THE DEVELOPMENT, IMPLEMENTATION AND VALIDATION OF A PLASMA-BASED HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ISONIAZID AND *N*-ACETYLISONIAZID:

AN ACETYLATOR STATUS POPULATION STUDY AT BREWELSKLOOF HOSPITAL

JENNIFER COCKCROFT

A project submitted to the Department of Pharmacology, University of Cape Town in fulfilment of the requirements for the degree

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Supervisors: Assoc. Prof. Peter Smith
Dr Helen McIlleron

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DECLARATION

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This thesis is presented for examination for the degree of Master of Science (MED).

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DATE: 17/05/2001

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ABBREVIATIONS

AcINH	*N*-acetylisoniazid
INH	isoniazid
HPLC	high performance liquid chromatography
TFA	trifluoroacetic acid
CH_3CN	acetonitrile
CV	coefficient of variation
r^2	coefficient of determination
SEM	standard error of the mean
SD	standard deviation
CI	confidence interval
RT	retention time
STD in MP	drug standard prepared in mobile phase
Cmax	maximum concentration
Tmax	time at which maximum concentration is reached
AUC	area under the curve
AUCT	total area under the curve
AUCINF	area under the curve extrapolated to infinity
Thalf	half-life
A	adenine
C	cytosine
G	guanine
T	thymine
PCR	polymerase chain reaction
NAT2	*N*-acetyltransferase 2 protein
NAT1	*N*-acetyltransferase 1 gene
NAT2	*N*-acetyltransferase 2 gene
NATP	*N*-acetyltransferase pseudogene
kb	kilobases

ABSTRACT

A novel high performance liquid chromatographic assay has been developed for the simultaneous determination of isoniazid and *N*-acetylisoniazid in plasma. Solid phase extraction involving C18 columns is used to extract the drug and the metabolite from 0.5 ml plasma. The analyte peaks are resolved using a C8 Spherisorb analytical column and ultraviolet detection at 270 nm. The assay is specific to the compounds, with consistent recovery of greater than 75% for isoniazid and over 90% for *N*-acetylisoniazid. The limits of detection in plasma are 300 ng/ml and 150 ng/ml for isoniazid and *N*-acetylisoniazid respectively. Linearity was conserved down to these concentrations.

This assay was used to generate pharmacokinetic data on 114 tuberculosis patients recruited for this study at Brewelskloof hospital, Worcester, South Africa. Using these data, various markers were investigated for the determination of acetylator phenotype, namely isoniazid half-life, isoniazid plasma level at three hours, and the ratio of metabolite to drug at three hours. The ratio of metabolite to drug at three hours proved to be the most reliable method for phenotype classification, this being confirmed during the genotypic portion of the study. Trimodality was evident, although the non-discrete separation of intermediate and rapid acetylators made this tentative.

The mean values of area under the concentration-time curve for each acetylator type were found to be significantly different, with rapid acetylators being potentially compromised in terms of exposure to isoniazid (slow *32.39 mg.l^{-1}.hr*; intermediate *21.25 mg.l^{-1}.hr* and rapid *16.04 mg.l^{-1}.hr*). Other pharmacokinetic parameters were bimodally distributed, homozygous and heterozygous rapid acetylators forming a single acetylator group. Codominance of the rapid and slow alleles was confirmed, the estimation of a mean intermediate elimination rate constant being within 7% of the observed mean.

The correlation of genotype to phenotype was found to be *88.2%* and the allelic distribution was determined to be acceptable using the Hardy Weinberg equation.

The incidence of raised liver enzyme levels was low in the study population with no relation to acetylator phenotype. Age and weight gain after two months of daily therapy did not correlate with phenotype. The slow acetylator population comprised of

a greater proportion of men, while women exhibited twice the number of rapid acetylators. No patient factors could be implicated in the apparent discordance of phenotype with genotype, and this suggests that there may be new allelic variants in this population.

This report provides validation and proves the usefulness of a novel HPLC plasma-based assay for determining isoniazid and *N*-acetylisoniazid levels in patients with tuberculosis.

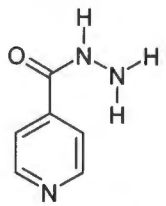
CHAPTER ONE

INTRODUCTION

1.1. HISTORY

Having been synthesised by Meyer and Mally in 1912, isoniazid (isonicotinic acid hydrazide) was not chemotherapeutically tested until forty years later.[1, 2, 3, 4]

It was introduced as an anti-tuberculosis agent in 1952. Bernstein and Lott *et al.* reported on the considerable anti-tuberculosis activity of isonicotinic acid hydrazide.[3]



"Nydrazid"

(Isoniazid)

Figure 1.1a. The chemical structure of "Nydrazid"

It was active in mice against the Ravenel strain of M. tuberculosis at a dose of 2 mg/kg, equivalent to 1/700 that of *para*-aminosalicylic acid, the therapy of choice at that time.[3] Grunberg and Leiwant *et al.* showed efficacy in mice infected with *M. tuberculosis* H37Rv.[5]

That same year, Rubin and Hassert *et al.*[2] published findings on the pharmacology of Nydrazid. Using mice, rats and dogs, acute, subacute and chronic toxicity studies were performed. The drug was found to be rapidly absorbed from the gastrointestinal tract, the early plasma peak drug concentrations being similar when administered orally or intravenously.

Selikoff and Robitzek[1] performed human clinical studies using isonicotinic acid hydrazide. Ninety-two patients with tuberculosis were treated with the drug and its isopropyl derivative with gratifying effects. Among other improvements significant weight gain was noted, as well as the complete elimination of fever in all cases within three weeks of treatment initiation. As far as clearing the sputum of the patient, 25% were negative after 4 to 15 weeks of therapy; while a further 28% showed a significant reduction in smear positivity. Most convincingly, there were no deaths due to the progression of tuberculosis, demonstrating the efficacy of these agents in human tuberculosis. Until this stage, tuberculosis had only been treated using

streptomycin, with the addition of para-aminosalicylic acid to prevent streptomycin resistance.[6]

Unlike certain other diseases, it was decided very early on that the use of monotherapy was a bad treatment choice, engendering the emergence of drug-resistant tuberculosis. Numerous clinical trials were run, trying to find the most beneficial combination of anti-tuberculosis agent for both the patient and the disease.[6]

Since its introduction, isoniazid has been one of the most powerful chemotherapeutic agents used in the treatment of tuberculosis. Isoniazid has remained one of the principal components of all first-line drug regimens. It has displayed a relatively low toxicity[1, 2] and is economically viable for third world treament.[7]

