Full Length Research Paper

Pharmacokinetics of isoniazid in Ethiopian children with tuberculosis in relation to the N-acetyltransferase 2 (NAT2) genotype

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Until recently, the dose of isoniazid used to treat tuberculosis was the same for all patient groups. However, the pharmacokinetic profile of isoniazid varies across different populations. A comparative, observational, single-dose, 5 h study was conducted evaluating the pharmacokinetics of isoniazid in Ethiopian children. Pharmacokinetic parameters for a dose of 5 mg/kg and NAT2 genotype were determined in 29 children with tuberculosis (<15 years, with mean age of 8.6). Initially, univariate analyses evaluated covariates that exhibited associations ($p < 0.2$) with isoniazid pharmacokinetic parameters. Covariates with associations, acetylator genotype ($p < 0.01$) and age ($p < 0.1$) were further analysed with multiple linear regression. Sixteen (55%) were genotyped as rapid and 13 (45%) as slow acetylators. Four rapid acetylators had 2 and 3 h post-dose concentrations of < 3 and 1.5 $\mu\text{g/ml}$, respectively. Multiple linear regression analyses revealed acetylator status to be the only predictor of k , area under the curve ($\text{AUC}_{2 \rightarrow 5\text{h}}$), and isoniazid concentrations at 2, 3, 4 and 5 h. The mean values of these variables were also found to differ between genotypes ($p < 0.0025$). These findings reaffirm that 5 mg/kg isoniazid dose may not provide adequate plasma drug levels in all paediatric patients. Thus, isoniazid dose for children should be higher than 5 mg/kg body weight to cover the diverse acetylation kinetics.

Key words: Acetylator status, paediatric tuberculosis, isoniazid, pharmacokinetics, NAT2, Ethiopia.

INTRODUCTION

Six decades after its introduction into clinical practice, isoniazid (INH) remains a main-stay agent of all first-line anti-tuberculosis (anti-TB) regimens owing to its remarkable bactericidal activity against the metabolically active organisms and relatively low toxicity profile. INH is

also a valuable component of anti-TB regimens as it prevents the emergence of resistant strains against companion agents (Donald et al., 2004, 2007; Mitchison 2000; Vilchze and Jacobs, 2000). The pharmacokinetics of INH in humans has been well characterized. After both

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oral and parenteral administrations, it is rapidly and completely absorbed and peak plasma concentrations usually occur within 1 to 2 h after oral administration (Weber and Hein, 1979). INH exhibits poor plasma protein binding pattern, and crosses biological membranes readily with appreciable distribution into body compartments (Schaaf et al., 2005). The main metabolic route of INH is acetylation to acetyl-INH by arylamine N-acetyltransferase-2 (*NAT2*) enzyme (Donald et al., 2007).

The common genetic polymorphism responsible for the pharmacogenetic variation as rapid and slow acetylator in INH metabolism involves the *NAT2* gene, and response of the different genotypes to INH differs considerably (Donald et al., 2007). It has been shown that *NAT2* genotype accounts for considerable variability in INH pharmacokinetics (Chen et al., 2009; Gumbo et al., 2007; Schaaf et al., 2005).

The pharmacokinetic profile of INH varies in different patient populations. This has been extensively studied in adults. However, the kinetic data for children particularly that embrace the acetylator genotype are scanty, and the available evidence is derived from few studies (McIlleron et al., 2009; Schaaf et al., 2005).

The one size fits all INH dosing approach, where both adults and children prescribed INH doses of 4 to 6 mg/kg/day for the treatment of tuberculosis was adopted for decades by many professional bodies including the World Health Organization (WHO) (2003). In recent years, however, there are some amendments in the recommendations that children should receive higher INH doses than adults. For instance, WHO (2010) and the South African national guidelines (2004 and 2009) have now changed the INH dose recommendations from 5 to 10 mg/kg, with a range of 10 to 15 mg/kg. On the other hand, National programs such as in Ethiopia (2010) still follow the classic one size INH dosing approach.

