Isoniazid and Acetylisoniazid Urine Concentrations as a Marker of Adherence to Isoniazid Preventive Therapy in Children

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DECLARATION

I, Veronica Asibi Amlabu, declare that the work on which this thesis is based is my original work unless stated otherwise and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree at this or any other university.

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ABSTRACT

The World Health Organization recommends the use of isoniazid preventive therapy to reduce the incidence of tuberculosis disease in populations at risk for developing the disease. Adherence to isoniazid preventive therapy is needful for efficacy. A urine assay known as the Arkansas test is widely used to monitor the ingestion of isoniazid. However, this test is limited by a drop in sensitivity with increasing time post isoniazid dose. Furthermore, results from the test can be affected by observer variation in colour changes, concomitant medications, urinary dilution, and presence of other substances in urine. Moreover, the Arkansas test results have not been evaluated against objective measurement of isoniazid and acetylisoniazid urine concentrations. This study seeks to measure the urine concentrations of isoniazid and acetylisoniazid at different time points after a dose in children, in order to establish reference ranges for liquid chromatography tandem mass spectrometry measurements of urine isoniazid and acetylisoniazid, as well as the Arkansas method. The reference ranges would provide a standard which can be used to estimate the probability of adherence to prior doses.

Urine samples were collected from forty-one children on daily and intermittent isoniazid preventive therapy at different time points. Isoniazid and acetylisoniazid urine concentrations were determined using liquid chromatography tandem mass spectrometry measurements. Effect of age, weight, gender, dosing time, dosing frequency and urine creatinine on isoniazid and acetylisoniazid concentrations were evaluated. Arkansas test was carried out and results were evaluated using liquid chromatography tandem mass spectrometry measurements of urine isoniazid and acetylisoniazid.

Reference ranges for urine isoniazid and acetylisoniazid concentrations were established in the study population. No effect of age, weight, gender and dosing frequency was detected on isoniazid and acetylisoniazid urine concentrations. There was good agreement between Arkansas colour change and the concentrations of isoniazid and acetylisoniazid. Arkansas test is useful for monitoring adherence to 4 hours post isoniazid doses (prior to clinic attendance) in children on isoniazid preventive therapy. It is however not useful for monitoring adherence to 24 hours post isoniazid doses.

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1. INTRODUCTION

In areas with high tuberculosis (TB) incidence, an important cause of morbidity and mortality in children is TB (Andvord, 2002; World Health Organization, 2008). Isoniazid Preventive Therapy (IPT) is recommended by World Health Organization (WHO) for children at risk of developing TB disease as a strategy to control and prevent tuberculosis (TB) (World Health Organization). For efficacy and to prevent the development of resistance, there is a need for adherence to therapy. The different methods used to monitor and assess adherence to IPT including self-reports, pill counts, electronic monitoring devices and urine assays all have their limitations (Perry *et al.*, 2002).

The Arkansas test method, a colorimetric method, is widely used to monitor the intake of isoniazid (INH). It identifies the presence of INH and its metabolites in urine. Results from the test can however be affected by factors such as observer variation in interpretation of colour changes, interference from concomitant medications, presence of other substances in urine, urine dilution and a major limitation of the test is a decrease in sensitivity with increase in time post INH dose. Previous studies on European adult populations showed sensitivity of greater than 90% up to 24 hours post INH dose and similar sensitivity was only achieved up to 12 hours post dose in black African adult population (Hanifa *et al.*, 2007; Schraufnagel *et al.*, 1990). The Arkansas test result however, has not been evaluated against objective measurement of INH and acetyl INH (AcINH) urine concentrations.

This study seeks to measure the urine concentrations of INH and AcINH at different time points after a dose in children, in order to establish reference ranges for liquid chromatography tandem mass spectrometry measurement of INH and AcINH, as well as the Arkansas methods. The reference ranges will provide a standard which can be used to estimate the probability of adherence to prior doses.

1.1 AIM

To establish clear reference ranges of urinary INH in the study population.

1.2 OBJECTIVES

1. To evaluate INH urine concentrations of children who have been on daily dosing and intermittent dosing for prophylaxis at different time points using mass spectrometry measurement of INH and AcINH, photography and Arkansas method.

2. To evaluate effect of age, weight, dosing time and frequency, and urine creatinine (a marker of urine dilution) on urine INH/AcINH concentrations.
3. To compare concentrations of INH and AcINH with the colour changes using Arkansas method at different times and frequency of dosing.
4. To propose a cut-off Arkansas colour for adherence to last dose for the 4, 24, 48 and 72-hour time points.

2. LITERATURE REVIEW

2.1 TUBERCULOSIS (overview)

TB is a transmissible bacterial infection which affects the lungs primarily (pulmonary TB), but can also be disseminated to other parts of the body (extra pulmonary TB) including the skin, bones, nervous system, digestive and urogenital tracts, lymph nodes through the blood stream and lymphatic system (Kumar *et al.*, 2012; Mitchell *et al.*, 2007). The disease is caused by *Mycobacterium tuberculosis (M. tuberculosis)* which was first identified and described by Robert Koch in 1882 (Huebner *et al.*, 1993; Nobel Foundation, 1905). As estimated by WHO in 2010, a third of the world's population is infected with the organism (World Health Organization, October 2012). WHO reported an estimate of 8.7 million new cases of TB and 1.4 million deaths in 2010 (World Health Organisation, 2011). The worldwide distribution is shown in figure 1.1. Majority of the population in many Asian and African countries test positive to tuberculin tests while with the United States, only 5 – 10% of the population test positive (Kumar *et al.*, 2012; Mitchell *et al.*, 2007).

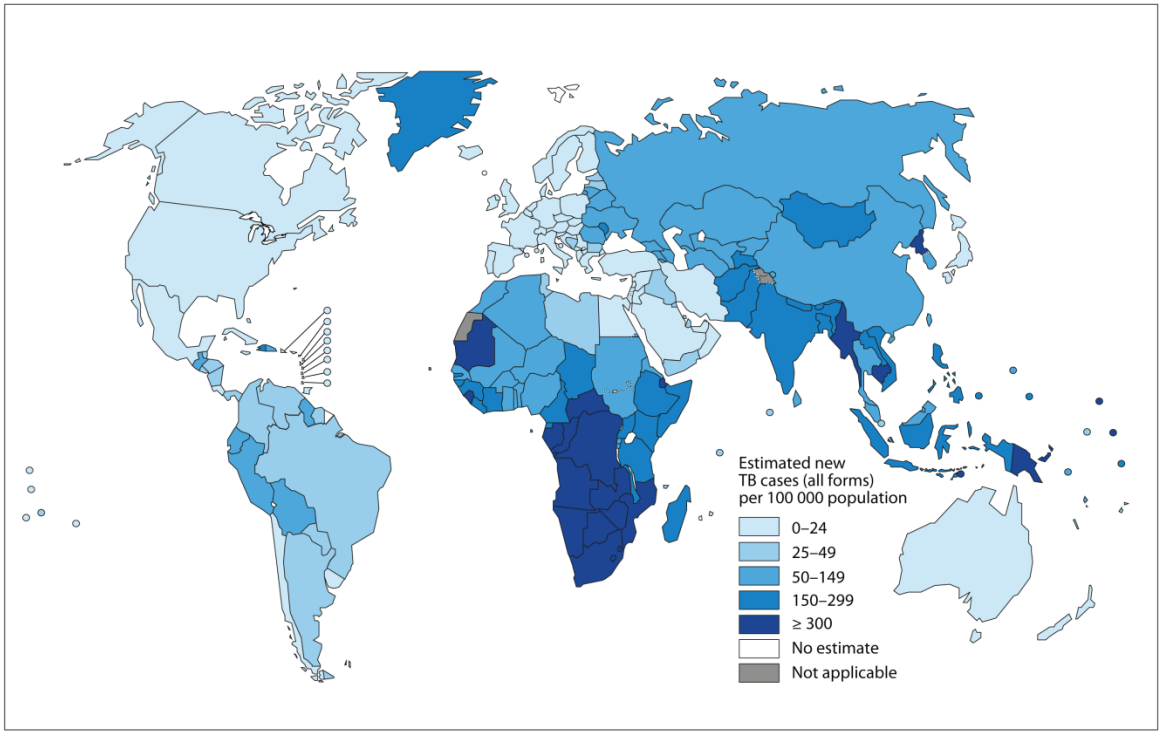

Figure 1 The estimated geographical distribution of TB incidence in 2010 (World Health Organisation, 2011).

2.1.1 TRANSMISSION

M. tuberculosis is transmitted by air droplets when people, usually adults with active pulmonary TB disease, cough, sneeze, laugh or spit (Lawn *et al.*, 2011; Van Soolingen *et al.*, 1997). Those who are latently infected are thought to be non-infectious (Kumar *et al.*, 2012). Transmission occurs when droplet containing *M. tuberculosis* is inhaled and transverse the mouth or nasal passages, upper respiratory tract and bronchi to reach the lungs' alveoli (CDC, 2011a).

M. tuberculosis transmitted to children can become latent or develop to pulmonary, disseminated or severe forms of TB disease including TB meningitis, and miliary TB. Young age and human immunodeficiency virus (HIV) infection are the most important risk factors for severe or disseminated TB disease (Marais *et al.*, 2004; Nelson *et al.*, 2004).

2.1.2 LATENT TB INFECTION AND ACTIVE TB DISEASE

TB infection in children could be seen as covering the spectrum from recent infection with *M. tuberculosis,* before an immune response is mounted, to an established state of equilibrium (Seddon *et al.*, 2012). In latent TB, the cells of the immune system which includes macrophages, T lymphocytes, B lymphocytes and fibroblasts aggregate to form granulomas with lymphocytes surrounding the infected macrophages. The granuloma prevents dissemination and growth of the mycobacteria. This makes the infection inactive with no symptoms and in a situation that can persist for years. However, it can reactivate and progress to disease in some people, especially in the setting of diminished immunity (Grosset, 2003).

The active disease occurs when the immune system is too weak to stop bacterial growth and the bacteria invade and replicate within endosomes of alveolar macrophages in the lungs. Active disease when developed requires prompt diagnosis and treatment to prevent spread of infection. Table 1 below summarizes the characteristics of latent TB infection and TB disease.

Table 1 Characteristics of latent TB infection and TB disease. Adapted from (Bass Jr *et al.*, 1990).

Latent TB infection	TB disease
Result of skin or blood test usually indicates TB infection. However, there may be false positive results.	Result of skin or blood test usually indicates TB infection. There may however be false negative results
Chest X-ray may or may not be normal. Sputum test is usually negative	Abnormal chest X-ray or positive sputum smear or cultures
Infected individual does not feel sick	Individual usually feels sick and may have symptoms such as coughing, fever and weight loss
Individual cannot spread TB bacteria to others	Individual with respiratory or pulmonary disease can spread TB bacteria to others
Treatment of latent TB infection prevents progression to TB disease	Needs treatment of TB disease

2.1.3 SIGNS, SYMPTOMS AND DIAGNOSIS OF TB

Signs and symptoms of TB disease include a chronic unremitting cough, weight loss or failure to thrive, fever, fatigue, loss of appetite, unexplained pneumonia. A definitive diagnosis of TB is made by identifying *M. tuberculosis* bacteria in a clinical specimen taken from the patient (CDC, 2011b). In children, it is often not possible to bacteriologically confirm TB and diagnosis is frequently made on a composite of history, clinical and immunological grounds (Khan *et al.*, 1995; World Health Organization, 2006). Investigations such as immunological tests (Mantoux tuberculin skin test), chest radiography, computed tomography scans are used also in diagnosis. The tuberculin skin test (TST) (Huebner *et al.*, 1993) is the primary screening test for the diagnosis of latent TB. In this test, purified protein derivative (PPD), a material derived from *M. tuberculosis* is injected into the skin of an individual and the reaction on the skin as indicated by the degree of induration is evaluated after about 48-72 hours (Huebner *et al.*, 1993). A person is said to be positive to the TST (PPD positive) if there is a strong skin reaction. TST is positive in HIV-infected children if induration is 5 mm or greater while an induration less than 5 mm is said to be TST negative (Huebner *et al.*, 1993). A positive TST is an indication of TB infection (recent or latent). In children, TB diagnosis is a challenge due to the limitations of the TST and Mycobacterial culture (Hesseling *et al.*, 2002).

2.1.4 RISK FACTORS FOR TB

Those at high risk for developing TB disease include:

- HIV-infected individuals - HIV infection is the most powerful factor that predisposes an individual to progress from latent TB to the active disease (Corbett *et al.*, 2003). The lifetime risk of progression from latent TB infection to active disease is about ten percent in the general population and five to fifteen percent yearly in HIV-infected persons with the risk increasing as immune deficiency worsens (Aaron *et al.*, 2004). People co-infected with HIV and TB are about 21-34 times more likely to develop TB disease compared with those who are HIV-uninfected (World Health Organization, 2011). In high prevalence areas, the risk of developing TB disease in children less than 5 years has been shown to be 3.5 times higher than in adults (Van Rie *et al.*, 1999). Though the risk of TB declines after 2 years of age, children with severe malnutrition and HIV infection, especially those with severe immune suppression remain at high risk of TB (Moyo *et al.*, 2010). Up to 25% of South African children with HIV infection develop TB annually if they are not on antiretroviral treatment (ART) (Martinson *et al.*, 2009; Zar *et al.*, 2007). While ART reduces the incidence of TB in children, the risk remains higher than in HIV-uninfected children (Marais *et al.*, 2006). HIV-infected children have been shown to have higher mortality than those who are HIV-uninfected when they develop TB (Walls *et al.*, 2004).
- Children and babies in contact with TB patients are at risk for developing TB. In high prevalence areas, the risk of developing TB disease in children less than 5 years has been shown to be 3.5 times higher than in adults (Van Rie *et al.*, 1999). Young children or children with impaired immunity frequently experience severe forms of TB including disseminated disease and meningitis.
- Elderly people (Stead *et al.*, 1983).
- Patients receiving certain medication treatments such as anti-cancer chemotherapy, corticosteroids, transplant anti-rejection medication.
- Individuals born where TB is prevalent such as in Africa, Asia, Latin America**.**
- Individuals exposed to TB through their jobs such as health care workers.
- People who inject illicit drugs (Graham *et al.*, 1992; Perlman *et al.*, 1995).
- People with other conditions such as diabetes, silicosis, cancer which weaken the immune system.