Various formulations of isoniazid have been developed with the intent to better drug exposure. A significant amount of time and research went into developing matrix isoniazid[8], a slow release preparation, which would hopefully decrease the number of doses required during intermittent therapy, easing the treatment period. However, the reduced saturation of the liver enzyme responsible for metabolising isoniazid necessitated the dosage to be increased to 40 mg/kg in rapid acetylators.[8] Side effects in rapid acetylators were more pronounced, due to this increase. It was later observed that rapid acetylators fared significantly worse than slow acetylators when given ordinary isoniazid with matrix isoniazid during once-weekly intermittent therapy, even with an increased dosage.[6] This was associated with the observed inhibition of the anti-tuberculous activity of isoniazid by the metabolite monoacetylhydrazine generated from the matrix isoniazid.[9]

The genetic hypothesis of acetylator status has been known since before the implementation of the drug in treatment regimens. During studies on the pharmacokinetics of isoniazid in the early 1950's, a large interindividual variation in metabolism was seen.[10] Initially the acetylation distribution was found to be bimodal, but more recent research has proven it trimodal, making a definite distinction between heterozygous and homozygous rapid acetylators, confirming initial expectations by Sunahara[11] and Dufour[12] This work has been studied extensively and confirmed by genetic work beginning on twins and families in the early 1960's,[13] but progressing at a phenomenal pace in the beginning of the last decade.

Apart from dealing exclusively with isoniazid and acetylator status; there are many

other drugs associated with this polymorphism. These include caffeine, procainamide, hydralazine, enflurane, dapsone, and sulphadimidine.[14,15] Various methods exist for the determination of acetylator phenotype. [reviewed in 16]

The isoniazid[17, 18, 19, 20, 21] and sulphadimidine[17, 22, 23] tests were the first to be used regularly. Price-Evans and White (1964) used isoniazid half lives for phenotyping but later, sulphadimidine replaced isoniazid because it was easier and required less time to determine acetylator status using one sample of either plasma or urine.[23] However, it was shown that urine testing left larger room for error than blood sampling. Du Souich and McClean *et al.*[24] highlighted this as well as a number of changes in nonmetabolic parameters, which could affect phenotyping using urine sampling with sulphamethazine and other agents, as opposed to a 6 hour blood sample.

Caffeine has been used as a metabolic probe to test for NAT2 acetylator phenotype. Acetylator phenotype is measured by determining the peak height ratio of two caffeine metabolites, using high performance liquid chromatography.[25] This test has often been compared to those using sulphasalazine[25] and sulphadimidine[26], and has been shown to produce identical results.

The types of **assays**, which have been used to assay biological samples, include colorimetric and high performance liquid chromatographic systems. Both have shown equivalent efficiency. The dapsone method[27] has been used frequently for determining acetylator status. This method can easily be implemented in both the clinical as well as the population screening setting. The method for phenotyping relies on the ratio of monoacetyldapsone to dapsone in plasma, and this remains constant in the bloodstream for up to 48 hours. Thus the timing of the sample need not be precise.[16] The assays used include HPLC methods, but also a number of other chromatographic methods. The question then was whether acetylation occurred bimodally or trimodally.

Today it is generally accepted that the distribution is trimodal, genetically and phenotypically, but the separation between the heterozygous rapid from the homozygous rapid acetylators tends not to be as discrete as that between the homozygous slow and the other two types.[28]

Isoniazid is still today regarded as an extremely powerful weapon against tuberculosis, in terms of its early bactericidal activity (EBA = 0.72).[7, 29] The maximal levels attained after a standard dose has been observed to be 16 times higher than

the minimal inhibitory concentration (MIC), much higher than that for other agents.[7] Thus, a decrease in plasma levels should not affect the overall killing ability significantly, provided that the decrease is slight. In the absence of kinetic vs. outcome-related studies, application of absolute levels to clinical outcomes is difficult.

Many different populations have been subjected to acetylator phenotyping, with evidence of certain population trends, such as the predominance of rapid acetylators in people of Asian descent. This epidemiology was later applied to genotypic analysis, linking various mutant alleles to certain origins.[30]

Table 1.1i. The acetylator phenotype distribution in various populations using isoniazid

Population	Phenotypic Results			Reference
	% Slow	*% Intermediate*	*% Rapid*	
Japanese	11.5	44.4	44.1	Sunahara and Urano *et al.* 1961[11]
Thai	27.8	52.8	19.5	
Ainu	12.8	36.0	51.2	
Korean	10.8	44.6	44.6	
Ryukuyuan	14.5	51.6	33.9	
Caucasian American	58.10	35.24	6.67	Dufour and Knight *et al.* 1964[12]
Black American	50.86	43.96	5.17	
Japanese	9.57	38.76	51.67	
Ethiopian Amharas	83.0	-	17.0	Russell and Russell 1973[31]
Lapps	28.57	-	71.43	Ellard and Gammon *et al.* 1973[32]
Lapps	37.0	-	63.0	Tiitinen and Mattila *et al.* 1973[33]
Finns	56.0	-	44.0	
Black South Africans	27.0	-	73.0	Buchanan and Strickwold *et al.* 1976[20]
West Africans	40.0	-	60.0	Salako and Aderounmu 1977[34]
Japanese	86.84	-	13.16	Horai and Ishizaki *et al.* 1982[35]
Indians	58.66		41.33	Gurumurthy and Krishnamurthy *et al.* 1984[36]
South African Coloured	35.0	45.0	20.0	Parkin and Vandenplas *et al.* 1997[37]

1.2. BREWELSKLOOF HOSPITAL

Brewelskloof hospital is situated just outside Worcester in the Breede Valley, north of Cape Town. Not officially designated as a tuberculosis hospital, it has become devoted to caring largely for patients with TB. It is a poor facility with few resources, but still manages to deal with the ever-increasing load of tuberculosis sufferers.

In the beginning of 1999, the Department of Pharmacology, University of Cape Town was approached to assay TB drug levels from a selected group of patients at Brewelskloof hospital. It had been noted that the relapse rate/treatment failure rate was increasing, and the investigation into this began at the level of medication efficacy.

Following this, the Department of Pharmacology, in collaboration with the hospital, initiated a pharmacokinetic study, the aims of which included determining the relationship between the pharmacokinetics of anti-tuberculosis therapy and treatment outcome. This acetylator status study formed part of the patient characteristics determination, and assessing their role in the pharmacokinetics of anti-tuberculosis drug therapy.