In selecting the optimum dosage regimen, extrapolation of the findings from adult studies to children would be irrational. The pharmacokinetic profile of the drug in children who are efficient metabolisers could significantly differ from that in adults. This study therefore attempted to assess the pharmacokinetic aspects of INH in children prescribed 5 mg/kg INH dose for treatment of tuberculosis (TB) in Ethiopia.

MATERIALS AND METHODS

Study design

The study was undertaken in Paediatric ward of Tikur Anbessa teaching hospital in Addis Ababa, Ethiopia. Children between 1 to 15 years of age admitted for treatment of newly diagnosed TB (all forms) were recruited after written informed consent obtained from parents/guardians. Verbal assent was also obtained for children above age of 12 years. Patients were excluded if they had already

been on an anti-TB regimen, were severely ill, had clinical evidence of liver damage or anaemia, showed sustained dissent or if consent was not obtained. An earlier study by Schaaf et al. (2005) has indicated the average mean values for 2 h post dose plasma concentrations of INH in rapid (fast and intermediate) and slow acetylator children to be 4.5 and 8.6 µg/ml, with average standard deviations of 1.974 and 1.81, respectively. Win Episcope software adopted from Epi Info was used to compute the sample size using means and standard deviations from the above study. Accordingly, a sample size of 14 (7 rapid and 7 slow acetylators) was calculated to assess the effect of acetylator genotype on INH pharmacokinetic variables, with a confidence interval of 95%. The effect size was 2.1, and to detect this effect size a power of 90% was assumed. The actual sample size was increased until the required number of the study subjects in both categories was attained.

A standard INH powder obtained from Sigma-Aldrich Inc. was used for pharmacokinetic analysis. INH was administered orally at a nationally prescribed dose of 5 mg/kg body weight to overnight fasted treatment-naïve children. Blood specimens of 1.5 to 2 ml each were collected at 2, 3, 4, and 5 h after dosing and were centrifuged (3000 *g* × 3 min) at 0°C immediately after each collection to separate the plasma samples. The plasma samples were then stored at -80°C until analysis. The multi-drug anti-TB regimen was initiated on the day following the 5 h INH administration. A 2 to 3 ml blood sample was further taken for genotyping assay on the sixth day after the first sample collection and was stored at -20°C.

Plasma INH concentration determination

INH concentrations were quantified by established high performance liquid chromatography (HPLC) method (Seifart et al., 1995). The accuracy of the HPLC method in terms of average percentage recovery was determined after five repeated injections at nine appropriately spaced calibration points and was found to be 98.8% (concentration value (CV) ± 2.2%), with a percent recovery of 96.7% (CV ± 3.1%) at lower limit of quantification (0.5 µg/ml). The intra-sample coefficient of variations of the concentration values were also less than 5%.

NAT2 genotype determination

Genomic DNA (gDNA) was extracted from the whole blood by using the ArchivePure™ DNA Purification Kit (5 PRIME Inc.), according to the manufacturer's protocol. A DNA fragment spanning 866 bp, which contained the whole coding region of the *NAT2* gene, was amplified from gDNA by polymerase chain reaction (PCR) with two previously reported primers (O'Neil et al., 2000) 5'-GGCTATAAGAACTCTAGGAAC-3' and 5'-AAGGGTTTATTTGTTCTTATTCTAAAT-3'. A combination of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and allele specific PCR was applied for analyzing *NAT2**4, *NAT2**5, *NAT2**6, *NAT2**7, *NAT2**14, and *NAT2**17 alleles on the basis of previously described methods (Cascorbi et al., 1995) with some modifications. Subjects that are either homozygous or heterozygous to the wild *NAT2**4 allele were categorized as rapid acetylators, whereas those with homozygous or two heterozygous mutant alleles were taken as slow acetylators.