2.1.5 PREVENTION OF TB

Prevention of TB relies on satisfactory adherence to the treatment regimen by people with infectious TB, vaccination of children in TB prevalent countries, and TB preventive therapy in persons infected with HIV (Lawn *et al.*, 2011). The bacilli Calmette Guerin (BCG) vaccine is currently given to babies at birth as part of the routine vaccine schedule particularly in countries where TB is highly prevalent (McShane, 2011). The vaccine has been reported to confer variable protection against contracting pulmonary TB and immunity decreases after about ten years, however, it is thought that it offers some protection against developing extra pulmonary TB (Lawn *et al.*, 2011; McShane, 2011). BCG vaccine has been reported to offer a protective estimate of 73% (range 67% - 79%) against TB meningitis and 77% (range 58% - 87%) against miliary disease in children (Trunz *et al.*, 2006).

2.1.6 TREATMENT OF TB

Treatment of most forms of pulmonary and extrapulmonary TB requires a minimum of six months with multiple anti-TB medications, usually four drugs (INH, rifampicin, ethambutol and pyrazinamide) in the intensive phase and two drugs (INH and rifampicin) in the continuation phase (Swaminathan *et al.*, 2010). The drugs mentioned above are first-line anti-TB drugs. Second-line drugs are used when situations like resistance to first-line therapy, multidrug-resistant TB (MDR-TB) or extensively drug-resistant TB (XDR-TB) arise. They include aminoglycosides, polypeptides, fluoroquinolones, thioamides, and cycloserine. (Caminero *et al.*, 2010; Goldman *et al.*, 2007; Shah *et al.*, 2007). Third-line drugs include macrolides, thioacetazone (Hari *et al.*, 2010; Rajeev *et al.*, 2011). Single drugs are used only in latent TB or chemoprophylaxis. The use of single drug for treatment of active disease results in the rapid development of resistance and treatment failure (Committee, 1948; Wang *et al.*, 2006). The different drugs in the TB regimen have different modes of action which is a reason for their combination.

2.2 ISONIAZID

Isoniazid (INH) is an important drug in the treatment of TB and the only medication approved by World Health Organization (WHO) for TB preventive therapy (World Health Organization, 2011). The drug has powerful bactericidal activity against metabolically active organisms and could also suppress the growth of non-multiplying organisms (Schaaf *et al.*, 2005).

Absorption: When taken orally, it is well absorbed from the gastrointestinal tract. In patients co-infected with TB and advanced HIV disease, there is concern that HIV may reduce anti-TB drug concentrations however; there exist limited evidence to this in children.

Distribution: INH is readily distributed throughout the body (Greenberg *et al.*, 1962). It diffuses into all body tissues and fluids including cerebrospinal fluids, breast milk and saliva (Kucers *et al.*, 1979). It is not appreciably bound to plasma protein.

Elimination: INH is eliminated mainly by metabolism. The primary route is acetylation by N-acetyl transferase enzyme found in the liver and small intestine (Weber *et al.*, 1979). INH is trimodally eliminated. The rate of elimination is determined by genetic polymorphisms of the arylamine N-acetyl transferase 2 gene (Parkin *et al.*, 1997). Schaaf *et al.* taking into account the N-acetyl transferase 2 enzyme showed that younger children eliminate the drug faster than older children and as a group, faster than adults (Schaaf *et al.*, 2005). In children less than 13 years given 10 mg/kg INH, Schaaf reported elimination rate constants (per hour) of 0.254, 0.513 and 0.653 for homozygous slow, heterozygous fast and homozygous fast acetylators from INH concentrations obtained at 2, 3, 4 and 5 hours post INH dose which was higher than those of adult patients given a similar 10 mg/kg INH dose. N-acetyl transferase 2 genotype is a determinant of INH concentrations in adults and children (Parkin *et al.*, 1997; Schaaf *et al.*, 2005). Studies involving children aged 3 months to 13 years with slow, intermediate and fast genotypes given variable INH doses (from <4 to > 12 mg/kg) showed peak concentrations (C_{max}) of 4.05, 2.63 and 1.54 mg/L respectively (McIlleron *et al.*, 2009).

Very little of INH is excreted unchanged in urine and the greater proportion is acetylated to AcINH. AcINH is hydrolysed to isonicotinic acid and monoacetyl hydrazine. Isonicotinic acid conjugates with glycine to give isonicotinyl glycine while monoacetyl hydrazine undergoes further acetylation to give diacetyl hydrazine. Some of the unmetabolised INH conjugates to alpha-ketoglutarate and pyruvate to hydrazones (Hughes *et al.*, 1955). Figure 2 shows the breakdown products of INH.

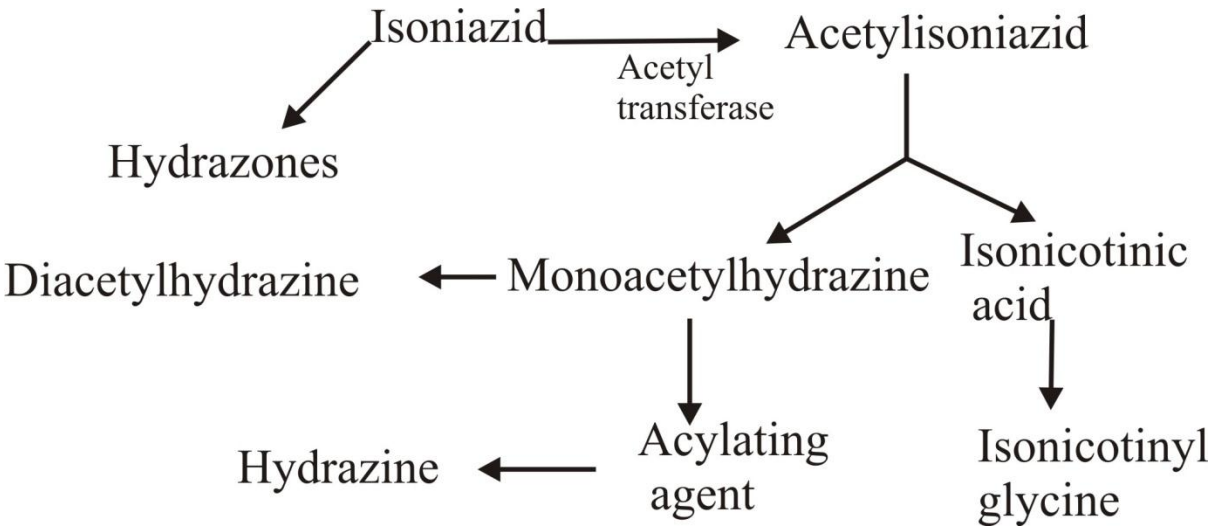

Figure 2 Flow chart showing elimination products of INH. Adapted from (Hughes *et al.*, 1955).

75-90% of INH is excreted in the urine within 24 hours mainly as metabolites. INH is generally well tolerated by children but rarely, hepatic toxicity, neurologic and gastrointestinal side effects may occur (Feja *et al.*, 2005).

2.3 TREATMENT OF LATENT TB INFECTION (TB PREVENTIVE THERAPY)

TB preventive therapy involves the use of anti-TB drugs singly or in combinations to prevent progression of latent TB infection to active disease. A meta-analysis was carried out using randomized controlled trials in which HIV-infected participants were randomly allocated to different regimens to assess the effect of preventive therapy on the incidence of TB and reduction of mortality. The different regimens compared were:

- Any anti-TB drug versus placebo
- INH + rifampicin versus placebo
- Rifampicin + pyrazinamide versus placebo
- INH + rifampicin + pyrazinamide versus placebo

Any anti-TB drug versus placebo reduced the risk of active TB by 32% (RR 0.68 95% CI 0.54 – 0.85). This effect was seen more in individuals with a positive TST (RR 0.38, 95% CI 0.25-057) than in those with a negative test (RR 0.89 95% CI 0.64-1.24). Efficacies for all regimens were alike but INH monotherapy stood out (Akolo *et al.*, 2010). Unlike other short course multidrug regimens, it was less associated with discontinuation of therapy due to adverse effects. INH alone versus placebo among subjects with a positive TST status showed

a reduction in mortality however, overall, there is no evidence that TB preventive therapy reduces mortality significantly (Akolo *et al.*, 2010).

2.3.1 INH PREVENTIVE THERAPY

IPT, intensified case finding and infection control form the 3 I's strategy of WHO to reduce the burden of TB among people living with HIV. IPT utilizes INH alone for patients infected with HIV to prevent the incidence of TB when active TB has been excluded (Cotton, 2011). Use of INH for TB preventive therapy has been known to reduce the incidence of TB in high risk individuals for over forty years (Comstock *et al.*, 1967; Ferebee *et al.*, 1963). Trials of treatment for latent TB infection conducted by Ferebee and colleagues in the 1960's established that INH administered for six to nine months was effective in preventing TB in adults and children with latent TB (Ferebee, 1970). Treatment of latent TB reduces the risk of developing active TB in HIV-infected individuals especially those with a positive TST (Akolo *et al.*, 2010). Studies suggest that IPT is protective in adults receiving antiretroviral therapy (ART) (Golub *et al.*, 2009; Golub *et al.*, 2007) and reduces TB incidence in HIV-infected adults (Bucher *et al.*, 1999; Grant *et al.*, 2005; Mwinga *et al.*, 1998; Pape *et al.*, 1993; Whalen *et al.*, 1997; Wilkinson *et al.*, 1998).

With good adherence, INH chemoprophylaxis was effective in preventing progression to disease in 69 – 93% of infected children (International Union Against Tuberculosis Committee on Prophylaxis, 1982). A double-blind study carried out by Zar and colleagues in Cape Town to assess the value of universal IPT in HIV-infected children prior to or in the absence of documented exposure to a source case showed a benefit in mortality comparing INH with placebo (11 (8%) versus 21 (16%)) (HR 0.46 95% CI 0.22-0.95, P=0.015). The incidence of TB was also lower in the INH group (5 cases, 3.8%) than in placebo (13 cases, 9.9%) (HR 0.28 95% CI 0.10-0.78, P=0.005) (20). Another study by Madhi and co-workers, a large multi-centre trial of 548 HIV-infected and 804 HIV-uninfected infants between 3-4 months of age in South Africa and Botswana showed no benefit of pre-TB exposure IPT when compared with placebo in reducing the incidence of TB and mortality (Madhi *et al.*, 2011). The disparity could be explained by difference in patient population and access to ART. Recently, an additive benefit of IPT and ART has been reported in HIV-infected children after 5 years of follow up. INH reduced the risk of TB disease by 0.22 (95% CI 0.09 – 0.53) compared with placebo. ART alone reduced TB risk by 0.32 (95% CI 0.07 – 1.55).

INH plus ART reduced the risk of TB disease by 0.11 (95% CI 0.04 – 0.32) (Frigati *et al.*, 2011).

2.4 RECOMMENDATIONS FOR IPT IN CHILDREN

WHO recommends IPT for six months in children living with HIV, greater than twelve months of age, unlikely to have active TB on symptoms based screening and have no contact with a TB case as part of a comprehensive package of HIV prevention and care service (World Health Organization, 2011).

In children with HIV who are less than twelve months of age, only those who have contact with a TB case and are evaluated for TB (using investigations) should receive six months of IPT if the evaluation shows no TB (World Health Organization, 2011).

All children living with HIV who have successfully completed treatment of TB disease should receive INH for an additional six months (World Health Organization, 2011).

IPT is recommended in South Africa for HIV-infected children and all children less than 5 years of age (regardless of HIV status), after exposure to a source case (Cotton, 2011).

2.5 ADHERENCE AND ASSESSING ADHERENCE TO IPT

Sub-optimal adherence and non-adherence in TB prevention and control are common important issues in people living with HIV and other populations at high risk of developing TB. Adherence on one hand is difficult because patients are asymptomatic, feel well, the dosing regimen is prolonged and medications can produce side effects (Ailinger *et al.*, 2008). Also, most people on IPT are already on other ARV's therefore increasing the burden of pills being taken. On the other hand, people already taking ARV's might be more adherent since once they have the habit of taking their medications, it could enhance their adherence compared to someone whose only medication is INH. Limited data exist on adherence to treatment and chemoprophylaxis regimens in children in settings highly burdened by TB (Van Zyl *et al.*, 2006). However, adherence to IPT has been reported to be poor in different populations at risk and studies have shown non-adherence rates to be 8 - 44.2% (Szakacs *et al.*, 2006; Van Zyl *et al.*, 2006). Van Zyl *et al.* reported that adherence to anti-TB treatment in children less than five years of age was significantly better than adherence to chemoprophylaxis (82.6% versus 44.2%, $p<0.001$) and that adherence to unsupervised chemoprophylaxis was poor (Van Zyl *et al.*, 2006). Adherence to therapy is necessary for

efficacy of therapy. Monitoring, to detect non-adherent patients, would allow for prompt counselling to be offered to the non-adherent patients identified.

There is no gold standard for assessing adherence (Starr *et al.*, 1999)**.** Frequently used methods are discussed below alongside their limitations. They include self-reports/caregiver reports, pill counts, use of electronic monitoring devices and urine assays for INH metabolites.

2.5.1 SELF-REPORTS

Many studies assessing patients' compliance to IPT use self-reports as a means of measuring adherence. Self-reports are cheap, easy to obtain and do not demand as much labour as some other methods. It could also give valid adherence reports (Blumberg *et al.*, 2005) however, it is not completely reliable as it can be affected by forgetfulness and social desirability bias (Starr *et al.*, 1999). In a study that compared self-reports and other methods for monitoring adherence to IPT, self-reporting appeared to overestimate adherence (Starr *et al.*, 1999). In children where caregiver reports are depended upon, assessing self-report can be a challenge when children arrive with different caregivers for study visits (le Roux *et al.*, 2009).