This work is still ongoing, with the hope to follow up all patients meeting research criteria up to two years, in order to gauge response, via certain study-determined markers.

Figure 1.2a. Brewelskloof Hospital, Worcester, Breede River Valley.

AIMS

The aim of this study is to develop the means to produce a comprehensive acetylator status study of in-patients at Brewelskloof hospital, producing criteria for phenotyping and validating the technique by genotypic analysis.

OBJECTIVES

1. Develop a high performance liquid chromatography plasma-based assay for the simultaneous determination of isoniazid and N-acetylisoniazid.
2. Conduct the pharmacokinetic study at Brewelskloof hospital, producing pharmacokinetic data on 100+ tuberculosis patients.
3. Use pharmacokinetic markers of isoniazid and N-acetylisoniazid to determine acetylator phenotype of patients, viz; half-life, Cmax, AUCI, AUCT and INH:AcINH ratio at 3 hours.
4. Compare acetylator phenotype obtained by each marker with the NAT2 genotype of the respective patient.
5. Compare acetylator phenotype obtained with clinical response and toxicity.

CHAPTER TWO

ASSAY DEVELOPMENT

2.1. INTRODUCTION

Numerous methods to determine isoniazid and *N*-acetylisoniazid have been developed and published since the introduction of isoniazid. Various assay types have been implemented, such as high performance liquid chromatography, fluorometry and colorimetry.

Ellard and Gammon developed methods of determination in serum and in urine, working from various other methods already published.[38] Isoniazid and *N*-acetylisoniazid were determined via fluorometry and colorimetry, one being more specific at low concentrations of isoniazid, and the other at higher concentrations. Utilising chemical methods to derivatise and extract compounds, extremely sensitive assays were produced, allowing very low concentrations to be analysed, but the efficiency is encumbered by a long assay procedure, using many different reagents.

Svensson and Muchtar *et al.* published a method, which utilised ion pair high performance liquid chromatography to determine the concentrations of isoniazid and *N*-acetylisoniazid in plasma and urine.[39] They originally designed this method because they experienced reduced sensitivity and found the spectrophotometric method to be time-consuming. The extraction procedure employs ultrafiltration, a simple technique used to rid the sample of unwanted protein contaminants. The method produced a simultaneous detection of isoniazid and *N*-acetylisoniazid. It does involve a derivatisation step, converting isoniazid into propionylisoniazid, before resolving the compounds on a reverse phase HPLC analytical column, using 1-dodecylsulphate as a counter ion.

Fluorescence detection is commonly used in the determination of isoniazid in biological samples. Ioannou[40] developed a simplified technique for the fluorometric detection of isoniazid and *N*-acetylisoniazid. However, the detection of *N*-acetylisoniazid was indirect in that total hydrazides were measured after acid hydrolysis. This would not be adequate for the proposed study.

Methods have been described for the liquid phase extraction of isoniazid and *N*-acetylisoniazid from plasma. Hutchings and Monie *et al.* described a comprehensive

method for the recovery of these two compounds from heparinised plasma.[41] The method is not excessively time-consuming and provides adequate recoveries. The detection system includes ultraviolet detection at 266nm, resolving both peaks in a single run on HPLC, using a nitrile analytical column, in a mobile phase of (0.01M) phosphoric acid in acetonitrile : water (20:80).

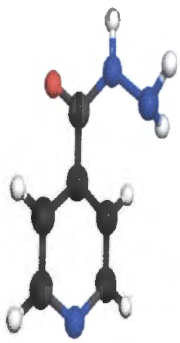


Figure 2.1a. The chemical structure of isoniazid

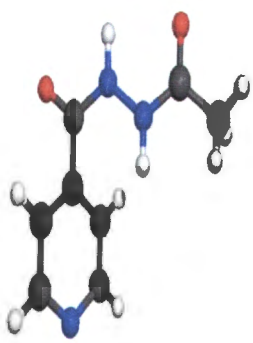


Figure 2.1b. The chemical structure of *N*-acetylisoniazid

Although a number of methods exist for measuring isoniazid and *N*-acetylisoniazid levels in plasma, it was necessary for the purposes of this study to develop a method, which would allow assaying of a large number of samples in a relatively short period of time. This would facilitate the speedy generation of pharmacokinetic data. The ideal option would be to avoid time-consuming steps such as derivatisation and acid hydrolysis. A method of choice would allow the simultaneous extraction of both drug and metabolite, coupled with a simultaneous resolution on HPLC.

2.2. MATERIALS AND METHODS

Materials

Isoniazid (INH) (Batch no. 960805) was obtained from Hoechst Marion Roussel (Johannesburg, South Africa). A standard of *N*-acetylisoniazid (AcINH) was generously donated by Dr Gordon Ellard, which he had prepared personally. HPLC-grade acetonitrile, methanol and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Deionised and distilled water was obtained via a Milli-Q Water system. C18 BondElut extraction columns were purchased from Analytichem International (Harbour City, CA, USA).

HPLC equipment consisted of a Spectra-Physics (San Jose, CA, USA) set-up, comprised of a pump Model SP8800, a degasser SCM400, an autosampler SP8780 and an integrator SP4290, which was ultimately connected to a variable wavelength UV detector Linear UVIS 200 Model 0200-4000 Linear Corp (Reno, NV, USA).

Methods

HPLC resolution was developed using the method described in the publication by Smith and Van Dyk *et al.*[42] The method uses a mobile phase system consisting of 3% acetonitrile in 0.06% TFA. A reversed phase C8 analytical column (Spherisorb, 150 X 4.6 mm ID, 5 μm - Supelco, USA) linked to a C8 precolumn was used with a flow rate of 1.5 ml per minute. UV detection was at 270 nm. The retention time for isoniazid varied between 2.80 and 3.00 minutes. This method was altered in various ways, including flow rate, mobile phase composition and pH to optimise the set-up for the simultaneous resolution of isoniazid and *N*-acetylisoniazid.