Pharmacokinetic parameters

The AUCs over the interval 2 to 5 h (AUC_{2-5h}) were calculated from INH concentration-time plot by trapezoidal rule. The apparent first

Table 1. Clinical and demographic features of children with tuberculosis assessed for acetylation status.

Clinical or demographic features	Number	%
Age group (years)		
≤ 4	6	20.7
4-9	8	27.6
9-15	15	51.7
Sex		
Male	16	55.2
Female	13	44.8
Body Mass Index		
< 18.5	15	51.7
18.5-25	14	48.3
Type of TB		
Pulmonary	14	48.3
Extrapulmonary	8	27.6
Disseminated	7	24.1
Extrapulmonary TB		
Peripheral TB lymphadenitis	6	75
TB pericarditis	1	12.5
TB spondylitis	1	12.5
Concomitant drug use		
Yes	5	17.2
No	24	82.8
Rapid acetylators alleles		
4*/4*	2	6.9
4*/5*	6	20.7
4*/6*	7	24.1
4*/14*	1	3.5
Slow acetylators alleles		
5*/5*	3	10.3
5*/6*	8	27.6
5*/7*	1	3.5
6*/7*	1	3.5

order elimination rate constant (k , h^{-1}) of the individual patients were obtained from slope of linear regression of the natural logarithm of INH concentration at time t ($\ln C_t$) and t from 2 to 5 h.

Statistical analyses

The INH pharmacokinetic data were summarized as mean value of the groups with respective standard error of the mean. The covariate

effects of age, weight, sex, acetylator genotype, type of TB and concomitant drug use on the pharmacokinetic variables (k , AUC_{2-5h} , and 2 to 5 h INH concentrations) was assessed by linear regression analyses. A logarithmic transformation was used on k values to reduce skewness and to improve the distribution of residuals. A multiple linear regression was further performed to evaluate the effects of covariates that were found to be possibly associated ($p < 0.2$) with INH pharmacokinetics in the univariate analyses or that have literature support. Colinearity diagnostics were employed to rule out possible multicollinearity between variables. Student t -test was used for unpaired data to test statistically significant group mean differences. Levene's test was also used to assess the homogeneity of group variance prior to a t -test parametric procedure. The relation between age and pharmacokinetic parameters were reassessed across the stratified genotypes by linear regression. Significance was set at $p < 0.05$. The statistical analyses were performed with statistical package for social sciences (SPSS), version 15.

Ethical considerations

The study protocol was reviewed and approved by the Armauer Hansen Research Institute/All Africa Leprosy, Tuberculosis and Rehabilitation Training Centre (AHRI/ALERT) and National Research Ethics Review Committees.

RESULTS

Patient characteristics

A total of 29 newly diagnosed inpatient paediatric TB patients with a mean age of 8.7 years (1.5 to 14.8 years) were enrolled in the study. Table 1 summarizes details of demographic and clinical features of the participants.

Genotyping

The presence of single nucleotide polymorphisms (SNPs) at positions 191, 341, 434, 590, and 857 of *NAT2* gene was tested for all 29 patients. Among the SNPs tested, *NAT2**5 (341T > C) was the most frequently seen SNP with occurrence rate of 36.2%, followed by *NAT2**6 (590G > A) which occurred in 27.6% of the cases. The least frequent SNPs were *NAT2**7 (857G > A) and *NAT2**14 (191G > A), with frequencies 3.5 and 1.7%, respectively. The wild type *NAT2**4 that lacks any of these SNPs was observed with an occurrence rate of 31%. The genotype data also showed that *5/*6 was the most frequent haplotype, which occurred in 8 (28%) of the patients followed by *4/*6 and *4/*5 with 7 (24%) and 6 (21%) occurrence rates, respectively. Accordingly, 16 children (55%) with a median age of 9 years were genotyped as rapid acetylators, and 13 (45%) with a median age of 11 years as slow acetylators. Among the rapid acetylators, only 2 (13%) were homozygous (fast) and the rest 14 (87%) were heterozygous (intermediate)

Table 2. Degree of associations obtained between INH pharmacokinetic variables and acetylator status, age and weight from multiple linear regression analyses.

