± 13.079 and 31.57 ± 11.742, respectively) when the adverse events appeared in 210 patients, and within the normal range (i.e., 31.90 ± 22.815 and 28.53 ± 18.195, respectively) when the adverse events resolved in 234 patients. AST and ALT values were raised, 1–2-fold above the upper limits of normal (i.e., 46.62 ± 14.593 and 41.13 ± 10.131, respectively) when the adverse events appeared in 47 patients,

Table 1
Demographic characteristics of patients enrolled in DOTS therapy.

Patients Demography		Center 1	Center 2	Center 3	Center 4	Center 5	Center 6	All Centers
Age (Year)	Men Mean ± (SD)	29.29 ± (15.195)	31.09 ± (13.552)	34.00 ± (16.504)	28.45 ± (14.873)	33.40 ± (15.415)	29.85 ± (8.732)	30.60 ± (14.435)
	Women Mean ± (SD)	31.89 ± (16.193)	23.00 ± (11.205)	23.14 ± (10.518)	25.00 ± (9.808)	34.62 ± (15.538)	39.14 ± (25.354)	27.57 ± (14.485)
	Average Mean ± (SD)	30.50 ± (15.700)	27.90 ± (13.209)	28.38 ± (14.665)	26.34 ± (12.973)	33.88 ± (15.231)	32.26 ± (14.875)	29.30 ± (14.514)
Weight (kg) Mean ± (SD)		30.50 ± (15.700)	46.70 ± (10.648)	43.63 ± (10.213)	43.12 ± (10.361)	43.70 ± (9.040)	48.25 ± (10.557)	45.30 ± (10.550)
Height (cm) Mean ± (SD)		161.11 ± (5.599)	160.99 ± (13.018)	153.58 ± (19.870)	158.07 ± (12.137)	159.64 ± (14.730)	163.69 ± (7.981)	157.89 ± (7.698)
BMI (kg/m^2) Mean ± (SD)		8.77 ± (4.13)	17.83 ± (2.915)	18.69 ± (4.674)	17.07 ± (3.011)	17.16 ± (2.916)	16.75 ± (2.920)	16.86 ± (4.160)

and 1–2-fold above the upper limits of normal when the adverse events resolved in 56 patients (i.e., 47.98 ± 19.725 and 34.23 ± 12.311, respectively). AST and ALT values were 2–3-fold above the upper limits of normal when the adverse events appeared, but displayed lower values when the adverse events resolved, and normal values in subsequent follow up tests in 59 patients (i.e., 99.27 ± 32.046, 53.54 ± 13.319, and 33.73 ± 9.662) and 42 patients (i.e., 73.84 ± 27.432, 43.55 ± 11.990, and 23.43 ± 12.377), respectively. AST and ALT values were 3–5 times above the upper limits of normal when adverse events appeared, lower when the events resolved, and nearly normal in follow up tests in 35 patients (i.e., 165.70 ± 70.312; 78.44 ± 22.166 and 44.22 ± 12.788) and 19 patients (i.e., 177.16 ± 49.344; 98.54 ± 18.443 and 48.44 ± 12.788), respectively (Table 2).

Baseline alkaline phosphate (ALP) levels were found to be 227.23 ± 91.864. Post-treatment ALP values were within the normal range (i.e., 229.14 ± 105.653 and 229.14 ± 105.653, respectively) when adverse events appeared and/or resolved in 228 patients. Post-treatment ALP values were raised 1–2-fold above the upper limits of normal when adverse events appeared and/or resolved in 69 patients (i.e., 303.96 ± 117.055 and 268.66 ± 123.864, respectively). ALP values were 2–3-fold above the upper limits of normal when adverse events appeared and/or resolved, but reduced to nearly normal in a follow up test in 43 patients (i.e., 441.00 ± 192.470, 349.34 ± 178.406, and 281.45 ± 155.677). In 11 patients, ALP values were greater than 3-fold above the upper limits of normal when adverse events appeared and/or resolved, but lower or nearly normal in follow up tests (i.e., 577.22 ± 212.798, 391.11 ± 157.225, and 314.33 ± 167.443) (Table 2).