Sample preparation began with adopting the form of solid phase extraction as per the method of Smith and Van Dyk *et al.*[42] Working with plasma, the blood collection was performed using Vacutainer PST™ Gel and lithium Heparin tubes, which were immediately placed on ice before being spun down at 3 000 rpm to pellet the erythrocytes. Centrifugation occurred within 15 minutes of collection and the plasma was stored at -80°C until required. BondElut C18 extraction columns were washed twice with 2 ml HPLC grade methanol, followed by two washes of 2 ml water and 2 ml 0.05 M potassium phosphate (pH 4.5) (phosphate buffer). This served to precondition the columns, facilitating the binding of applied drugs. Five hundred microlitres of plasma was thawed and loaded onto each column. This was allowed to bind for 5 minutes and the unbound material was pulled through and discarded. The columns were then washed again with 1 ml 0.05 M phosphate buffer, removing excess plasma and unwanted waste. The bound drugs were then eluted with 0.5 ml acetonitrile and 0.5 ml methanol, the eluents subsequently being pooled. Of this, 500 μl was evaporated down to dryness using a Vacuum centrifuge at 37°C for 60 minutes and reconstituted in 500 μl 3% acetonitrile in 0.06% TFA. Twenty microlitres was then injected onto the HPLC for resolution. Isoniazid and pyrazinamide were detected together on the same system at 270 nm.

The method of Hutchings and Monie *et al.* [41] utilises a **liquid phase extraction**. One millilitre of spiked plasma is placed into a glass test tube. Five hundred microlitres of 0.5M phosphate buffer (pH 7.4), saturated with sodium chloride, is added to this. The sample is extracted using a mixture of 10 ml chloroform:butane-1-ol (70:30) (AnalaR, BDH Lab supplies), while gently mixing for 10 min. The mixture is separated by centrifugation at 3 000 rpm for 6 minutes. The top layer is then aspirated and the coagulated protein layer removed. Back extraction of the organic layer is performed using 500 µl phosphoric acid, mixing for 10 min. Two hundred microlitres is then injected onto the HPLC.

Stock solutions of both compounds were prepared in water and stored at -80°C in aliquots of 100µl. The plasma to be spiked was supplied by the Blood Transfusion unit at Groote Schuur Hospital and maintained at -20°C until required. Thawing took place at room temperature. The plasma was then vortexed (Vortex-Genie-2, Scientific Industries) and spun down at 3 000rpm for 6 min in a bench top centrifuge (Sigma 3E-1) to remove the fibrin and other particulate matter. Aliquots of 500 µl plasma were spiked with the standard stock solutions, to a final concentration of 10 µg/ml for each drug. Various methods of extraction were initially investigated, including liquid and solid phase extraction.

Method optimisation involved running 10 µg/ml standards of each analyte on the system described above. HPLC methods required changing the concentration of acetonitrile, altering the percentage TFA used, thus altering the pH of the mobile phase, changing the ratio of TFA to acetonitrile and altering the flow rate. Sample preparation methods required investigation into the preconditioning and washing buffer and the elution solvents. Having developed a potential assay system, tests such as storage stability, temperature stability, recovery analysis, sensitivity, detection limits and linearity were performed.

Assay validation was performed in order to test the accuracy and efficiency of the developed assay. Attributes such as specificity, recovery, stability (related to temperature), linearity, standard curves and sensitivity were investigated.

2.3. HPLC RESOLUTION

2.3.1. RESULTS

The method of Smith and Van Dyk *et al.*[42] was applied in an attempt to resolve the standards of isoniazid and *N*-acetylisoniazid prepared in mobile phase. Using a mobile phase of 4% acetonitrile to 96% (0.06%) TFA solution on a C8 analytical column, resolution of the two compounds was not achieved. N-acetylisoniazid was not retained adequately on the column.

In an attempt to increase the retention times of both analytes, the concentration of acetonitrile was reduced to 1%, while the pH remained unchanged. This only served to shift both peaks later, and did not improve the resolution.

The pH of the mobile phase often affects the retention times of specific compounds. Different concentrations of TFA were used to vary the pH and to gauge the effect of pH on the elution of drug and metabolite.

Table 2.3.1a. The effect of pH on the retention of isoniazid and *N*-acetylisoniazid

Mobile Phase pH	*TFA %*	*Drug*	*Retention Time (min)*
1.9	0.06	INH	3.83
		AcINH	3.62
1.75	0.15	INH	3.91
		AcINH	4.49

Using a mobile phase of CH_3CN : 0.15% TFA (1:99) the pH of the TFA solution played a large role in determining the retention time of *N*-acetylisoniazid. *N*-acetylisoniazid was affected more than isoniazid when the pH was lowered, as is seen when comparing the relative retention times above. The change in retention time for isoniazid was +0.5 min, compared to a change of +1.76 min for *N*-acetylisoniazid.

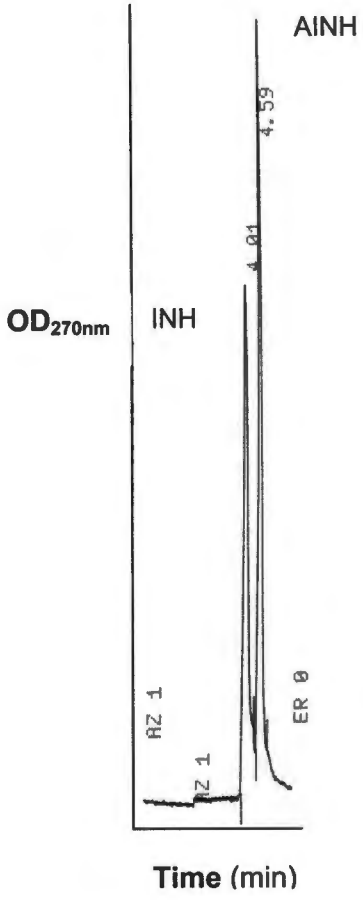


Figure 2.3.1a. A standard of 2.5 μg/ml isoniazid (4.01 min) and 2.5 μg/ml *N*-acetylisoniazid (4.59 min) in 1% CH_3CN:99% (0.15%) TFA at pH 1.75 at $OD_{270\ nm}$.

2.3.2. DISCUSSION AND CONCLUSIONS

The C8 reverse phase analytical column, when used with a mobile phase of pH 1.75, resolved the two peaks.

This demonstrated the increased sensitivity of *N*-acetylisoniazid to a lower pH. When running at a pH of 1.9, the *N*-acetylisoniazid is eluted before the isoniazid (Table 2.3.1a.). After adjusting the pH to 1.75 by increasing the strength of the TFA solution the order of elution was reversed. Isoniazid displayed a retention time of 3.91 min, which was followed by *N*-acetylisoniazid at 4.49 min.

Since isoniazid has one of its pKa values at 1.8,[43] going below this pH will produce a protonated form of isoniazid. Above pH 1.8, the compound will have a negative charge associated with the loss of a proton from the carboxyl group.