2.5.2 PILL COUNTS

Pill counts rely on patients remembering to bring left over tablets to the clinic. In one of the few trials to perform pill counts regularly, they showed an association between good adherence defined as taking more than 80% of doses on each monthly visit and treatment efficacy (International Union Against Tuberculosis Committee on Prophylaxis, 1982). A limitation of this method is that it could give false adherence measures as patients could manipulate left over tablets (Starr *et al.*, 1999). In children, caregivers sometimes have to repeat medication doses when a child vomits, spits out the medicine or there is spillage and this can lead to overestimation of adherence (le Roux *et al.*, 2009).

2.5.3 USE OF ELECTRONIC MONITORING DEVICE

This measures adherence indirectly and depends on data obtained from electronic device in a container lid that records the date and time container was opened (Ailinger *et al.*, 2008; le Roux *et al.*, 2009). The best correlation between measures of adherence and treatment effect has been shown with these devices in patients with hypertension or diabetes (Cramer, 2004). Measures could be affected when patients open their containers more often than they need to,

especially when they had a prior knowledge that information would be obtained from the lid cover or less often than required like taking out pills for more than one dose (Ailinger *et al.*, 2008; Starr *et al.*, 1999). Other limitations include technical problems which could also occur when using electronic monitoring devices and high cost of device (Ailinger *et al.*, 2008).

2.5.4 URINE ASSAYS

The sure way to establish the intake of a drug is by demonstrating its presence or that of its metabolites in a body fluid such as urine, saliva or blood (Ellard *et al.*, 1980). This can be done either quantitatively or qualitatively but unlike quantitative methods, qualitative methods only reflect recent drug ingestion and do not quantify or differentiate parent compounds and their metabolites (Sirgel *et al.*, 2006). Quantitative methods on the other hand are expensive, labour intensive and require a well-equipped laboratory with trained personnel (Sirgel *et al.*, 2006). Tandem mass spectroscopy with high performance liquid chromatography because of its sensitivity and specificity is a rapidly growing method for analysis of drug in different matrices but it is impractical for continuous monitoring of adherence (Ng *et al.*, 2007). Urine assays are non-invasive and are most suitable for compounds which are polar since the concentrations in urine are likely to be higher than concomitant salivary levels (Ellard *et al.*, 1980).

To monitor the ingestion of INH, urine tests are easily applicable, inexpensive, non-invasive and collection of urine samples is acceptable by patients and parents (Palanduz *et al.*, 2003). These make it easy to frequently perform urine tests and possibly use it for evaluating compliance. The drawbacks however are that the tests do not give information about dose ingested, demonstrates only recent drug intake and does not necessarily reflect overall adherence (Palanduz *et al.*, 2003; Starr *et al.*, 1999). Also, urine test samples may be diluted due to excessive intake of fluids close to sample collection time or concentrated due to decreased fluid intake. Creatinine concentration is checked during standard urine drug tests to know if test sample is diluted or not.

2.5.4.1 URINE CREATININE

Creatinine is a metabolic by-product of muscle metabolism (Karriem-Norwood, 2012). It is chiefly filtered out of the blood by the kidneys with little or no tubular reabsorption, and is passed out of the body in urine in relatively constant quantities over a 24 hour period with "normal" liquid intake. Creatinine concentration is checked during standard urine drug tests

using a urine creatinine test which measures the amount of creatinine in the urine as an indicator of urine water content or as a marker identifying a specimen as urine**.** Greater than normal intake of water will increase the urine water content (lowering the creatinine level) consequently diluting the amount of drug in urine. Conversely, a limited intake of water can lead to an abnormally concentrated urine specimen (as occurs with dehydration) resulting in elevated creatinine levels. Urine creatinine test can be done alone or with other tests that determine the relative amounts of other substances being excreted in the urine. Random urine creatinine levels have no standard reference ranges. Normal random urine creatinine concentrations range from 40-300 mg/dL in males and 37-250 mg/dL in females (Redwood Toxicology, 2013). In pharmacokinetic studies, concentration of the drug relative to the creatinine is used as a way to standardize measurement and account for the problem of varying dilution due to varying intake of fluids by the individual.

2.6 QUALITATIVE URINE TESTS

Different qualitative urine tests have been developed to monitor intake of INH. A method for detecting N-AcINH in urine was described by Eidus and Hamilton (1964). A more sensitive method for determination of INH metabolites- isonicotinic acid and isonicotinylglycine was reported by Ellard and Greenfield. The method could be used for urine samples collected from about 1-24 hours from patients ingesting 300 mg of INH daily (Ellard, 1977). Kilburn reported the use of reagent impregnated strip to detect the presence of isonicotinic acid and isonicotinylglycine using potassium thiocyanate, chloramine-T and barbituric acid (1972). Currently, a frequently used test is the Arkansas test.

2.6.1 ARKANSAS TEST

The Arkansas test is a widely used urine test which objectively identifies the presence of INH and its metabolites in urine (Perry *et al.*, 2002). It was developed by Potts, Cozart and Reagan and later reported by Henderson (Schraufnagel *et al.*, 1990). It is simple, quick, inexpensive, reliable and gives immediate visual results that can be easily read which makes it useful in evaluating drug compliance in patients being treated for latent TB infection with a single daily dose of INH (Eidlitz-Markus *et al.*, 2003). With its repeated use as a follow up method, it could be useful in better characterizing adherence (Guerra *et al.*, 2010; Perry *et al.*, 2002).

Reagents utilised for the test are readily obtainable and have a shelf-life of at least six months at room temperature (Schraufnagel *et al.*, 1990). The test gives visual colours and colour

change with positive tests varies (Guerra *et al.*, 2010; Hanifa *et al.*, 2007; Schraufnagel *et al.*, 1990). Colours which have been reported as positive include dark blue or turquoise, blue, purple, blue-green, green (Hanifa *et al.*, 2007; Palanduz *et al.*, 2003; Schraufnagel *et al.*, 1990). White, yellow, orange, light lemon and no colour change have been reported for negative results. These colours were read at variable times; Schraufnagel reported development of colour within two minutes, other studies read colours at times ranging from 1-30 minutes (Guerra *et al.*, 2010; Hanifa *et al.*, 2007; Perry *et al.*, 2002). Those which utilised dipstick and other devices that contained the reagents took longer times to read developed colours.

The Arkansas test has been validated in different populations and has been reported to have high sensitivity and specificity. Schraufnagel reported a sensitivity and specificity greater than 97% and 98% respectively (Schraufnagel *et al.*, 1990). A sensitivity/specificity of >95%/98% was reported by Renata and colleagues in the fifth minute when an isoscreen utilizing Arkansas method was used to measure adherence to IPT in an outpatient hospital population 24 hours post INH observed dose. In this study, there was a difference in the sensitivity of the test when colour changes were read at the first and fifth minutes with the fifth minute having a higher sensitivity than the first minute (84% and 95%; first and fifth minute) however, specificity remained the same (Guerra *et al.*, 2010). Hanifa and colleagues reported sensitivities of 93.4% (95%CI 88.2-96.8) at 12 hours and 77% (95%CI 69.1 - 83.7) at 24 hours post INH dose in a black African adult male population. Lower sensitivity at 24 hours post dose than those reported by other studies was thought to be due to a higher proportion of fast acetylators in the black African population (Hanifa *et al.*, 2007).

Factors that could affect the Arkansas test result include:

1) Misinterpretation of colour changes on which the test is based particularly when test produces a weak colour. Objectively evaluating concentrations with colours produced from the Arkansas test would help to establish a cut-off for defining a positive or negative result as well as, value for telling about last dose (few hours before visit) or dose prior to that.
2) Concomitantly administered medications; one study reported a pink colour result in a single urine sample when INH and aspirin were concomitantly administered (Kilburn *et al.*, 1972). Another study also reported a pink colour in a single urine sample when INH, pyridoxine and multivitamin were co-administered (Hanifa *et al.*, 2007).

3) Presence of other substances in urine such as nicotine.
4) Variability in the amount of INH metabolites in urine (Ellard *et al.*, 1980).
5) Natural background colour of the urine sample (Ellard *et al.*, 1980).
6) Inaccuracies inherent with the test.

A major limitation of this test is that sensitivity drops with increasing time post INH dose making it difficult to know a patient who has not been compliant for even a few days (Hanifa *et al.*, 2007; Szakacs *et al.*, 2006). Sensitivities of greater than 90% have been reported at post INH ingestion times less than or equal to 12 hours and even up to 24 hours in European populations (Hanifa *et al.*, 2007; Kilburn *et al.*, 1972; Schraufnagel *et al.*, 1990). Lower sensitivities of 76% and 85% were reported by Elizaga and Perry respectively at 48 hours post INH dose (Elizaga *et al.*, 1997; Perry *et al.*, 2002). Perry and colleagues utilized recall interval to validate the Arkansas test which can be limited by forgetfulness. This study validates the Arkansas method by evaluating it against objective measurement of INH and AcINH urine concentrations particularly in children. The method chosen for the quantification of INH and AcINH in this study is the High Performance Liquid Chromatography tandem Mass spectrometry. The technique is described below.

2.7 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY (HPLC-MS/MS)

2.7.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) is used to separate a mixture of compounds with the purpose of identifying, purifying and quantifying the individual components of the mixture. It involves the introduction of the sample mixture to be separated into a column containing sorbents (also known as stationary phase). The individual components of the mixture are moved through the column at different velocities by a liquid forced through the column by high pressure (this technique is also called high pressure liquid chromatography). The interaction (chemical/physical) between the molecules of the sample mixture and the stationary phase aids in the separation of the components. The different components of the sample are then identified by a detector at the end of the column base on the time each component reaches the detector after injection. The intensity of each component is measured by the detector and the end result is presented as a chromatogram as shown in Fig. 3**.**

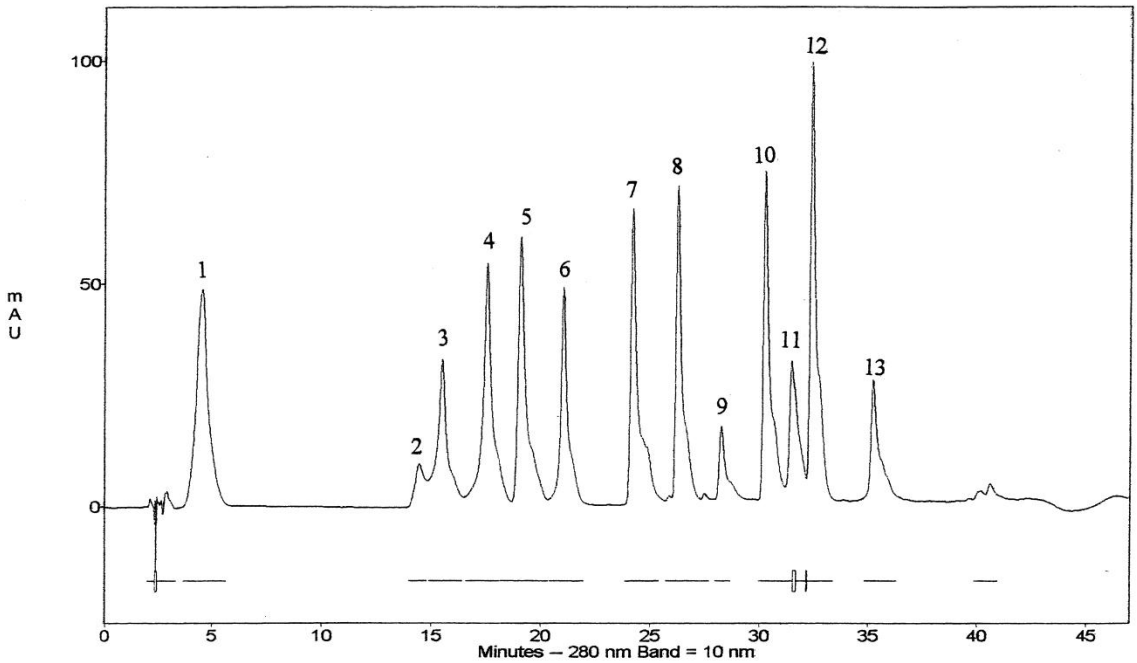

Figure 3 HPLC chromatogram of the flavonoid and phenolic acid standards at 280 nm. Peaks: 1=gallic acid; 2=gentisic acid; 3=(+)-catechin; 4=chlorogenic acid; 5=caffeic acid; 6=(-)-epicatechin; 7=p-coumaric acid; 8=sinapic acid; 9=benzoic acid; 10=p-anisic acid; 11=myricetin; 12=3,4,5-trimethoxycinnamic acid; 13=quercetin. Reprinted from (Chen *et al.*, 2001).

2.7.2 MASS SPECTROMETRY (MS)

This is an analytical technique that measures the mass-to-charge ratio of charged particles. It is a technique with the ability to provide elemental analysis and molecular weight of a compound, and it can also be used to identify various fragments of a molecule (Snyder *et al.*, 2011). The method of operation can be explained using the diagram in figure 4.

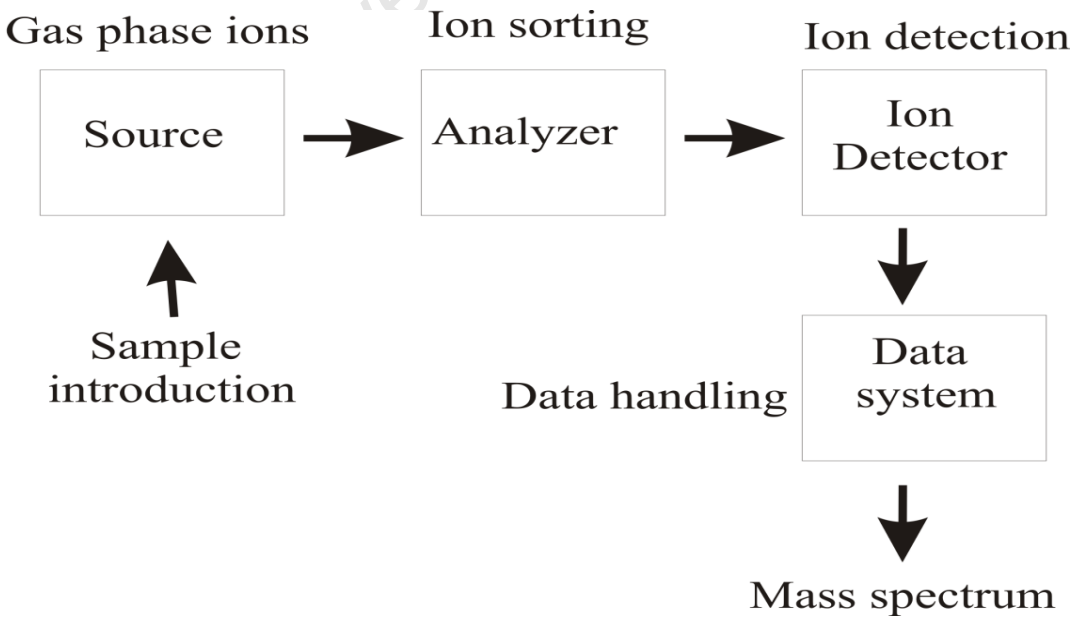

Figure 4 Illustration of the different stages of MS. Adapted from (Stahlberg).