Variables	2 h INH conc. [$\beta \pm SE^*$ (Sr^{2**})]	3 h INH conc. [$\beta \pm SE^*$ (Sr^{2**})]	4 h INH conc. [$\beta \pm SE^*$ (Sr^{2**})]	5 h INH conc. [$\beta \pm SE^*$ (Sr^{2**})]	AUC2-5 h [$\beta \pm SE^*$ (Sr^{2**})]	K (log) [$\beta \pm SE^*$ (Sr^{2**})]
Slow acetylator status	1.50 $\pm$ 0.37 ^a (0.349)	1.31 $\pm$ 0.33 ^a (0.31)	1.10 $\pm$ 0.26 ^a (0.336)	0.91 $\pm$ 0.24 ^a (0.291)	3.74 $\pm$ 0.91 ^a (0.341)	-0.15 $\pm$ 0.05 ^a (0.262)
Age (years)	0.65 $\pm$ 0.10 (0.009)	0.14 $\pm$ 0.09 (0.053)	0.13 $\pm$ 0.07 (0.066)	0.12 $\pm$ 0.07 (0.065)	0.37 $\pm$ 0.25 (0.044)	-0.02 $\pm$ 0.01 (0.046)
Weight (kg)	0.07 $\pm$ 0.04 (0.001)	0.02 $\pm$ 0.03 (0.012)	0.04 $\pm$ 0.03 (0.029)	0.03 $\pm$ 0.03 (0.029)	0.08 $\pm$ 0.10 (0.014)	-0.01 $\pm$ 0.01 (0.057)

*Regression coefficients $\pm$ Standard error ** Semipartial correlations ^a $p < 0.01$.

acetylators.

Pharmacokinetics

HPLC chromatograms with well resolved peaks were obtained for all samples. INH concentration at 2 h after dosing, which is frequently quoted in the literature as a convenient reference point, varied from as low as 1.76 μ g/ml to as high as 7.67 μ g/ml (Figure 1). A 2 and 3 h post-dose INH plasma concentration of 3 to 5 μ g/ml (Peloquin et al., 1996) and 1.5 μ g/ml (Schaaf et al., 2005), respectively have been suggested as a required range for optimal bactericidal effects. Even though the above validated therapeutic ranges for INH were achieved in majority (86%) of the study subjects, four (14%) had 2 and 3 h concentrations less than 3 and 1.5 μ g/ml, respectively. Among these four, one even failed to achieve a concentration of 2 μ g/ml at 2 h.

Univariate regression analyses showed that there was no association ($p > 0.2$) between INH pharmacokinetic parameters and the covariates: weight, sex, type of TB and concomitant drug use. Acetylator genotype ($p < 0.01$) and age ($p < 0.1$), were however found to be strongly and weakly associated, respectively with the kinetic parameters. Since several pharmacokinetic studies in

children strongly support the relationship between weight and drug elimination (Serrano et al., 1999; Trenque et al., 2004; Laer et al., 2005; Aumente et al., 2006; El-Tahtawy et al., 2006; Anderson and Holford, 2008), the effect of weight on INH pharmacokinetics was reassessed with acetylator genotype and age using multiple regression analysis. The analyses revealed that only acetylator status was found to be a strong predictor of all pharmacokinetic parameters (k, AUC_{2→5h}, and 2 to 5 h INH concentrations), while age and weight failed to make any contribution to the model (Table 2). For this model, the correlation coefficient (R) was significantly different from zero ($p < 0.01$) for all set of variables. The adjusted R² values indicate that around 40% of the variability in INH concentrations could be explained by the model. Even though there appeared to be a considerable overlap in the observed variables between the genotypes, plasma INH concentrations and AUC_{2→5h} were higher and elimination rate constants lower in the slow compared to the rapid acetylators. For instance, the lowest 2 h INH concentration (1.76 μ g/ml) was observed in a rapid acetylator subject, and the highest (7.67 μ g/ml) was noted in a slow acetylator. Comparison of the 2 h plasma INH concentrations for the two genotypes is illustrated in Figure 1. The homogeneity of group means