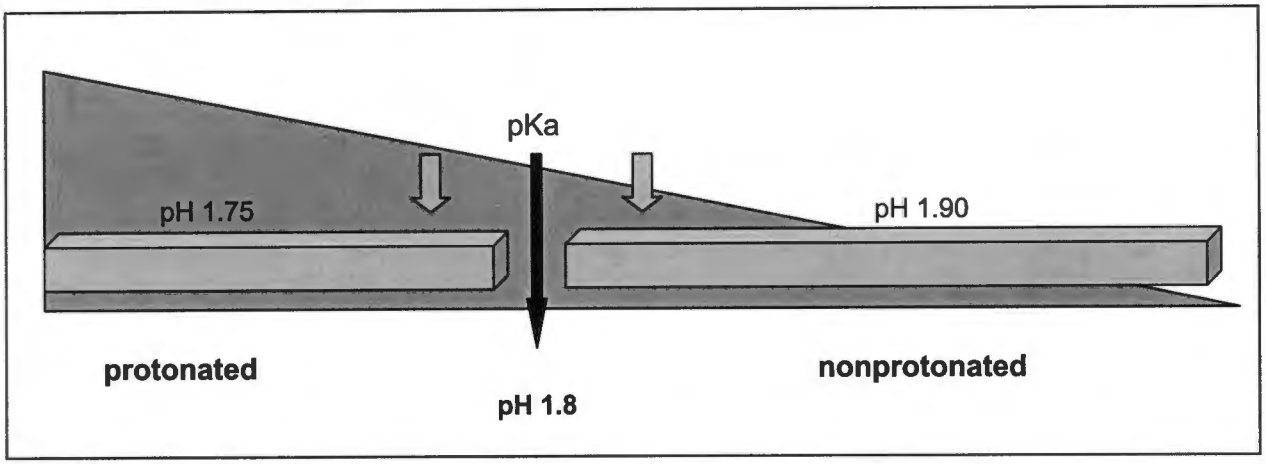


Figure 2.3.1c. The protonation of isoniazid relative to the first pKa value.

The pKa values of *N*-acetylisoniazid are not known. However, it is clear that lowering the pH to 1.75 significantly increased the retention of the compound when compared to isoniazid. The central structure of *N*-acetylisoniazid consists of a pyridine ring (Figures 2.1a and 2.1b). The structures differ at the substituted group at C1. It is most likely due to this structure that *N*-acetylisoniazid responds better to a pH reduction. The substituted group in *N*-acetylisoniazid is an ether, while in isoniazid it consists of a primary amine and a secondary amide.[14,15] The presence of the two carbonyl groups in *N*-acetylisoniazid could be the cause of the heightened pH effect, the carbonyl groups being responsible for the increased resonance withdrawal of *pi* electrons, thereby deactivating the aromatic ring to a greater extent that in isoniazid.[44]

The two carbonyl groups, now in possession of greater electronegative force, would exert a greater inductive effect on the C8 carbon chains in the column, producing a positively charged carbon atom, allowing the compound to adhere more strongly, and be eluted later. Of course, this would all depend upon the exact pKa values associated with the functional groups in question.

Using a C8 Spherisorb Analytical column and a mobile phase comprised of CH_3CN (1) : 0.15% TFA (99), peaks were well resolved, producing adequate resolution at 10 μg/ml concentration. (See figure 2.3.1a.).

The use of low pH in mobile phases may have previously been cause for concern due to the instability of the silica at low pH. However, in recent years a wide range of HPLC analytical columns with stability in the pH range 1-13 has been produced.

2.4. SAMPLE EXTRACTION

Various methods of sample extraction from plasma were attempted, with varying degrees of success. Ultimately, the extraction procedure used by Smith and Van Dyk *et al.*[42] was modified for the analysis of *N*-acetylisoniazid.

Pooled drug free plasma was spiked with known concentrations of isoniazid and *N*-acetylisoniazid. In order to determine the optimum extraction procedure, it was decided to concentrate on the extraction of *N*-acetylisoniazid initially, the method for isoniazid already established.

2.4.1. RESULTS AND DISCUSSION

The solid phase extraction method of Smith and Van Dyk *et al.* was attempted initially for extracting isoniazid and *N*-acetylisoniazid simultaneously from 1 ml spiked plasma (Section 2.2). The results presented in table 2.4.1a below indicate that *N*-acetylisoniazid fails to bind adequately to the C18 extraction columns.

Table 2.4.1a. The recoveries of INH and AcINH, using the extraction method of Smith and Van Dyk *et al.*[42]

Analyte (10μg/ml)	Peak Area (Mobile Phase)	Peak Area (Plasma)	Recovery (%)
INH	487 125	271 690	56.16 ± 0.38
AcINH	0	0	0

In an attempt to improve the binding of both analytes, the pH of the plasma was lowered with 5M HCl and the pH of the preconditioning buffer was altered. This failed to improve the binding of either compound.

Liquid phase extraction, using the method of Hutchings and Monie *et al.*[41] (see section 2.2) was attempted. The high concentration of NaCl successfully precipitated most of the protein out of the plasma, forming a large mass at the interface of the aqueous and the organic layers. Both the isoniazid and *N*-acetylisoniazid were extracted into the chloroform:butan-1-ol mixture, but with unsatisfactory recovery.

The recovery of isoniazid reached 85.29%, but the *N*-acetylisoniazid recovery remained low (Table 2.4.1b.).

Table 2.4.1b. The recoveries of isoniazid and AcINH using the liquid phase extraction method of Hutchings and Monie *et al.*[41]

Analyte (10μg/ml)	Peak Area (Mobile Phase)	Peak Area (Plasma)	Recovery (%)
INH	498 358	305 267	61.25
AcINH	315 022	9 455	3.00
INH	498 358	425 054	85.29
AcINH	315 022	51 874	16.47

It is clear that this method improved the recovery of *N*-acetylisoniazid, but the results were still unacceptable. Following this, assay development returned to solid phase extraction.

In order to establish if *N*-acetylisoniazid was being removed from the solid phase extraction column by the washing buffer, the wash step was eliminated. Modifying the ***plasma pH*** and the ***preconditioning/washing buffer pH*** had failed to produce a significant increase in yield of both drugs. Varying the pH of the washing buffer failed to retain the compound.

Following the elimination of a buffer wash after applying the spiked plasma to the column, significant recoveries of *N*-acetylisoniazid were recorded. This showed that *N*-acetylisoniazid was not binding strongly enough to withstand a buffer wash.