Source

After a sample is introduced in to the source, the sample is vaporised (if not in gaseous state) since mass spectrometry requires freely moving atoms or molecules. An electron beam ionises the sample forming charged particles.

Analyser

In the analyser, an electromagnetic field separate the ions by their mass-to-charge ratio. Using the measure of the particle, only ions of the right mass are allowed into the detector.

Ion detector

The abundance of each mass is detected by varying the magnetic field.

Data system

Finally, the ion signal is processed and a mass spectrum is given as an output.

All samples that are to be analysed using MS must first be separated before it is inputted. If mixtures are not separated, the output will be a spectral of overlapping peaks. For this reason, a technique such as HPLC that separates mixtures into its various components can be used in tandem with MS/MS.

2.7.3 HPLC-MS/MS

The use of the sensitive ability of HPLC in tandem MS/MS (selective ability) (Matuszewski *et al.*, 2003) can be applied to samples requiring high sensitivity and selectivity. It is a technique that is being used in various fields. Some investigators have used this technique to detect traces of illicit drugs (Berset *et al.*, 2010; Boleda *et al.*, 2007; Zuccato *et al.*, 2008). It has been used to quantify and identify different collagen types (Pataridis *et al.*, 2009), and its sensitivity, selectivity and precision for the study of nine environmental phenols in urine using automated online column switching system has been shown (Ye *et al.*, 2005). Recently it has been used to quantify INH and AcINH in plasma with excellent precision and accuracy with calibration curves exhibiting linearity within a range of 1 – 250 ng/ml of INH and 0.05 – 50 ng/ml of AcINH (Ng *et al.*, 2007). In this study, it is used to quantify INH and AcINH in urine with calibration range for both compounds between 0.125 µg/ml and 20 µg/ml.

3. STUDY POPULATION AND METHODS

3.1 SETTING AND PARTICIPANTS

This study is embedded in a large IPT study in an established research facility at Red Cross War Memorial Children's Hospital (RCCH), University of Cape Town. Children who were participating in the main study, aged sixteen years and below, HIV-infected on ARVs and randomized to receive daily or intermittent (thrice weekly) doses of INH or placebo as prophylaxis were eligible to participate in the study. Parents or legal guardians had already given a full written consent to participation in the main study and study was approved by the Faculty of Health Sciences Human Research Ethics Committee (HREC ref no. 299/2005). The ethics committee also approved a review of the main study for extra urine sampling. Caregivers gave a verbal informed consent. Caregivers and children were offered some form of compensation for their time and transport fare.

Assays were conducted in an established research laboratory which is recognized internationally for TB, HIV and Malaria outputs and is accredited by South African National Accreditation System (an internationally recognized agency for the development of and conduct of HPLC-MS/MS assays on biological samples).

3.2 STUDY PROCEDURES

The study pharmacist generated a list of children who had been allocated to INH or placebo from the main study, distributed between the two regimens (daily and thrice weekly INH or placebo in a dose of 8-12 mg/kg depending on whether a quarter or half a tablet was required) in a ratio of three to one (3:1) for INH: placebo. The rest of the study team (including those who read the colours observed from the colorimetric assay) remained blinded with respect to allocation of INH or placebo. A case record form (CRF) and standard operating procedures (SOP) were designed for data capturing and sample collection on the clinical site.

Appointments were made with caregivers of study participants who were reminded in advance by the study nurse of the scheduled sampling visit day(s). The study nurse contacted the participants' caregivers during the week of the urine sampling date to ensure that they were aware of the expected time of arrival at the study site on the day

of urine sampling; to record the exact time and dose of the last dose of INH/placebo and any other drug taken within the last two days prior to sampling; not to give child any drug before arrival on the day of urine sampling.

At visit, the following information were gathered: weight, height, date of birth of the study participant, concomitant medications, time of last meal before dose at visit, whether or not child vomited after dose of INH and time of vomit.

Dose administration and urine sampling:

In the intermittent arm, children were asked to come once on a Monday when they would have had their doses approximately 72 hours earlier (on a Friday) and urine samples were collected on arrival. Then they ingested a dose in the clinic (under the observation of a nurse) and 4 hours later, urine samples were taken. The same patients were asked to come on a Wednesday which would be approximately 48 hours after the dose taken at the clinic and again, urine samples were taken on arrival.

In the daily arm, children were asked to come once on a Wednesday when they would have had their doses approximately 24 hours (on a Tuesday) and urine samples were collected on arrival. They then ingested a dose in the clinic (under the supervision of a nurse) and 4 hours later, urine samples were taken.

INH dose prior to clinic visit (dose before 72 hours and 24 hours urine sampling) was given by child's caregiver. Adherence to this dose was maximized through caregiver phone call. Time and dose taken was verified and recorded by the study nurse. INH dose at clinic visit (dose before 4 hours and 48 hours urine sampling) were observed by the study nurse.

4-hour time point for urine collection was chosen because it is a typical time after dose for children attending clinic visits.

The exact dates, days and times (to the nearest minute) of sampling (time urine sample was presented) were recorded on the case record form.

Duplicate urine samples (each 1ml) were pipetted into appropriately labelled eppendorf tubes, placed in a suitable cool box with dry ice and on the same day transferred with a sample inventory form for each sample to the pharmacology laboratory at Groote Schuur Hospital (GSH) where they were stored in -80 degrees freezer until INH and

AcINH concentrations determination and Arkansas testing. The rest of the urine sample collected was decanted into tubes, labelled and sent to the RCCH chemical pathology laboratory for urine creatinine measure.

3.3 LABORATORY METHODS

3.3.1 COLORIMETRIC DETERMINATION OF INH AND AcINH METABOLITES IN URINE

The Arkansas test was carried out according to the procedure described by Schraufnagel (1990). 30 mg of barbituric acid was weighed into tubes. This was followed by the addition of 140 µL of urine, 70 µL of chloramine T (14 g/dl) used within one hour of preparation and 70 µL of potassium cyanide (5 g/dl). The test tubes were agitated vigorously by hand and allowed to stand for 10 minutes. Photographs of colour produced by each sample were then taken in a white box with the camera and test tube positions approximately uniform for each sample. Two observers initially read the colours independently in a subset of the data which included 48 samples. Range of colours reported included dark blue, blue, violet, green, light blue, pale blue, green, and yellow but because of the excellent concordance between observers and limited resources, a subsequent decision to rationalize to one observer was made. Colours were read by comparing photographs taken with a colour chart and recorded. The test was carried out on samples in batches of 10.

Control samples for HPLC-MS/MS assay were obtained by spiking urine (obtained from individuals who were not taking INH) with INH and AcINH. The dilutions were carried out as stated below and the limit of quantification for INH and AcINH obtained. 200 µL was pipetted from 1 mg/ml INH in methanol into a clean tube. 4.8 ml of urine was then added to it to make 5 ml solution containing 40 µg/ml INH. Other dilutions were done with the equations shown below.

1 ml of 40 µg/ml INH + 1 ml urine → 20 µg/ml INH

1 ml of 40 µg/ml INH + 3 mls urine →10 µg/ml INH

1 ml of 40 µg/ml INH + 7 mls urine → 5 µg/ml INH

1 ml of 5 µg/ml INH + 1 ml urine → 2.5 µg/ml INH

1 ml of 5 µg/ml INH + 4 mls urine → 1 µg/ml INH

1 ml of 1 µg/ml INH + 1 ml urine → 0.5 µg/ml INH

1 ml of 0.5 µg/ml INH + 1 ml urine → 0.25 µg/ml INH

1 ml of 0.25 µg/ml INH + 1mls urine → 0.125 µg/ml INH

1 ml of 0.125 µg/ml INH + 1 ml urine → 0.0625 µg/ml INH

1 ml of 0.0625 µg/ml INH + 1 ml urine → 0.03125 µg/ml INH

1 ml of 0.03125 µg/ml INH + 1 ml urine → 0.015625 µg/ml INH

Similar dilution as above was also carried out for AcINH. The limit of quantitation was 0.125 µg/ml for both INH and AcINH.

3.3.2 DETERMINATION OF INH AND ACINH BY HPLC-MS/MS

INH and AcINH were analyzed using an HPLC/MS/MS based assay. Urine samples were centrifuged to remove any particulate matter and then diluted tenfold with water. 10 µL was injected onto the column. Gradient chromatography was performed on a Phenomenex, Luna 5 µm PFP(2), 100 A, 50 mm × 2 mm analytical column, using acetonitrile and 5 mM ammonium acetate as mobile phase, and was delivered at a flow rate of 300 µl/min. An AB Sciex API 3200 Q-Trap mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 131 to the product ions at m/z 121 for INH and the protonated molecular ions at m/z 180 to the product ions m/z 138 for INH. The calibration range for both compounds was between 0.125 µg/ml and 20 µg/ml.

HPLC-MS/MS conditions were as follows:

Curtain gas: 25

Ion spray voltage: 5500

Temp: 500 °C

Gas 1: 50

Gas 2: 60

Declustering potential for INH: 31

Declustering potential for AcINH: 66

Collision energy for INH: 25

Collision energy for AcINH: 31

The standard curves used to extract the concentrations from peaks produced by the mass spectrometer are shown in figures 5 and 6. The curves were as expected.

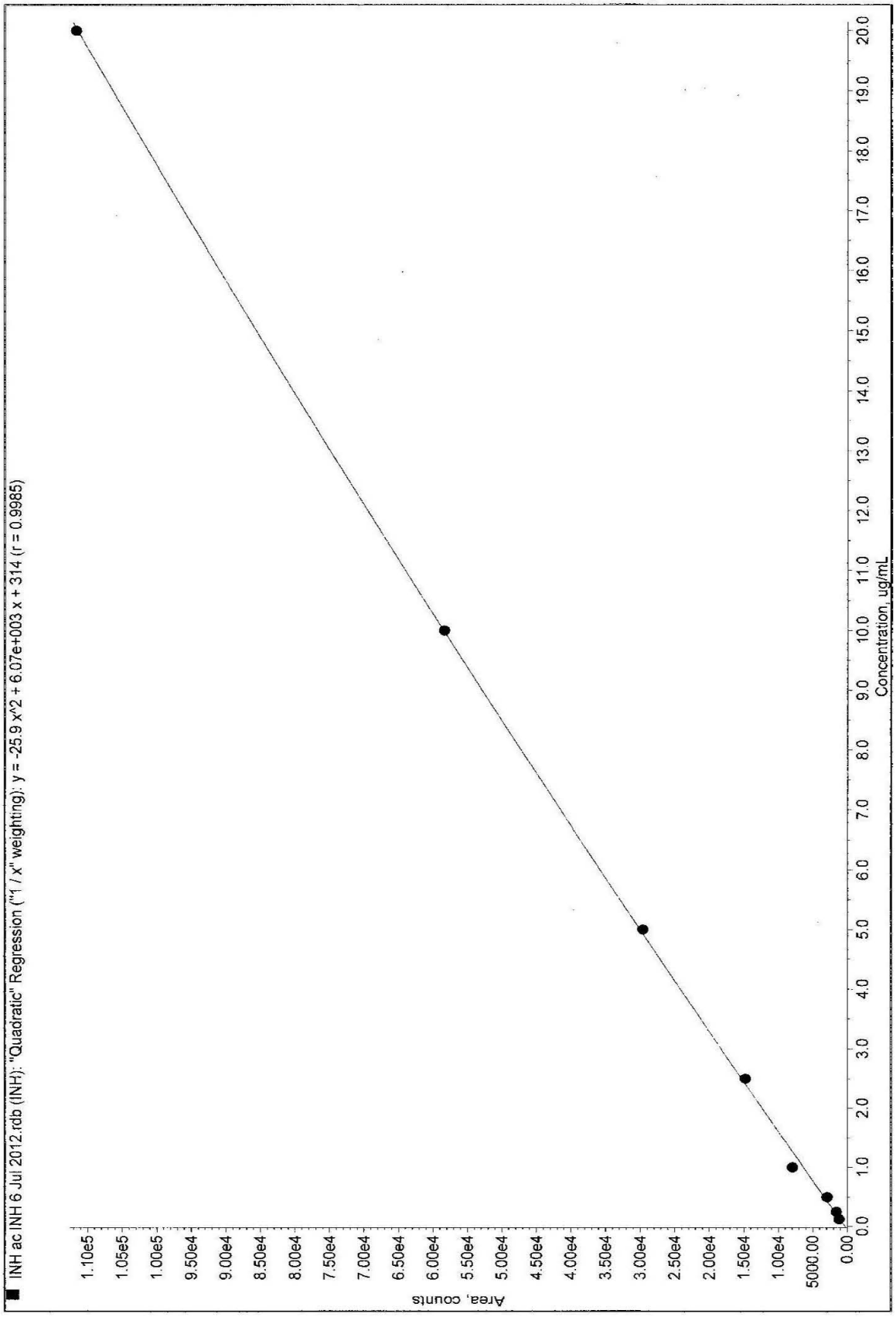

Figure 5 Area count Standard curve used for extraction of INH concentration.