The baseline uric acid level (Mean ± SD) was found to be 5.85 ± 1.391, and post-treatment uric acid levels were within the normal range in 265 of the patients who reported adverse events both when the event occurred and when it resolved (i.e., 5.35 ± 0.625 and 4.56 ± 0.968, respectively). Post-treatment uric acid levels were 6–8 mg/dL when adverse events appeared and/or resolved, but subsequently returned to normal in 52 patients (i.e., 7.68 ± 0.821, 6.21 ± 0.533, and 5.42 ± 0.750). Post-treated uric acid values were greater than 8 mg/dL in 34 patients when their adverse events appeared and resolved, and subsequently returned to normal (8.48 ± 0.532, 7.23 ± 0.651, and 6.79 ± 0.893, respectively). Discontinuation of one or all anti-tuberculosis drug(s) was only required in 11 patients, in whom blood uric acid levels reached greater than 8 mg/dL (Table 2S).

Baseline male and female hemoglobin values were 12.02 ± 1.364 and 10.70 ± 1.640, respectively. Post-treatment mean hemoglobin values in 200 male patients who reported adverse events were 11.23 ± 1.332 when the adverse event occurred, and 12.42 ± 2.186 when it resolved. In the 151 female patients who reported adverse events, these values were 10.57 ± 2.759 when the event

Table 2
Biochemical Investigations of Liver Function in Patients Experiencing ADE.

Tests	Biochemical Liver Function Test Results		
	ALT (Mean ± SD)	AST (Mean ± SD)	ALP (Mean ± SD)
Baseline values (n = 1011)	23.77 ± 15.198	29.40 ± 13.752	227.23 ± 91.864
After DOTS initiation (n = 351)	**ALT values after adverse drug events**		
ALT/SGOT (U/L) Mean ± SD	Appeared	Resolved	Follow up Tests
Normal (n = 210)	26.58 ± 13.079	31.57 ± 11.742	–
1–2-fold above UNL (n = 47)	46.62 ± 14.593	41.13 ± 10.131	–
2–3-fold above UNL (n = 59)	99.27 ± 32.046	53.54 ± 13.319	33.73 ± 9.662
3–5-fold above UNL (n = 35)	165.70 ± 70.312	78.44 ± 22.166	44.22 ± 12.788
After DOTS initiation (n = 351)	**AST values after adverse drug events**		
AST/SGPT (U/L) Mean ± SD	Appeared	Resolved	Follow up Tests
Normal (n = 234)	31.90 ± 22.815	28.53 ± 18.195	–
1–2-fold above UNL (n = 56)	47.98 ± 19.725	34.23 ± 12.311	–
2–3-fold above UNL (n = 42)	73.84 ± 27.432	43.55 ± 11.990	23.43 ± 12.377
3–5-fold above UNL (n = 19)	177.16 ± 49.344	98.54 ± 18.443	48.44 ± 12.788
After DOTS initiation (n = 351)	**ALP value after adverse drug events**		
ALP (U/L) Mean ± SD	Appeared	Resolved	Follow up Tests
Normal (n = 228)	229.14 ± 105.653	229.14 ± 105.653	–
1–2-fold above UNL (n = 69)	303.96 ± 117.055	268.66 ± 123.864	–
2–3-fold above UNL (n = 43)	441.00 ± 192.470	349.34 ± 178.406	281.45 ± 155.677
greater than 3-fold above UNL (n = 11)	577.22 ± 212.798	391.11 ± 157.225	314.33 ± 167.443

ALT = Alanine transaminase, AST = Aspartate transaminase, ALP = Alkaline phosphatase, SGOT = Serum glutamate oxalo-acetate transaminase, SGPT = Serum glutamate pyruvate transaminase, U/L = Unit per liter, UNL = Upper normal Limit.

occurred, and 11.21 ± 1.372 when it resolved (Table 3S). The baseline mean total leukocyte count was 8579.41 ± 3204.443 cells/mm^3. Post-treatment total leukocyte counts when adverse events occurred, and when symptoms of the adverse events

resolved were 9732.35 ± 2542.412 cells/mm^3 and 9075.00 ± 3786. 129 cells/mm^3, respectively (Table 3S).