Analysis of the buffer washes confirmed that a major loss of *N*-acetylisoniazid was occurring at this step. Recoveries from plasma after eliminating the buffer wash step exceeded 50% (Mean = 57.37; σ = 3.81%) (Table 2.4.1c.). Adjustment of the pH of the spiked plasma prior to application to the column had no effect on yield.

Table 2.4.1c. *N*-acetylisoniazid recoveries using different preconditioning buffers

Buffer pH	RT (min)	Peak Area	Recovery (%)
1.5	5.36	166 622	**57.57**
3	5.41	145 978	**50.43**
5	5.37	176 391	**60.94**
7	5.39	175 899	**60.77**
8	5.38	165 377	**57.14**
STD in MP	5.35	289 438	**100.0**

Preconditioning the columns with a range of pH buffer at different pH's prior to the application of the sample had no effect on recovery (Figures 2.4.1a. and 2.4.1b.)

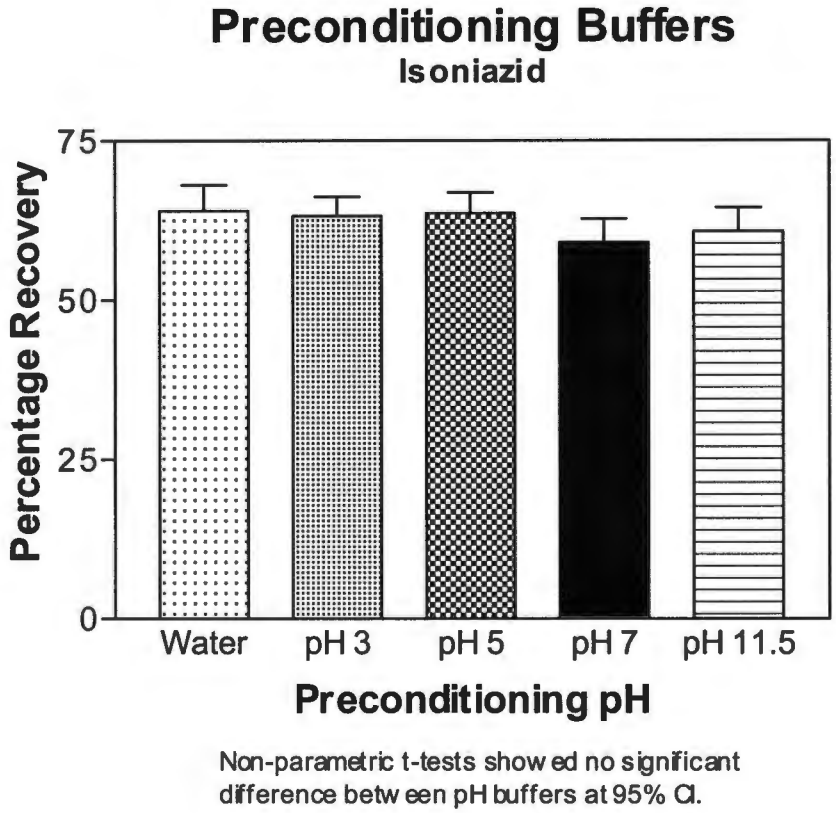


Figure 2.4.1a. The effect of various buffers on the recovery of isoniazid (n=5, Mean ± SEM).

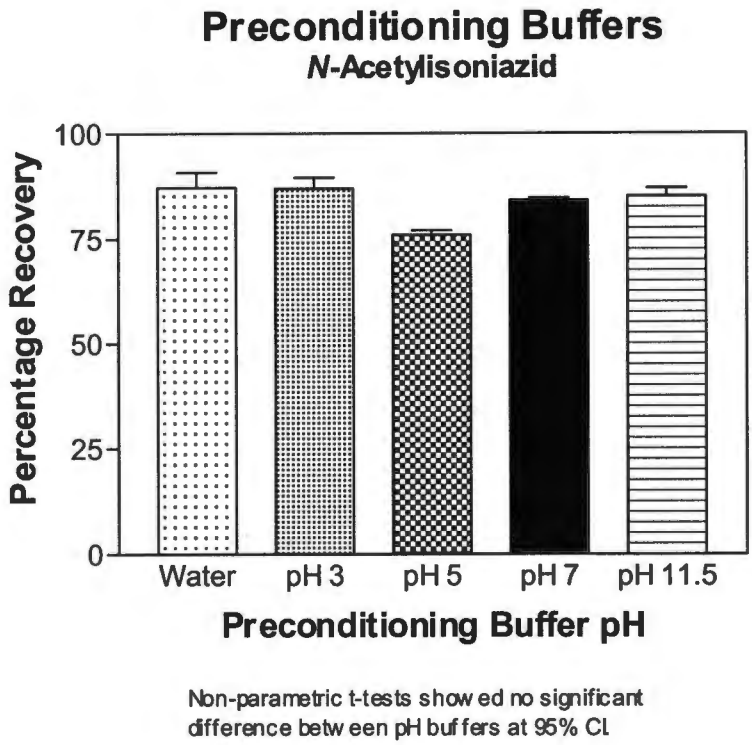


Figure 2.4.1b. The effect of various buffers on the recovery of *N*-acetylisoniazid (n=5, Mean ± SEM).

Recovery was maximised using water for preconditioning instead of a phosphate buffer. The mean recovery of isoniazid when using water as the preconditioning wash and eliminating any subsequent washing, was 82.87% ± 1.453% and 91.98% ± 2.858% for *N*-acetylisoniazid.

Elution of the drugs from the solid phase extraction columns without inclusion of a wash step frequently resulted in some protein precipitate in the eluate. This was removed by centrifugation and the supernatant used for analysis.

The HPLC program set up to elute isoniazid at 4.00 minutes and *N*-acetylisoniazid at 4.50 minutes at a flow rate of 1 ml/min, following solid phase extraction was as follows:

Table 2.4.1d. Program set up on the ternary HPLC pump including end-of-run wash

Time (min)	*% (100%) CH_3CN*	*% (0.15%) TFA*
0.0	1.0	99.0
4.0	1.0	99.0
5.0	20.0	80.0
9.0	20.0	80.0
10.0	1.0	99.0
13.0	1.0	99.0

The figures shown below (figures 2.4.1c., 2.4.1d. and 2.4.1e.) indicate the results of application of the solid phase extraction method determined above, coupled with the HPLC resolution method described in section 2.3. The peak appearing after 5 minutes represents the extraneous plasma proteins being washed off the column by the 20% acetonitrile wash. The run time of the assay was determined using the flow stability gauge to determine when the flow has returned to an acceptable level of stability following the acetonitrile wash (20%). A flow stability of below 10 was determined adequate and this was observed in the consistency (i.e. steady baseline and uniform retention times) of the subsequent assays.