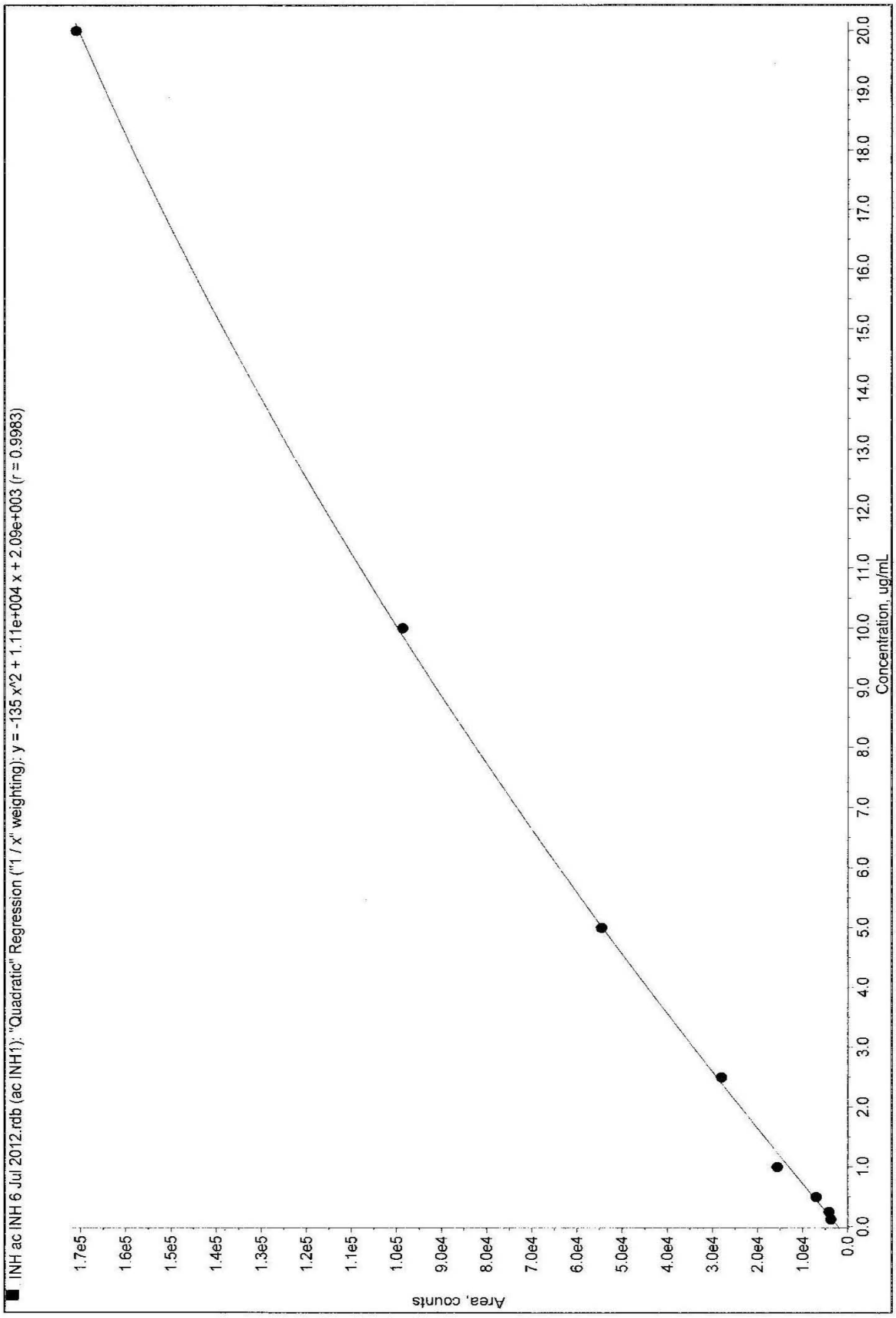

Figure 6 Area count Standard curve used for extraction of AcINH concentration.

3.4 STATISTICAL ANALYSIS

Based on the distribution of the data which was examined and showed non-normal distribution, it was summarized as median and interquartile range (IQR). The nonparametric Wilcoxon rank-sum test was used to determine whether the 4-hour urine concentrations for the daily and intermittent arms where statistically significantly different. Univariate regression analysis was used to determine the association between covariates and urine concentrations of INH and AcINH. Quantile regression analysis was used to measure the increase in urine concentrations of INH and AcINH for each unit increase in urine creatinine. Regression analysis was also used to measure the increase in the urine concentrations of INH and AcINH, respectively, for each colour compared to colour 1 (the reference). Analysis was carried out using Stata, version 11.0 and Origin 8.6 softwares.

4. RESULTS

4.1 PATIENT CHARACTERISTICS

A total of 41 children; 22 females, 19 males with median age 7.7 years (IQR, 6.6 - 9.5 years) were selected for the study. 24 were on daily and 17 on intermittent dosing schedules. 31 children participants received prophylactic INH and 10 placebo. Of the 31 children who received INH, 18 received it daily and 13 intermittently.

Each of the 24 children on daily dosing schedule submitted a 4-hour and 24-hour urine sample. 16 of the intermittent arm children submitted all three 4, 48 and 72 hours' urine samples. The 17th child could not submit a 72-hour urine sample because her caregiver could not make it to any of the Monday's urine sampling schedule. Altogether, 36 samples from the INH daily arm and 38 samples from the INH intermittent arm were assayed. Of these, 46 were from female children and 28 from male children. The results from children taking placebo were excluded in the final analysis as they all showed negative for both colorimetric and mass spectrometry assays. The age and weight (median (IQR)) for children whose samples were used in analysis was 7.7 years (IQR, 6.1-8.5 years) and 21.3 kg (IQR, 17.2 - 28.8 kg) respectively. The characteristics of the children and number of samples produced are summarized in table 2.

Table 2 Patient characteristics.

Characteristics	Daily	Intermittent	Total
Gender			
Male	13	6	19
Female	11	11	22
Median age (years)	7.65	7.7	7.7
Number of children on INH	18	13	31
Number of children on placebo	6	4	10
Number of samples collected at 4 hours	18	13	31
Number of samples collected at 24 hours	18	-	18
Number of samples collected at 48 hours	-	13	13
Number of samples collected at 72 hours	-	12	12
Number of samples used for analysis	36	38	74

The participating children were on other medications and this was noted. The different medications used concomitantly with the INH medication and the number of patients on each medication are shown in table 3. All the children that participated in this study were on Antiretroviral drugs and a few were taking multivitamin concomitantly with INH but no pink colour result was seen from the Arkansas test on those samples like previously reported by Hanifa (2007).

Table 3 Concomitant medications used by study population.

Concomitant drug	No. of patients
Lopinavir / Ritonavir	27
Lamivudine	34
Stavudine	7
Abacavir	15
Zidovudine	17
Didanosine	3
Efavirenz	10
Tenofovir	1
Nevirapine	1
Sodium valproate	3
Cotrimoxazole	3
Vitamin B complex	7
Folic acid	1
Multivitamin	8
Oxybutynin	1

4.2 ARKANSAS TEST RESULTS

The Arkansas test results were available for all the samples. For the INH group (children on prophylactic INH), several colours were observed which includes dark blue, blue, light blue violet, light blue, pale blue/green and yellow. All samples from the children randomized to placebo showed a yellow colour or no colour change with the Arkansas test. Previous studies have reported similar colours; Schraufnagel et al reported dark blue for positive and yellow for negative (1990), Hanifa (2007) and Schmitz (2010) reported blue and green colours for positive while yellow and no colour change were considered negative. The colours observed in this work are coded from 1 to 6 as shown in table 4.

Table 4 Table showing Arkansas test colours observed and their codes.

Colour observed	Codes
Yellow/no colour change	1
Pale blue/green	2
Light blue	3
Light blue violet	4
Blue	5
Dark blue	6

Fig. 7 is the colour chart used to read the colours and Fig. 8 shows representative photographs of the Arkansas test result on clinic samples.

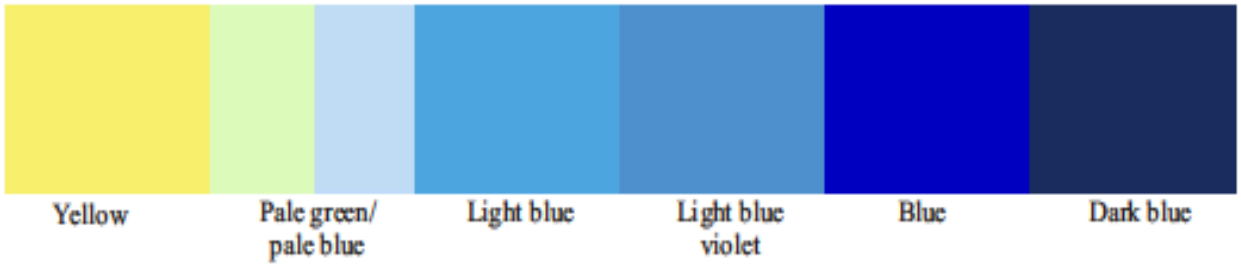

Figure 7 Colour chart used for the Arkansas test.

Figure 8 Representative clinic samples showing colours produced by the Arkansas test.

Figures 9 and 10 are bar charts showing the frequency of the different colours produced by the Arkansas test for the daily and intermittent arms at the different sampling times. For the daily arm, blue, dark blue and light blue violet were the predominant colours at 4 hours, while yellow, pale blue/green and light blue were the more common at 24 hours. Light blue colour was seen in two 4-hour samples even though the concentrations of INH and AcINH were high (concentrations were similar to other 4-hour samples). One yellow colour and one pale green colour were seen at 4 hours. The yellow at 4 hours came from a child whose 24

hours sample also showed yellow colour. The pale green colour at 4 hours came from a child whose 24 hours sample showed yellow.

In the intermittent arm, light blue violet, blue and dark blue were the predominant colours observed at 4 hours which is similar to that of the daily arm. No light blue and pale blue/green colours were observed and only one sample showed yellow at 4 hours. At 48 hours, yellow was the predominant colour with the other colours observed as pale blue/green (2 samples) and light blue (1 sample). None of the predominant colours (dark blue, blue and light blue violet) at 4 hours was observed at 48 hours. Only yellow or no colour change was observed for all the 72 hours samples.

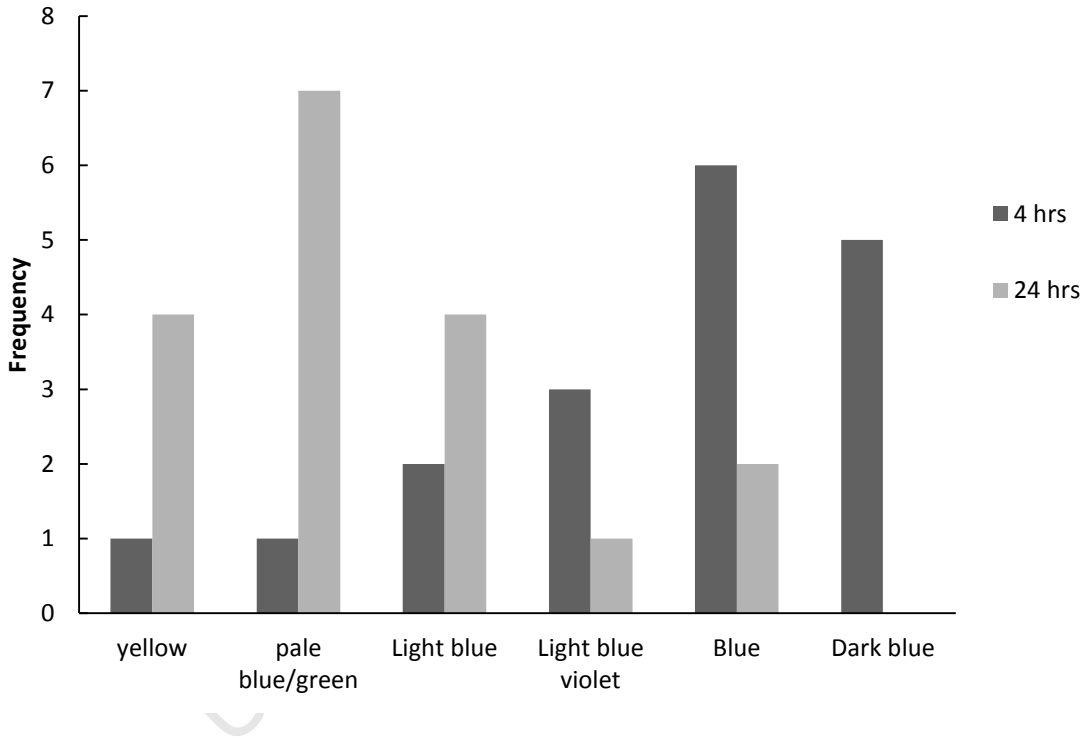

Figure 9 Bar chart showing colours observed from the Arkansas test and their frequencies of occurrence at 4 and 24 hours sampling times in the daily arm.