were tested for each pharmacokinetic variable with 95% confidence, and there was a statistically significant difference in each of the variables between the genotypes ($p < 0.01$ in each instance) (Table 3). In addition, all four observed INH plasma levels below the target ranges occurred in rapid acetylators, with a rate of 25%, and the two homozygous rapid acetylators were amongst them. The proportions of male and female were 9 (56%) and 7 (44%) for rapid acetylators and 7 (54%) and 6 (46%) for slow acetylators, respectively. Thus, tests of homogeneity of group means of pharmacokinetic variables of the two sexes were performed within the genotypes by using a two tailed t-test, with 95% confidence. The values of k, AUC_{2→5h}, and 2, 3, 4, and 5 h INH concentrations were not significantly different between males and females in both genotypes.

The effect of age on INH pharmacokinetics was further examined by linear regression analysis after stratifying the study subjects into acetylator genotypes. In both genotypes, fitting straight line regressions on age for k, AUC_{2→5h} and INH concentration at the time points of 2, 3, 4, and 5 h was performed. In slow acetylators, with increasing age, a significant decline in transformed k rest of the pharmacokinetic variables was observed. At 95% confidence interval, the deviation

Table 3. The mean first order elimination rate constant (k), the area under the curve (AUC), and the mean plasma INH concentrations at 2, 3, 4, and 5 h after dosing with 5 mg/kg INH in rapid and slow genotypes among children with tuberculosis assessed for acetylation status.

Pharmacokinetic variable	Genotype (n)		p*	
	Rapid (16)	Slow (13)		
K (h ⁻¹)	0.51±0.04	0.34±0.02	0.0024	
AUC _{2→5} (µg × h/ml)	5.53±0.53	9.62±0.76	<0.0001	
INH concentration (µg/ml)	2 h	3.46±0.21	5.03±0.31	0.0002
	3 h	2.12±0.20	3.57±0.28	0.0002
	4 h	1.35±0.15	2.57±0.22	<0.0001
	5 h	0.87±0.13	2.05±0.28	0.0003

Data are mean $\pm$ standard error of the mean, n is number of study subjects; k- elimination rate constant; AUC_{2→5h}-area under the plasma concentration curve over the interval 2 to 5 h; *p values by student two-tailed t-test to evaluate equality of the pharmacokinetic variables between the slow and rapid acetylators groups.

Figure 1: Bar charts showing 2 h Plasma INH concentrations ($\mu\text{g/ml}$). Slow (A) and rapid (B) acetylators in 29 children with TB after an INH dose of 5 mg/kg. [INH]-isoniazid concentration, *homozygous rapid acetylators

of slope from zero was statistically significant ($p < 0.05$ in each instance). However, the relations obtained in rapid acetylators were neither uniform nor were the slopes significantly different from zero.

DISCUSSION

The influence of acetylator genotype and age on INH pharmacokinetics of children has been well described (McIlleron et al., 2009; Schaaf et al., 2005). In this study, the INH pharmacokinetic profile of the study subjects was best predicted by acetylator genotype, while age contributed to a lesser extent only in the univariate model.