The blank sample shown in the figure below (Fig. 2.4.1c.) indicates an interfering plasma peak, which merged with the isoniazid on occasion. Running an extracted blank plasma sample with each patient set enabled an integration correction to be made to the reported peak area of isoniazid, when this peak did not separate.

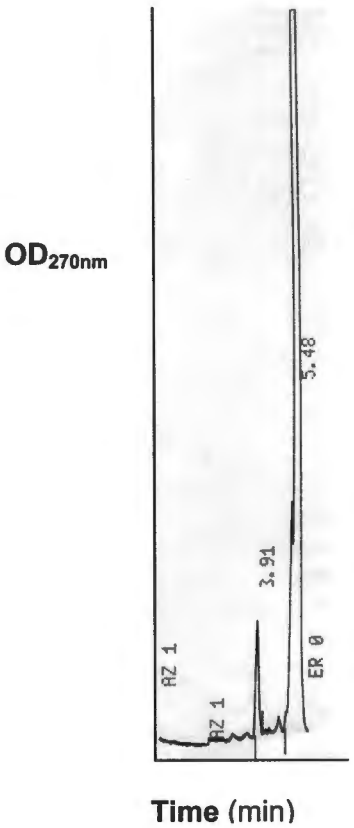


Figure 2.4.1c. An extracted blank plasma sample

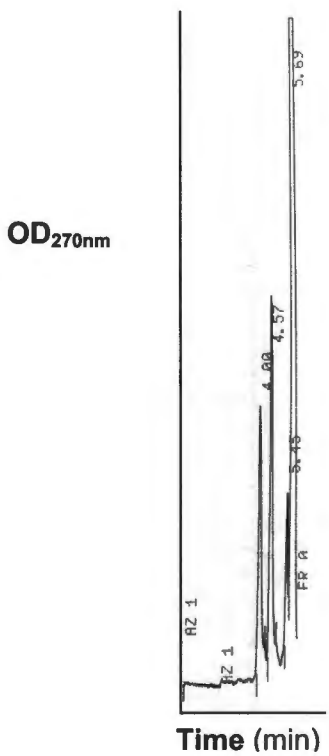


Figure 2.4.1d. An extracted control of 2.5 μg/ml isoniazid (4.00 min) and 2.5 μg/ml *N*-acetylisoniazid (4.57 min).

2.4.2. FINAL CONCLUSIONS

An assay procedure has successfully been implemented which includes a simple solid phase extraction, simultaneous extraction of isoniazid and *N*-acetylisoniazid, which are then detected on the same HPLC system.

HPLC RESOLUTION

- **Column:** S5 C8 Spherisorb (25cm X 4.6mm I.D.; 5μm particle size)
- **Mobile Phase:** CH_3CN (1) : 0.15% TFA (99) - pH 1.75
- **Flow Rate:** 1.00 ml/min
- **Run Time:** 13.0 min
- **Wavelength:** 270 nm at 0.02 a.u.f.s. deflection
- A two minute wash using 80% acetonitrile is implemented after 5 minutes to clean up the column for the following injection.
- The integrator chart speed was set to 0.5 and the peak threshold at 200, setting a cut off limit of a peak area of 200.

SAMPLE EXTRACTION

1) Wash each SPE C18 column with methanol and water.
2) Precondition each column with 1.0 ml Milli-Q water.
3) Apply 500 μl plasma to each column, pull onto the column and allow to bind for 5 min.
4) Pull the plasma through the columns with the vacuum until dry.
5) Elute into labelled, clean, dry polypropylene tubes with aliquots of CH_3CN as follows:
    - 200 μl left for 2 min and eluted (repeated),
    - 100 μl left for 1 min and eluted.
6) Spin down eluent at 10 000 rpm for 5 min, to pellet the protein precipitate.
7) Remove 250 μl supernatant and transfer into a screw-capped glass vial, which has been acid-washed.
8) Evaporate the samples completely in a rotary evaporator at 37°C (± 45-60 min depending on the pump strength).
9) Reconstitute with 500 μl mobile phase, incurring a two-fold dilution. Vortex and inject 20 μl onto HPLC.

Coupled with the solid phase extraction method determined earlier, the wash was sufficient to clear up problems with protein-contaminated samples.

2.5. ASSAY VALIDATION

In order to create a working assay system, it is necessary to run a series of validation steps using the assay. Having set up the assay it was validated with respect to **detection limits** (or sensitivity); **linearity**; **specificity**; **recovery**; **precision** and **stability**.

2.5.1. RESULTS AND DISCUSSION

2.5.1.i. **A Standard curve was constructed** by spiking aliquots of drug free plasma with known concentrations of isoniazid and *N*-acetylisoniazid. Samples were simultaneously spiked with isoniazid and *N*-acetylisoniazid from 10 μg/ml down to 0.03125 μg/ml, extracted and analysed using the predetermined assay system (sections 2.3 and 2.4). Following this, linear regression curves were constructed using GraphPad Software Prism (Version 2.01) and the equations calculated.