Baseline neutrophil (N), lymphocyte (L), and eosinophil (E) counts were 64.59 ± 11.794, 18.40 ± 9.855, and 3.25 ± 1.581, respectively. No significance differences in pre-treatment or post-treatment DLC were reported among patients who developed adverse events. Post-treatment neutrophil, lymphocyte, and eosinophil counts when adverse events occurred were 77.32 ± 12.923, 19.40 ± 9.855, and 6.34 ± 1.438, respectively. Post-treatment neutrophil, lymphocyte, and eosinophil counts when symptoms of adverse events resolved were 70.98 ± 15.420, 19.65 ± 10.916, and 4.55 ± 1.820, respectively (Table 3S).

3.3. Plasma INH concentrations in tuberculosis patients

Blood samples were taken from of 36 patients after 1 week of standard DOT therapy for plasma drug concentration analysis using HPLC. Plasma INH concentrations were in the range of 1.17–4.10 μg/ml. The mean ± SD plasma concentration was 2.335 ± 0.887 (p-value < 0.0001; interval of difference 2.018 to 2.750 at 95% CI) Table 3. Plasma concentration verses patient number profiles are shown in Fig. 2.

Of the 36 patients, 11 had plasma concentrations in the 2.5–3.0 μg/ml range, 8 had plasma concentrations in the 1.5–2.0 μg/ml range, 7 had plasma concentrations in the 1.0–1.5 μg/ml range, 5 had plasma concentrations in the 3.0–3.5 μg/ml range, and 2 each had plasma concentrations in 2.0–2.5 μg/ml and 3.5–4.0 μg/ml ranges. Only one patient had a plasma concentration in the 4.0–4.5 μg/ml range (Fig. 3).

3.4. Acetylation patterns among tuberculosis patients receiving DOTS

Most of the people of India are reportedly fast acetylators. In this study, of 36 patients tested, 20 (55.56%) were slow acetylators and 16 (44.44%) were fast acetylators. The histogram is bimodal with an antimode at 2–2.5 μg/ml. In assigning phenotypes, a slow acetylator was defined as one whose plasma INH concentration was greater than the antimode, and a fast acetylator was one whose plasma INH concentration was less than the antimode. By these criteria, we found that 20 of 36 patients (55.56%) were slow acetylators and 16 of 36 (44.44%) were fast acetylators (Fig. 4).
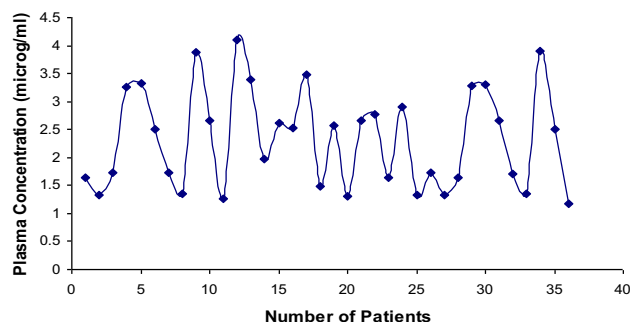

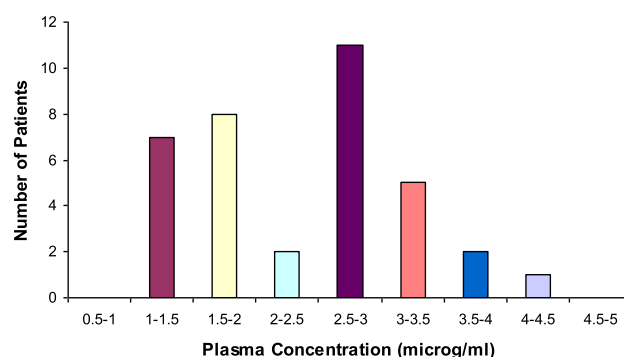**Fig. 2.** Plasma concentration of INH in individual tuberculosis patient.