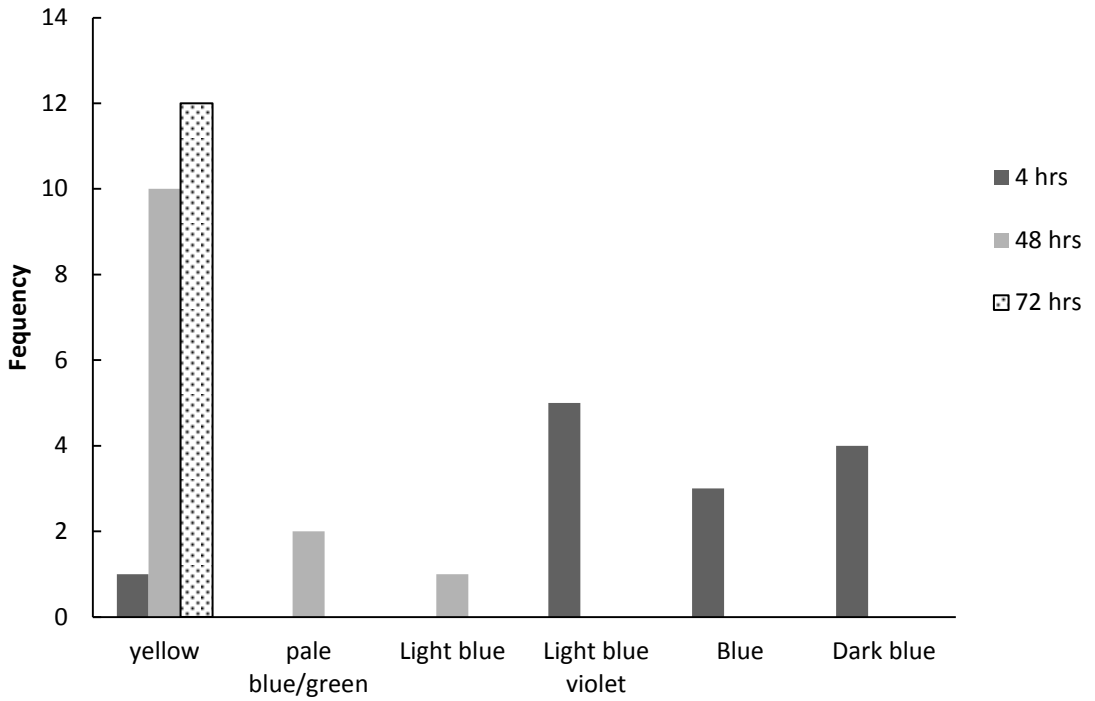

Figure 10 Bar chart showing colours observed from the Arkansas test and their frequencies of occurrence at 4, 48 and 72 hours sampling times in the intermittent arm.

There was a transition in the observed colours with increasing time post INH dose as shown in figure 11. In figure 11, darker colours (dark blue, blue and light blue violet) were only observed up to 24 hours post INH dose. At 48 hours post INH dose, only pale colours (pale blue and pale green) and predominantly yellow colours were observed. At 72 hours, only yellow colour was observed.

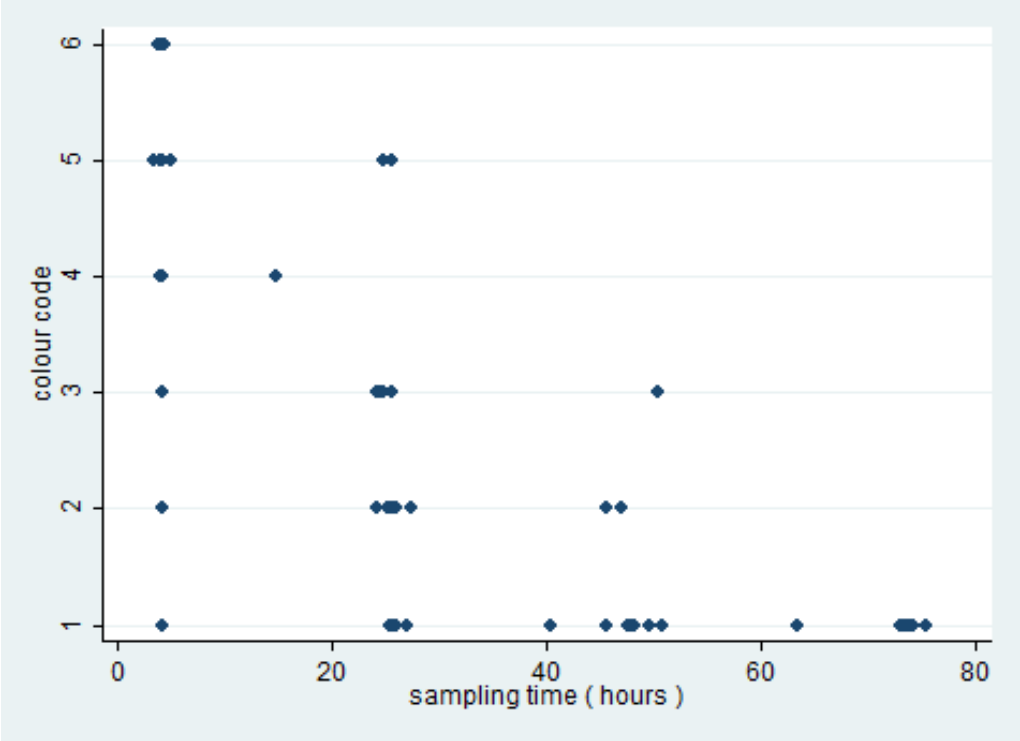

Figure 11 Scatter plot showing transition in Arkansas colour with increase in sampling time.

4.3 HPLC-MS/MS RESULTS

INH and AcINH urine concentrations were available for all the samples and are summarized and shown in table 5. The concentrations of INH and AcINH decreased with increase in time (4 hours to 72 hours) post INH dose (INH median concentration; 133.50 – 0.00 µg/ml, AcINH median concentration 116.00 – 0.00 µg/ml). At 4 hours, median concentration of INH was higher than that of AcINH but with increase in time post INH dose, median concentration of AcINH became higher than that of INH as shown in table 5.

Table 5 Urine INH and AcINH concentrations by HPLC-MS/MS at different sampling times, in daily and intermittent dosing schedules, for INH preventive therapy.

		Urine concentration (µg/ml) Median (IQR)		
Sampling time (hr)	**No. of samples (n)**	**INH**	**ACINH**	**INH + ACINH**
4	31	133.50 (45.75 – 273.00)	116.0 (36.05 – 272.00)	298.5 (112.25-536.0)
24	18	0.53 (0.34 – 4.90)	3.91 (1.39 – 9.95)	4.43 (1.67-18.8)
48	13	0.00 (0 – 0.20)	0.22 (0 – 0.37)	0.22 (0.14-0.54)
72	12	0 .00(0.00 – 0.07)	0.00 (0.00 – 0.18)	0.00 (0-0.26)

For INH, 1 sample obtained at 4-hour, 1 sample obtained at 24-hour, 7 samples obtained at 48-hour and 3 samples obtained at 72-hour points were below the limit of quantification for HPLC-MS/MS measurement carried out. Similarly, 1 sample obtained at 24-hour, 2 samples obtained at 48-hour, and 1 sample obtained 72-hour points for AcINH were below the limit of quantification. These samples whose concentrations fell below the limit of quantification were assigned a value of zero for the concentration.

Figures 12 and 13 show the urine concentrations for INH and AcINH respectively at each sampling times while figure 14 shows the combined urine concentrations of INH and AcINH at each sampling time.

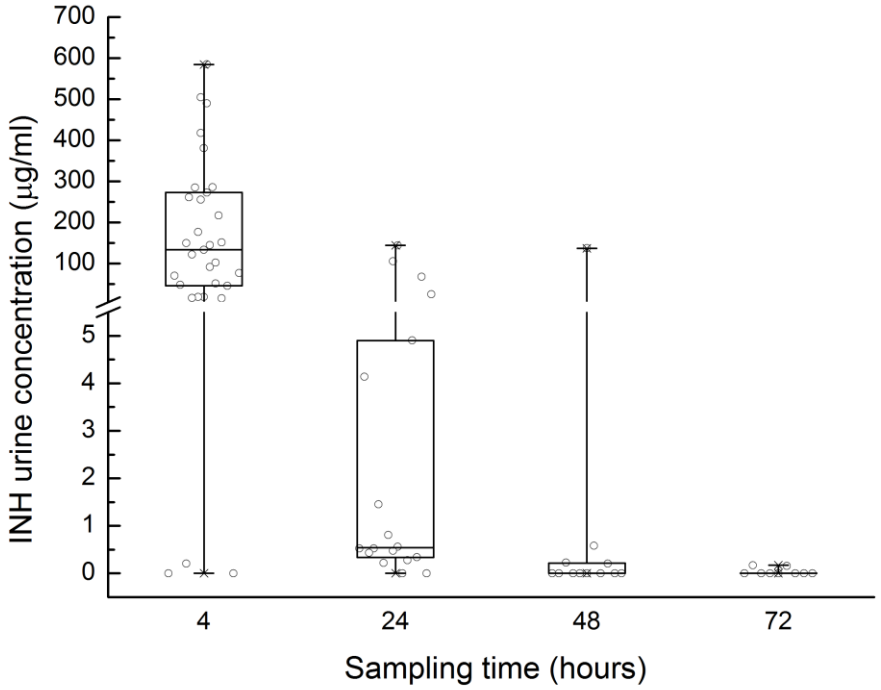

Figure 12 INH urine concentrations at each sample collection time in 31 children on INH preventive therapy. Horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles.

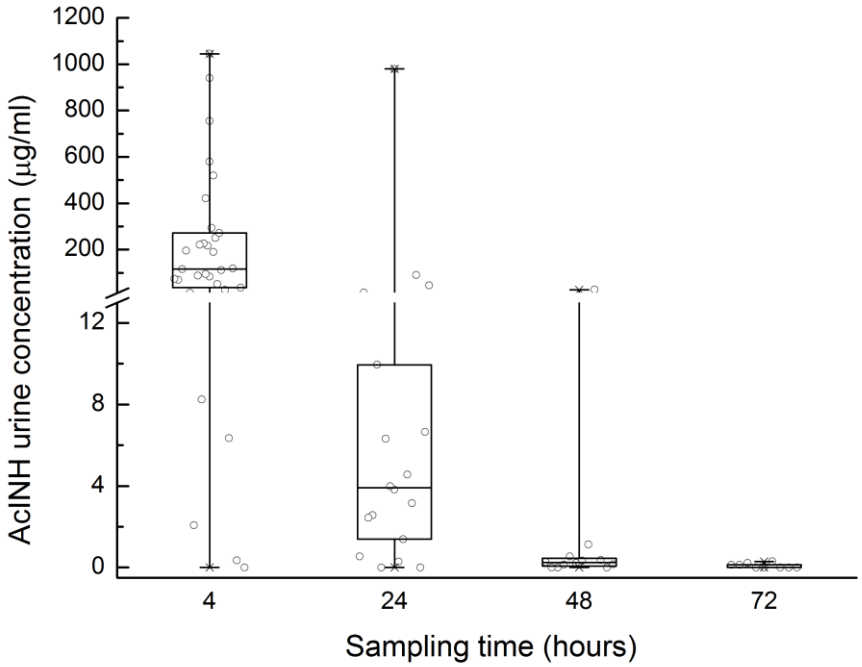

Figure 13 AcINH urine concentrations at each sample collection time in 31 children on INH preventive therapy. Horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles.

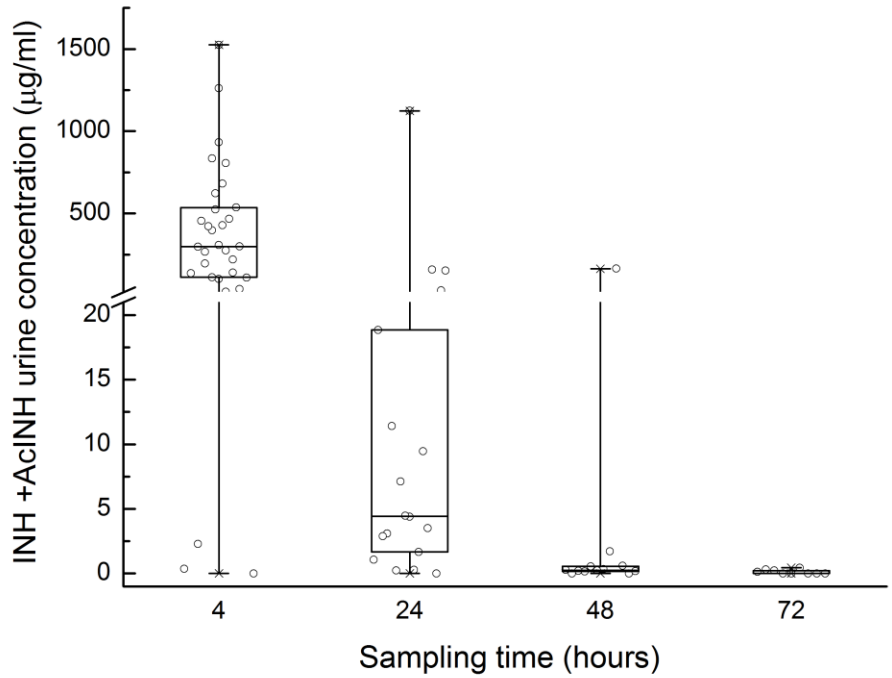

Figure 14 INH + AcINH urine concentrations at each sample collection time in 31 children on INH preventive therapy. Horizontal bars in boxes represent median concentration, whiskers represent minimum and maximum concentrations, boxes represent 25th and 75th percentiles.

Two samples from a child (15.9 years old) in the daily INH arm showed no levels for both INH and AcINH (both produced yellow colour for Arkansas test) at 4 and 24-hour time points and a 4-hour sample from a child (4.9 years) in the daily arm had low concentrations of 0.2 µg/ml and 2.07 µg/ml for INH and AcINH (pale green colour) respectively. A 4-hour sample from a child in the intermittent arm had a concentration of 0.352 for AcINH and for INH, concentration fell below the limit of quantification (yellow colour for Arkansas test). A 48-hour urine sample from a child showed light blue colour for Arkansas test with high concentrations of 137 µg/ml and 26.87 µg/ml for INH and AcINH respectively. These are more in line with 24 to 72-hour time points and recent ingestion of INH on the morning of urine collection and could be considered as outliers. It is likely these children with very low urine INH and AcINH concentrations did not take the dose at the allocated time (even though the doses were supposed to be observed) and the child with very high concentrations had his drug in the morning before coming to the clinic.

No significant effect of age, dose, weight and gender was detected on urine concentrations of INH and AcINH by univariate regression analysis. Urine creatinine was seen to be significantly associated with INH and AcINH concentrations by regression analysis ($P<0.001$ for both INH and AcINH). There was a 9.68 µg/ml (95% CI 4.51 – 14.84 µg/ml) change in INH concentration and 7.04 µg/ml (95% CI 4.96 – 9.12 µg/ml) change in AcINH concentration for each unit increase of creatinine. A plot of INH and AcINH urine concentrations against urine creatinine is shown in figures 15 and 16.