Furthermore, statistically significant differences in INH handling were observed between slow and rapid acetylators children. These results underscore the considerable differences in pharmacokinetic variables of INH between fast and slow acetylators children as reported elsewhere (McIlleron et al., 2009; Rey et al., 2001; Schaaf et al., 2005). Despite the statistically significant differences, a clear margin of drug exposure was not observed among some individuals between the genotypes. Such an overlap between the fast and slow acetylators children was also suggested by Cranswick and Mulholland (2005) in their commentary article on the report of Schaaf et al. (2005). In this study, 25% of the rapid acetylators children did not reach a 2 h concentration of 3 $\mu\text{g/ml}$ and a 3 h concentration of 1.5 $\mu\text{g/ml}$. The incidence of poor INH plasma levels was also higher in the homozygous than the heterozygous rapid acetylators. These findings, in conjunction with earlier published reports in children (McIlleron et al., 2009; Schaaf et al., 2005) point to the fact that rapid acetylators children would fail to achieve the

desirable concentrations with 5 mg/kg dose of INH, homozygous types being at a greater risk than the heterozygous ones. It is worth noting that the proportion of rapid acetylators that did not achieve the recommended plasma concentration varies with the dose of INH used. For instance, all homozygous rapid acetylators failed to achieve the desired concentration of INH in the present study as well as in the study published by McIlleron et al. (2009).

The proportion of homozygous rapid acetylators who did not achieve the desired concentration came down with increasing INH dose (McIlleron et al., 2009; Schaaf et al., 2005). A different pattern emerges with heterozygous rapid acetylators. The proportion that failed to achieve the desired concentration with 5 mg/kg dose of INH was 14% (present study) and 90% (McIlleron et al., 2009). However, all recruited heterozygous children had achieved the recommended concentration with increasing dose (McIlleron et al., 2009).

Moreover, earlier pharmacokinetic studies conducted in Ethiopian children also showed that drug levels within therapeutic ranges could be achieved with INH dose of 10 mg/kg (Eriksson et al., 1988). These findings reaffirm the notion that higher dosing of INH could diminish the incidence of low drug levels in fast acetylators. In addition, one of the currently recommended doses in children (10 mg/kg) is reported to sufficiently treat majority of the children and is not associated with unacceptable adverse reactions (Cranswick and Mulholland, 2005). Even though the data generated so far provide justification, at least in part, for the recommendation of higher mg/kg doses of INH in children, further studies are required to tailor the higher doses based on disease severity, age and acetylation status for optimal treatment outcomes, with the least possible risk of toxicity.

The study demonstrated that age contributed to the univariate pharmacokinetic model of the slow acetylator group, but not to that of rapid acetylators. The absence of the expected effects of age could in part be explained by strongly skewed age pattern of the study participants (with median age of 9) to the maturation point of NAT2, which is around 10 to 12 years (Pariente-Khayat et al., 1991). Another reason could be the smaller number of younger children within a genotype, as the sample size was not determined, taking into account age influences, which is a limitation of the current study. As age is more essential for defining pharmacokinetics in young children and infants compared with older children (Holford, 2010), further evaluation of INH pharmacokinetics in small children is necessary.

An interesting observation in this study was the low frequency of the NAT2*14 allele. This SNP is considered as a specific feature for black Africans (Hein et al., 2000; Osborne 2003). Surprisingly, this SNP was observed in

only one of the 29 Ethiopian study participants. The relative scarcity of this allele in the Ethiopian population was also reported by Yimer et al. (2006), where none of the 128 Ethiopian adults screened for the NAT2*14 allele expressed this SNP. Even though there is not complete absence of the allele in the population, as demonstrated by its presence in one of the study subjects, it nevertheless indicates that the prevalence rate of the NAT2*14 is very low, at least in the studied Ethiopian population.

Conclusion

Our study presents evidence that 5 mg/kg INH dose might not provide adequate plasma levels in paediatric TB patients who are rapid acetylators. Thus, in settings where dosage cannot be tailored based on acetylator status, children should receive a higher mg/kg body weight INH dose to ensure that the rapid acetylators, especially the homozygous, would attain an optimal INH plasma concentration.

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