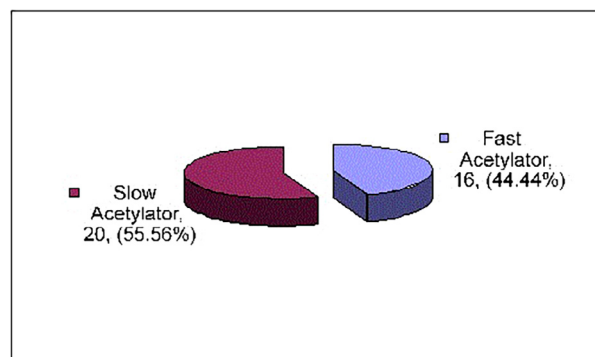**Fig. 3.** Distribution tuberculosis patients according to their plasma INH conc.

Fig. 4. Acetylation patterns among the tuberculosis patients receiving DOTS.

Table 3
Plasma INH concentrations in individual tuberculosis patients.

Patient's Identity	Plasma INH Concentration (μg/ml)	Patient's Identity	Plasma INH Concentration (μg/ml)
Patient-1	1.63	Patient-19	2.58
Patient-2	1.34	Patient-20	1.31
Patient-3	1.73	Patient-21	2.65
Patient-4	3.26	Patient-22	2.78
Patient-5	3.33	Patient-23	1.64
Patient-6	2.50	Patient-24	2.91
Patient-7	1.72	Patient-25	1.32
Patient-8	1.35	Patient-26	1.74
Patient-9	3.89	Patient-27	1.33
Patient-10	2.67	Patient-28	1.63
Patient-11	1.27	Patient-29	3.28
Patient-12	4.10	Patient-30	3.31
Patient-13	3.39	Patient-31	2.67
Patient-14	1.97	Patient-32	1.71
Patient-15	2.62	Patient-33	1.35
Patient-16	2.52	Patient-34	3.91
Patient-17	3.49	Patient-35	2.5
Patient-18	1.49	Patient-36	1.17

4. Discussion

The baseline liver function test results were either normal or slightly increased in the majority of patients who experienced adverse events. Totally, 351 patients experienced adverse effects. In 210 of these patients, AST and ALT values (expressed as Mean ± SD) were within the normal range (i.e., 26.58 ± 13.079 and 31.57 ± 11.742) when the adverse events occurred. These values were in the normal range (i.e., 31.90 ± 22.815 and 28.53 ± 18. 195) when the adverse events were resolved in 234 patients. AST and ALT values were 1–2-fold above the normal upper limit (i.e., 46.62 ± 14.593 and 41.13 ± 10.13) in 47 patients when the adverse event occurred, and in 56 patients (i.e., 47.98 ± 19.725 and 34.23 ± 12.311) when the adverse events were resolved. AST and ALT values were 2–3-fold above normal upper limits when adverse events occurred, lower but not normal when the adverse events were resolved, and normal in the subsequent test in 59 patients (i.e., 99.27 ± 32.046, 53.54 ± 13.319, and 33.73 ± 9.662) and

42 patients (i.e., 73.84 ± 27.432; 43.55 ± 11.990 and 23.43 ± 12.377), respectively. AST and ALT values were 3–5-fold above normal upper limits when adverse events occurred, lower but not normal when adverse events resolved, and normal in the subsequent test in 35 patients (i.e., 165.70 ± 70.312, 78.44 ± 22.166, and 44.22 ± 12.788) and 19 patients (i.e., 177.16 ± 49.344, 98.54 ± 18.443, and 48.44 ± 12.788), respectively. In a clinical trial, the median aspartate aminotransferase level was 3–10-fold above the normal upper limit in 16 participants taking INH for 9 months, and 2–10-fold above normal in 3 participants taking rifampin for 4 months. Corresponding alanine aminotransferase levels were 6–16-fold and 4–15-fold above normal upper limits, respectively (Menzies et al., 2008).