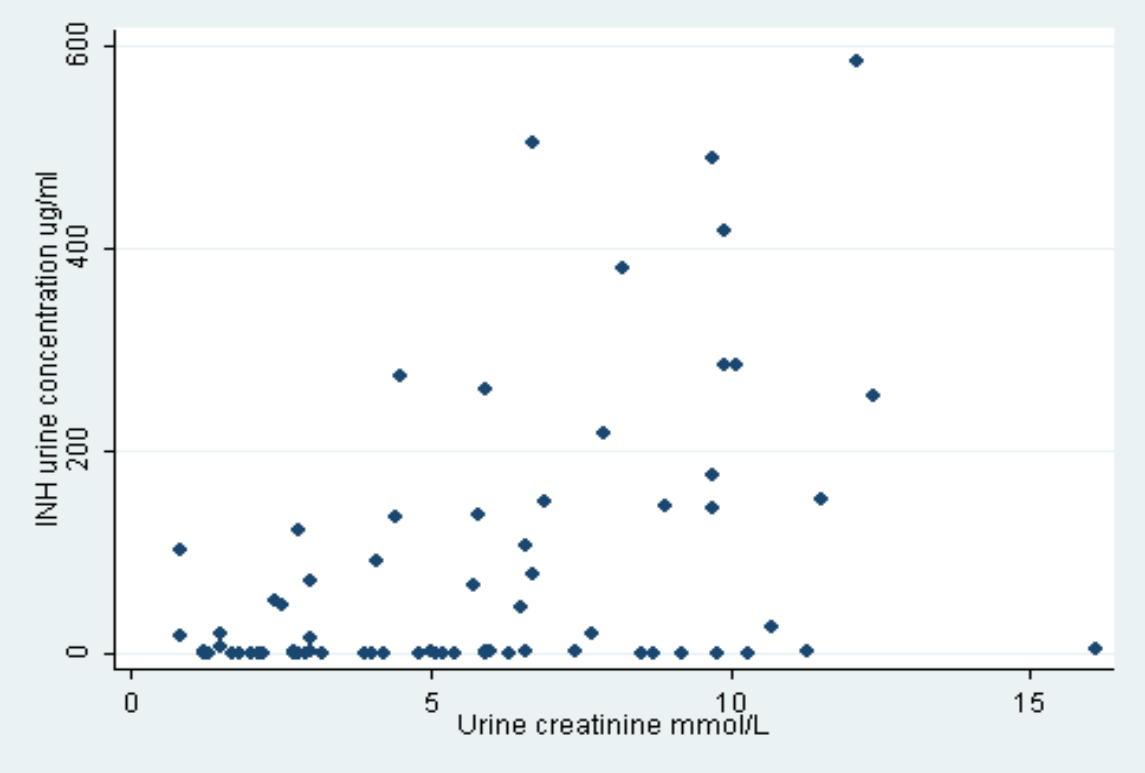

Figure 15 Plot of INH urine concentration against urine creatinine.

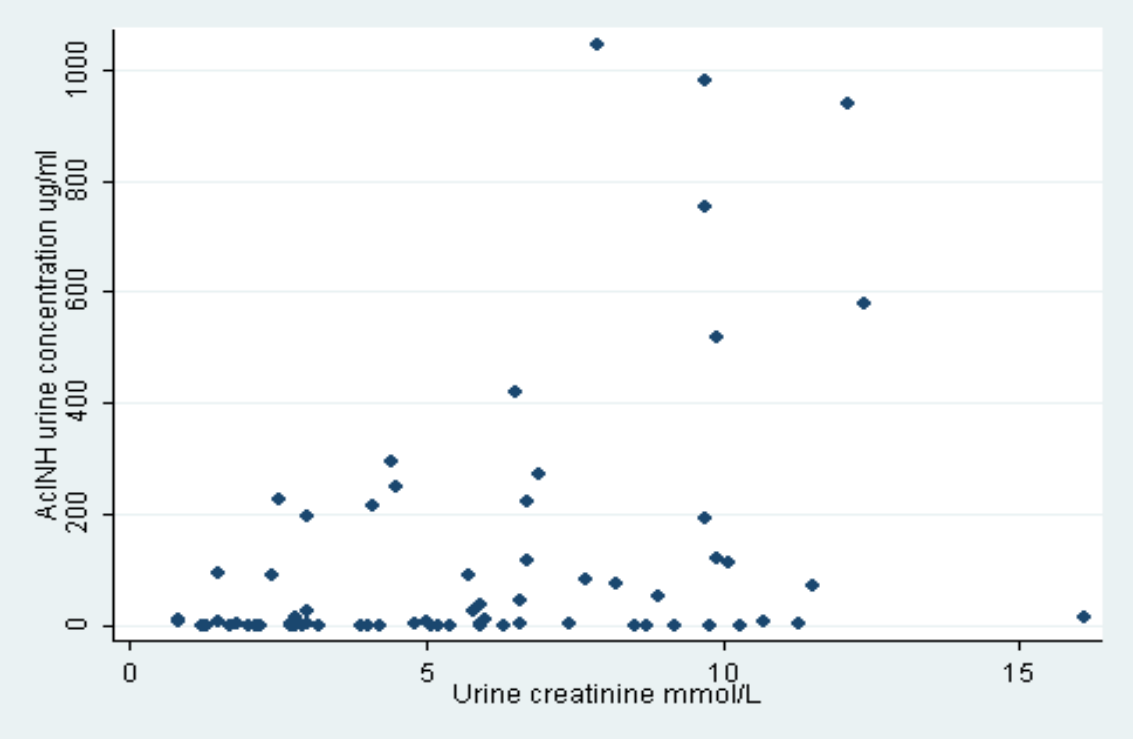

Figure 16 Plot of AcINH urine concentration against urine creatinine.

There was no statistically significant difference between the urine concentrations of INH and AcINH for the daily and intermittent arms at 4 hours (INH median (IQR) for daily arm

139.25 (18.30 – 273.00) µg/ml versus 122.00 (51.50 – 255.00) µg/ml for intermittent arm, p=0.8175; and AcINH, median (IQR) for daily arm 102.75 (36.05 – 227.00) versus 216.50 (69.00 – 421.00) µg/ml for intermitent arm, p=0.5165).

4.4 COMBINED ARKANSAS AND HPLC-MS/MS RESULTS

Figures 17, 18 and 19 show concentrations of INH, AcINH and a combination of INH and AcINH respectively for the different colours produced by the Arkansas test.

In figures 17 and 18, there was an increase in median concentrations of INH and AcINH as colour moved from yellow to dark blue. The unit increase in concentrations of INH and AcINH between each colour and yellow and their 95% CI values are shown in tables 6 and 7. For INH, the increase in median concentration was not seen between the light blue violet (colour code 4) and blue (colour code 5). Although we were unable to measure urine concentrations of other metabolites of INH, urine concentrations of INH and AcINH correlated with the different shades of blue colour produced by the Arkansas test by multivariate regression analysis (INH: R^2=0.4423, p<0.0005; AcINH: R^2=0.4606, p<0.005).

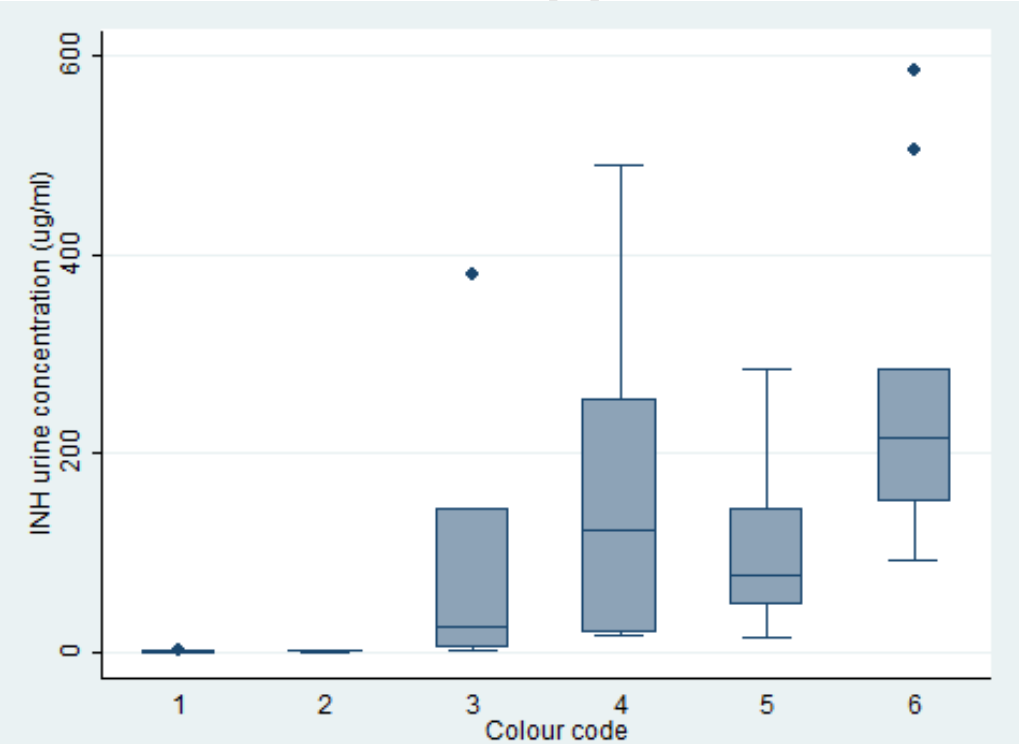

Figure 17 Box plot showing concentrations of INH for each Arkansas colour produced. The numbers 1 to 6 represent colours from yellow to dark blue as shown in Fig. 7.

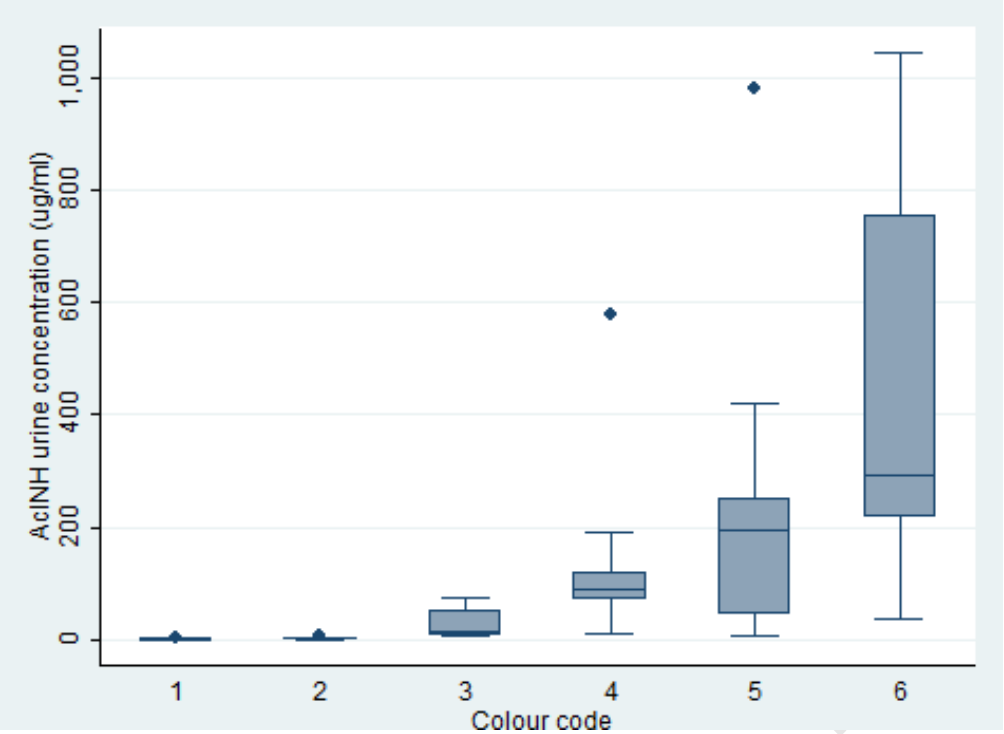

Figure 18 Box plot showing concentrations of AcINH for each Arkansas colour produced. The numbers 1 to 6 represent colours from yellow to dark blue as shown in Fig. 7.

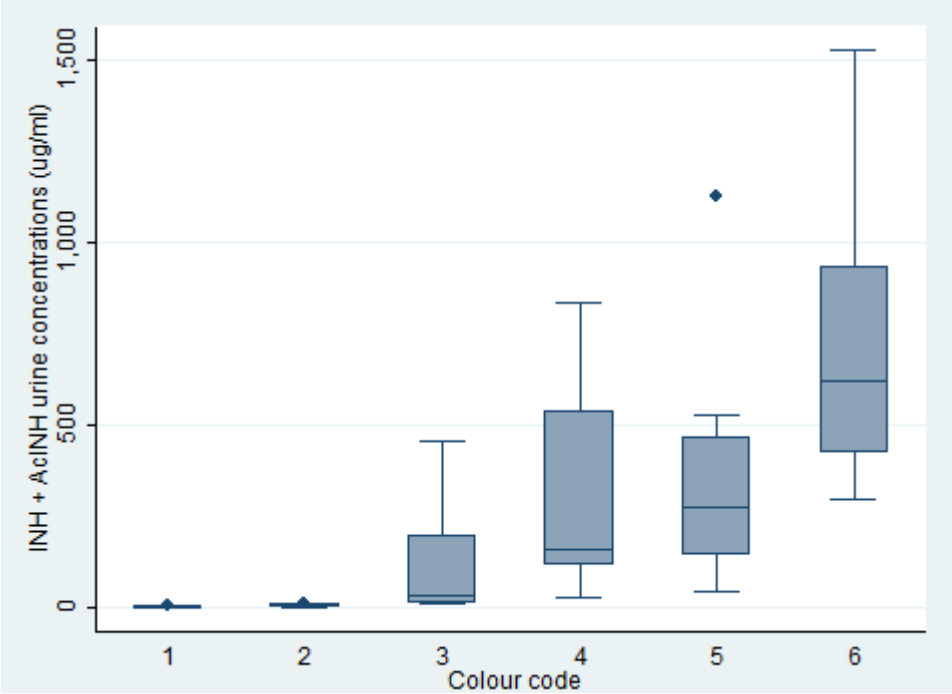

Figure 19 Box plot showing concentrations of INH+AcINH for each Arkansas colour produced. The numbers 1 to 6 represent colours from yellow to dark blue as shown in Fig. 7.