The median baseline alkaline phosphate (ALP) level was 227.23 ± 91.864. In patients undergoing DOT therapy, ALP values were within the normal range when adverse events occurred and/or resolved in 228 patients (i.e., 229.14 ± 105.653 and 229.14 ± 105.653). In 69 patients, ALP values were 1–2-fold above normal upper limits (i.e., 303.96 ± 117.055 and 268.66 ± 123.864) when adverse events occurred and/or were resolved. ALP values were 2–3-fold above normal upper limits when adverse events occurred and/or were resolved, but were reduced to normal in the subsequent test in 43 patients (i.e., 441.00 ± 192.470, 349.34 ± 178.406, and 281.45 ± 155.677). ALP values were > 3-fold above normal upper limits when adverse events occurred and/or were resolved, but reduced to normal in the subsequent test in 11 patients (i.e., 577.22 ± 212.798, 391.11 ± 157.225, and 314.33 ± 167.443).

In this study, the baseline uric acid level (Mean ± SD) was 5.85 ± 1.391. In patients undergoing DOTS, uric acid levels were within the normal range in 265 patients who reported adverse events (i.e., 5.35 ± 0.625 and 4.56 ± 0.968). Uric acid levels were 6–8 mg/dL when adverse events occurred and/or were resolved, and subsequently returned to normal in 52 patients (i.e., 7.68 ± 0.821, 6.21 ± 0.533, and 5.42 ± 0.750). After DOTS therapy initiation, uric acid levels were greater than 8 mg/dL in 34 patients upon occurrence of an adverse event, lower but above normal upon resolution, and normal upon subsequent measurement (8.48 ± 0.532, 7.23 ± 0.651, and 6.79 ± 0.893). Discontinuation of one or all anti-tuberculosis drug(s) was required in only 11 patients in whom blood uric acid levels exceeded 8 mg/dL.

Baseline hemoglobin values among male and female patients were 12.02 ± 1.364 and 10.70 ± 1.640, respectively. The mean hemoglobin value in treated male patients who reported an adverse event was 11.23 ± 1.332 when the event occurred, and 12.42 ± 2.186 when the event was resolved. In female patients, the mean level upon occurrence of the adverse event was 10.57 ± 2.759, and upon resolution was 11.21 ± 1.372. The baseline total leukocyte count was 8579.41 ± 3204.443. After initiation of DOTS therapy, the total leukocyte counts were 9732.35 ± 2542.412 cells/mm^3 when adverse events occurred, and 9075.00 ± 3786.129 cells/mm^3 upon resolution of symptoms. One clinical trial has reported a reduction in leukocyte count of at least 1.00 cells/mm^3 in 41% of participants taking rifampin (with a full set of measurements), and 10% had a reduced count of $\geq$ 2.50 cells/mm^3 (Menzies et al., 2008).

Baseline neutrophil (N), lymphocyte (L) and Eosinophil (E) counts were 64.59 ± 11.794, 18.40 ± 9.855, and 3.25 ± 1.581, respectively. Post-treatment neutrophil, lymphocyte and Eosinophil counts upon adverse event occurrence were 77.32 ± 12.923, 19.40 ± 9.855, and 6.34 ± 1.438, respectively. Post-treatment neutrophil, lymphocyte, and Eosinophil counts upon resolution of adverse event symptoms were 70.98 ± 15.420, 19.65 ± 10.916, and 4.55 ± 1.820, respectively. No significant differences were reported in patients who developed adverse events relative to baseline counts or those after initiation of DOTS therapy. One

clinical trial reported baseline neutrophil counts of 1.90×10^9 cells/L and 1.76×10^9 cells/L in patients entering the two treatment cohorts, which decreased to nadir levels of 0.66 and 0.81×10^9 cells/L, respectively, after 4 to 5 weeks of therapy. Both groups remained asymptomatic, and neutrophil counts returned to near-baseline levels within 2 weeks after therapy was stopped (Menzies et al., 2008).

We used an analytical method described in (Sadeg et al., 1996) with modifications to determine plasma INH concentrations in patients receiving DOT therapy. This method was modified and partially validated according to ICH guidelines, and was found to be suitable for these analytical procedures and stable throughout the experiment. Interference was greatly reduced with our analytical method, and found to be insignificant, as plasma samples were screened for interference by other biological components and no interfering peaks were seen at the retention time of INH. System selectivity was evaluated by injecting three lots of blank plasma. An interfering peak was observed at the retention time of the drug, but the interfering peak's area under the curve was insignificant relative to that of the drug peaks. Based on these 3 injections, the coefficient of variation (% CV) in area was 0.8%. The method was accurate and precise for plasma sample analysis, as intra–day variation ranged from 0.86% to 4.13%, and inter-day variation ranged from 1.38% to 6.04%. The intra-batch accuracy of the method ranged from 98.50% to 105.21%, and the inter-batch accuracy ranged from 100.37% to 108.28%. These parameters are in concordance with bioanalytical guidelines for analytical method validation. This method demonstrates linearity over a concentration range of 0.25–4 μg/ml, and total recovery was 96.21–102.89%. The analyte of interest (INH) was stable through three-freeze thaw cycles and for up to 40 h on bench top. Hence, this method is accurate, precise, linear, able to demonstrate stability, and partially passes the validation criteria in the bioanalytical guidelines document for analytical method validation. Therefore, it was used to analyze INH in plasma samples.

The patients' data, and the bimodal distribution of proposed parameters shows that the INH method allowed clear-cut classification of study participants into slow and fast acetylators. Computation of antimode allowed assignment of slow acetylator status to 20 patients (55.56%) and fast acetylator status to 16 patients (44.44%). Our results are consistent with an earlier study conducted on Gujarati and Marathi populations in Mumbai that found that 46.8% of participants were slow acetylators and 54.2% of participants were fast acetylators (Kshirsagar et al., 1987). Another study conducted at the All India Institute of Medical Science in New Delhi found that 66% of participants were slow acetylators, and 34% were fast acetylators (Singh et al., 1996). A similar study conducted among North Indian populations reported that 14.55% of participants were slow acetylators, 46.36% were intermediate speed acetylators, and 39.09% were fast acetylators (Gupta et al., 1984). Similar results have been reported in earlier studies that showed that subjects with low plasma levels after drug administration rapidly acetylate the drug in the liver (Evans et al., 1983; Salako and Aderounmu, 1977).

The rate of INH inactivation is genetically determined (Evans et al., 1983; Harris et al., 1958; Peter and Gordon, 1987). Marked racial differences in prevalence of rapid and slow INH inactivators have been reported by various investigators. Rapid inactivator frequencies range from 95% among Eskimo populations (Armstrong and Peart, 1960) to 32% among the Swedish (Hanngren et al., 1970). Reported INH rapid inactivator frequencies are 89% among Koreans and 36% among the Finns (Mattila and Tiitinen, 1967). These variations in incidence have been ascribed to ethnic differences. In the present study, slow and fast acetylators were evenly distributed among participants, so INH dosing should be modified on an individual basis according to each patient's acetylator

phenotype and/or plasma INH concentration to increase efficacy and minimize adverse effects.

5. Conclusions

Plasma INH concentrations among tuberculosis patients receiving standard DOTS therapy were determined using a validated HPLC method, and show distinct acetylation rate profiles. The histogram is bimodal with an antimode at 2–2.5 μg/ml. In the present study, slow and fast acetylators were roughly evenly distributed among the patients, suggesting that INH dosage should be modified on an individual basis according to each patient's acetylator phenotype and/or plasma INH concentration to increase drug efficacy, and minimize adverse events. Further studies, including more patients, are recommended to more broadly identify INH acetylation patterns among populations.

Acknowledgements

Declaration of Competing Interest

Faisal Imam, Manju Sharma, Khalid Umer Khayyam, Mohammad Rashid Khan, Mohammad Daud Ali, Wajhul Qamar have no conflicts of interest directly relevant to the content of this study.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2020.04.003.

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