Table 6 Table showing unit increase in concentration of INH between each colour and yellow for the Arkansas test and their 95% CI.

INH colour	Increase in INH concentration	95% CI
Yellow	Reference	-
Pale blue/green	0.32	-73.31 – 73.95
Light blue	99.67	15.20 – 184.10
Light blue violet	172.80	96.17 – 249.30
Blue	110.60	39.50 – 181.80
Dark blue	267.10	190.50 – 343.70

Table 7 Table showing unit increase in concentration of AcINH between each colour and yellow for the Arkansas test and their 95% CI.

AcINH colour	Increase in AcINH concentration	95% CI
Yellow	Reference	-
Pale blue/green	1.70	-127.20 – 130.60
Light blue	26.44	121.40 – 174.30
Light blue violet	138.20	4.09 – 272.20
Blue	233.50	109.00 – 358.00
Dark blue	465.70	331.60 – 599.80

The proportion of samples with detectable INH and AcINH on HPLC-MS/MS, and the proportion with a positive Arkansas test (dark blue, blue, light blue violet, light blue, pale blue/green colour change were considered as positives) at each sampling time are shown in table 8.

Table 8 Proportion of positives for Arkansas test and mass spectrometer measures of INH and AcINH.

	Daily		**Intermittent**		
	4 hr	24 hr	4 hr	48 hr	72 hr
	n = 18	n = 18	n = 13	n = 13	n = 12
Arkansas positivity, %	94.1 (17)	77.8 (14)	92.3 (12)	23.1 (3)	0 (0)
% with detectable INH (HPLC-MS/MS)	94.1 (17)	94.1 (17)	92.3 (12)	30.8 (4)	25 (3)
% with detectable AcINH (HPLC-MS/MS)	94.1 (17)	94.1 (17)	100 (13)	69.2 (9)	41.7 (4)

Figures in parenthesis represent the number of samples that tested positive and n represents the total number of samples.

From table 8, the Arkansas test gave similar positive proportion result to the mass spectrometer urine concentration measures at 4 hours post INH dose in the daily arm (94.1%). Proportion in the daily arm would have been 100% if results from the child's samples which showed negative for the Arkansas test and zero urine concentrations for INH and AcINH were excluded. The decrease in proportion for the intermittent arm (92.3% against 100%) was caused by a single sample which showed a yellow colour with low levels of INH and AcINH, 0 and 0.352 µg/ml respectively (these concentrations and colour observed are more in line with 24 to 72-hour time points).

At 24, 48 and 72 hours post dose, the proportions of positives for the Arkansas test dropped to 77.8%, 23.1% and 0% respectively. The proportions of positives were lower than those obtained from the mass spectrometric measurements as shown in table 8.

To determine an optimal cut-off Arkansas colour for adherence to last dose for each of the 4, 24, 48 and 72-hour time points, results from the Arkansas test and HPLC-MS/MS were used to draw a table under the following conditions:

1) The 4-hour results for the daily and intermittent arms were combined.
2) Proportions of positives for all samples are designated P_1. In a secondary analysis with results designated P_2, few outliers were excluded (outliers are reported in section 4.3). For the results taken as outliers, mass spectrometer measurements of INH and AcINH did not reveal that those children actually had their drugs before the 4 hours urine collection and since this dose was not strictly observed, there could be possible non-adherence.

3) Using the colour code in table 4, break points for each colour was defined in the following way; if a colour was chosen as negative, then colours with a higher number in the colour-code table were regarded as positive. All samples that fall in colours regarded as positives were then used to calculate the proportion of positives using that break point.
4) Since the results used for the table came from children in the INH group (children on daily or intermittent INH), the cut-off point which gave the highest proportion of positives was then considered the optimal cut-off Arkansas colour.

Table 9 shows the proportions of positives for different Arkansas colours and time points.

Table 9 Table showing the proportion of positives for different Arkansas colours and time points. P_1 represent proportion of positives for all samples and P_2 represent proportion without outliers.

	Proportion of positives (%)			
Time / **Break point**	**4 hours**	**24 hours**	**48 hours**	**72 hours**
Y – PB/G	P_1 93.5	P_1 77.8	P_1 23.1	P_1 0.0
(-) (+)	P_2 100.0	P_2 82.4	P_2 16.7	P_2 0.0
PB/G - LB	P_1 90.3	P_1 38.9	P_1 7.7	P_1 0.0
(-) (+)	P_2 100.0	P_2 41.2	P_2 0.0	P_2 0.0
LB - LBV	P_1 83.9	P_1 16.7	P_1 0.0	P_1 0.0
(-) (+)	P_2 92.9	P_2 17.6	P_2 0.0	P_2 0.0
LBV - B	P_1 58.1	P_1 11.1	P_1 0.0	P_1 0.0
(-) (+)	P_2 64.3	P_2 11.8	P_2 0.0	P_2 0.0
B - DB	P_1 29.0	P_1 0.0	P_1 0.0	P_1 0.0
(-) (+)	P_2 32.1	P_2 0.0	P_2 0.0	P_2 0.0
DB	P_1 0.0	P_1 0.0	P_1 0.0	P_1 0.0
(-)	P_2 0.0	P_2 0.0	P_2 0.0	P_2 0.0

Y=yellow, PB/G=pale blue/green, LB=light blue, LBV=light blue violet, B=blue, DB=dark blue. (+)=positive, (-) =negative.

At 4 hours with outliers, no 100% proportion of positives was obtained with any of the break points. However, similar proportions (93.5% and 90.3%) were obtained for yellow - pale blue/green and pale green/blue – light blue break points. Without the outliers, 100% proportion was obtained when yellow - pale blue/green and pale blue/green – light blue break points. With the similar proportions of positives obtained with yellow - pale blue/green and pale blue/green-light blue chosen as breakpoints, pale blue colour is chosen as cut-off for adherence to 4 hours post INH dose.

At 24 hours, proportions for yellow – pale blue/green colour was 77.8% (with outliers), 82.4% (without outliers) and differed greatly when pale blue/green – light blue colour (with, 38.9; without, 41.2%) was used as break point.

Proportions at 48 hours for all break points were ≤ 23.1% and 0% at 72 hours.

5. DISCUSSION AND CONCLUSION

This study describes ranges of urine concentrations of INH and AcINH from 4 to 72 hours after the last dose in children on INH preventive therapy. The Arkansas test colour changes were evaluated using mass spectrometric measurements of INH and AcINH. Urine concentrations of INH and AcINH decreased with increasing time post INH dose as shown in table 5. There was an excellent correlation between colour change with the Arkansas test and the amount of INH, AcINH and INH plus AcINH respectively (figures 17 – 19 and tables 6 - 7). A decrease in urine INH and AcINH concentrations coincided with the transition to paler colours with increasing time post INH dose as shown in figures 11 and 13. The decrease in concentrations could explain why the Arkansas test sensitivity for evaluation of last dose adherence declines with increase in time post INH dose although, the concentrations of other metabolites were not evaluated alongside. It is however expected that the concentrations of other metabolites also decrease with increase in time post INH dose since they are products of AcINH breakdown (Fig.2).

The similar proportion of positives produced by the mass spectrometer and Arkansas test as shown in table 8 and the reduction from 100% caused by only single sample in each arm shows the Arkansas test to be reliable at 4-hour post INH dose and can be useful for monitoring ingestion of morning doses in children during clinic visits. The Arkansas test however, is not useful for monitoring 24-hour and above post INH doses ingestion. HPLC-MS/MS is useful for monitoring up to 24-hour post INH dose ingestion but it is not useful at 48-hour post INH dose (tables 8 and 9). A more sensitive mass spectrometer is required for 48 hours and above (table 8).

With the similar proportions of positives obtained with yellow - pale blue/green and pale blue/green – light blue chosen as breakpoints, pale blue/green colour was chosen as optimal cut-off Arkansas colour for adherence to 4 hours post INH dose (table 6). However, there is need to confirm findings in larger rigorously conducted study.

The dark blue Arkansas colour seen only with the 4-hour urine samples and not with the other time points is useful in telling that patient ingested his drug in the morning (approximately 4 hours) before coming to the clinic (although urine concentrations of other metabolites were not determined in this study, also background urine colour might have an impact on the colours produced from the Arkansas test).

For the samples that showed very low urine INH and AcINH concentrations (two 4-hour samples - one of the samples came from a child in the daily arm (age, 4.9 years; INH and AcINH concentrations, 0.2 µg/ml and 2.07 µg/ml respectively; urine creatinine, 4.8 mmol/L), the other sample was from a child in the intermittent arm (age, 8.4 years; INH and AcINH urine concentrations, 0 and 0.352 µg/ml respectively; urine creatinine, 9.8 mmol/L)), there was no clear reason to why the urine concentrations were low. However, the children who produced these samples could possibly not have taken their drugs (since we did not strictly observe the doses), have impaired absorption or result may have been affected by their acetylator status. HIV infection could cause the impaired absorption of anti-TB drugs which can lead to a significant reduction in urine levels of INH and AcINH (Gurumurthy *et al.*, 2004). Variability in acetylator status due to differences in intestinal and hepatic N-acetyl transferase 2 enzyme activity due to genetic polymorphisms can also affect INH concentration.

In summary, I have addressed objectives 1 (To evaluate INH urine concentrations of children who have been on daily dosing and intermittent dosing for prophylaxis at different time points using mass spectrometry measurement of INH and AcINH, and Arkansas method) and 2 (To evaluate effect of age, weight, dosing time and frequency, and urine creatinine on urine INH/AcINH concentrations) for the study population. This however, should be confirmed in other populations which may be different for various reasons including N-acetyl transferase 2 genotype which was not evaluated in this study. Moreover, a larger sample size may be required to detect the effects of the relevant covariates. I compared the concentrations of INH and AcINH with the colour changes using Arkansas method (objective 3) and found that there was good agreement between colour change and the concentrations of INH and an even stronger association with AcINH concentrations. Objective 4 (To propose a cut-off Arkansas colour for adherence to last dose for the 4, 24, 48 and 72-hour time points): pale blue/green colour was proposed for non-adherence to 4 hours post INH dose. However, possible non-adherence within protocol could affect results. There is need to confirm findings in larger rigorously conducted study. None-the-less, my findings made a valuable contribution to the interpretation of adherence to INH doses using the Arkansas test. While it can reliably detect a recent dose (4 hours after dose), 24 hours after the dose it does not perform well. More studies are needed to assess the proportion of positives for the test between dose and 4 hours and at time points later- 4 hours up to 12 hours (during which clinic visits might occur).

In conclusion, ranges for INH and AcINH urine concentrations were obtained. The Arkansas test is useful in monitoring morning doses approximately 4 hours before clinic attendance of prophylactic INH in children. It can reliably detect a recent 4 hours after INH dose but does not perform well 24 hours after dose.

5.1 STUDY LIMITATIONS

A limitation to this study was our inability to strictly observe all the doses before urine sampling. Though we tried to optimize the use of phone calls to cover for this limitation, a few of the results may have been affected by non-adherence.

Sample size was limited. A large sample size may be required to detect effects of the relevant covariates on INH and AcINH urine concentrations.

A single observer read the colour which could create a strong chance of bias. However, the observer was blinded as to allocation of INH/placebo to reduce this bias.

Acetylator status, known to be an important determinant of INH concentrations was not determined for study cohort.

5.2 FURTHER WORK

This study evaluated INH and AcINH concentrations in urine samples collected at 4, 24, 48 and 72-hour time points. Further work is required to assess other time points between dose and 4-hour and later than 4-hour up to 12-hour (during which clinic visits might occur).

Concentrations of INH and AcINH agreement with colour changes using Arkansas test should be confirmed in other populations which may be different for various reasons including N-acetyl transferase 2 genotype.

APPENDIX A

Case Record Form

UIAE Project Patient's study ID ☐☐☐☐☐☐

PRINTED REPORT OF URINARY INH DATA

Weight ☐☐.☐ kg Height ☐☐☐ cm Date of birth d d m m y y y y

Treatment arm: Intermittent dosing ☐ Daily dosing ☐

Dose of tablets being taken INH / placebo ☐ mg

Date and time of dose prior to visit:

INH / placebo d d m m y y y y h h : m m

Date and time of dose at visit:

INH / placebo d d m m y y y y h h : m m

Time of last meal before dose at visit h h : m m

Did patient vomit after dose at or prior to visit? Y N State time patient vomited h h : m m

* If patient vomited up to 1 hour after dose prior to visit or at visit, urine samples should not be collected

CONCOMITTANT MEDICATIONS (Including ARVs)

Drug	Dose (mg)	Frequency

URINE COLLECTION

Date of collection d d m m y y y y Day of collection (Mon. – Fri.) ☐

Daily dosing arm

Collection	Time	ID number on collection tube
1st (24 hours post dose)	h h : m m	☐☐☐☐☐☐ - 1
2nd (4 hours post dose)	h h : m m	☐☐☐☐☐☐ - 2

Comment(s) _____

UIAE Project Patient's study ID ☐☐☐☐☐☐

PRINTED REPORT OF URINARY INH DATA

Intermittent dosing

Collection	Time	ID number on collection tube
1st (72 hours post dose)	h h : m m	☐☐☐☐☐☐ - 1
2nd (4 hours post dose)	h h : m m	☐☐☐☐☐☐ - 2
Day / Date of 3rd collection	Day ☐	Date d d m m y y y y
3rd (48 hours post dose)	h h : m m	☐☐☐☐☐☐ - 3

Comment(s) _____

NB: Samples should be stored at -80 degrees Celsius

Urine creatinine Date d d m m y y y y

Result:

Sample 1: _____

Sample 2: _____

Sample 3: _____

Forms completed by _____ _____
Name Signature

Where there protccols that had to be violated ? Y N

State reason (if yes) _____

_____

DECLARATION BY INVESTIGATOR

I declare that I have verified all the information written in this form. The information reported are correct and Changes made have been duly signed and dated.

Initials ☐☐

Signature
of investigator _____ Date d d m m y y y y

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