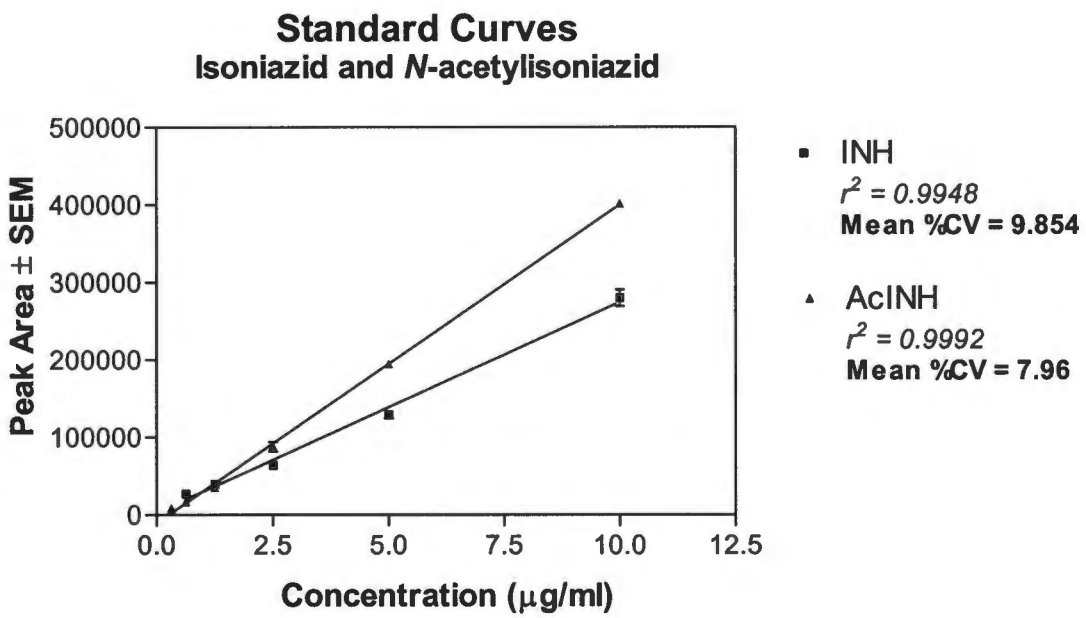


Figure 2.5.1i. Standard curves for isoniazid and *N*-acetylisoniazid. (n = 6)

Each point was prepared by serially diluting a stock solution of 10 μg/ml. The resulting curves demonstrated good linearity (r^2 = 0.9948 and 0.9992 for isoniazid and *N*-acetylisoniazid respectively within the range of concentrations tested.

Isoniazid:	$y = 27\,170x + 2\,911$
***N*-acetylisoniazid:**	$y = 41\,060x - 10\,660$

The equations from each curve may be used to convert peak areas of unknown concentration, into useful data. The coefficients of determination for each curve provided an estimate of the linearity of the assay. Both of these are above 0.990 so the assay was determined to be linear.

2.5.1.ii. ***Detection Limits*** were determined by extending the range of the standard curve down to 75 ng/ml for both compounds. Having completed this experiment in plasma, it was repeated in mobile phase. This was to determine the effect of any interfering plasma peaks. Peaks that were clearly visible and successfully integrated at the lowest recordable concentration, were determined to be the detection limit for that analyte. The data was plotted using GraphPad Prism (Version 2.01). ***Linearity*** was determined again using the same set of experiments. An estimation of linearity was gleaned from the resulting coefficient of determination for each analyte for each curve. The coefficients of determination were 0.9911 for isoniazid and 0.9927 for *N*-acetylisoniazid. These values compare closely with that obtained in the previous experiment (section 2.5.1.i). The experiment was performed in triplicate for each set of conditions.

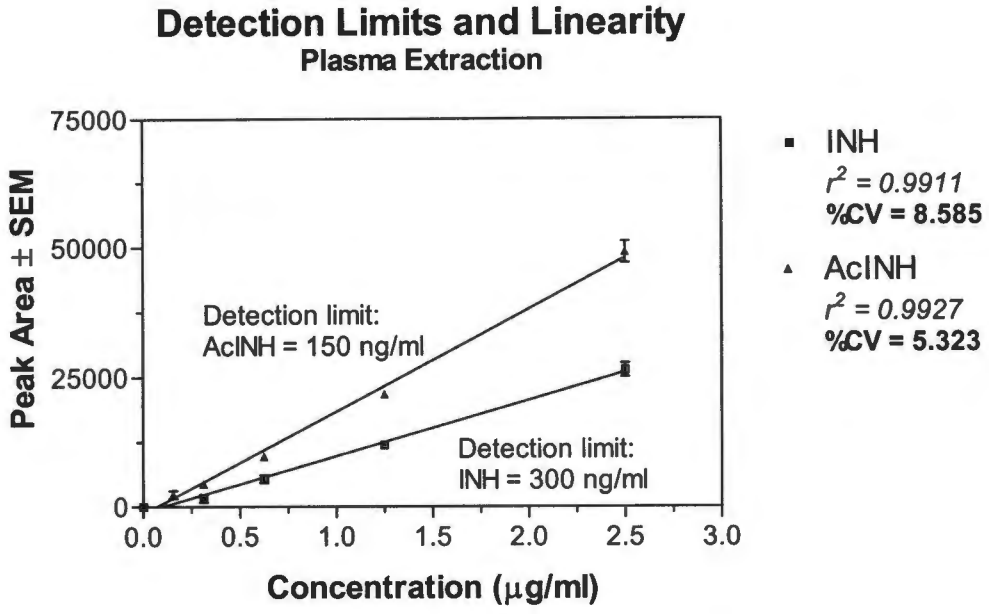


Figure 2.5.1.iia. Sensitivity and linearity testing using plasma extraction. (n = 3)

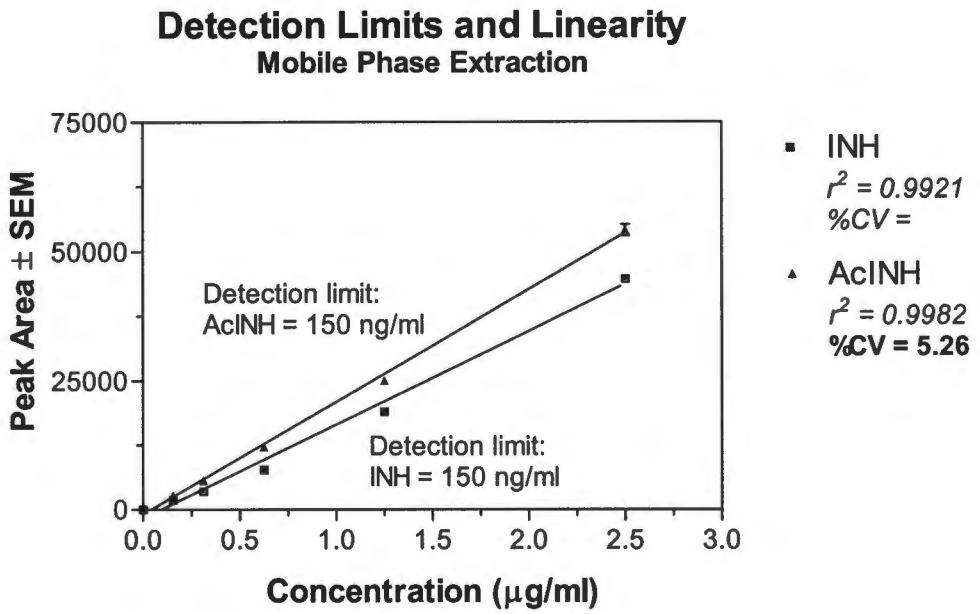


Figure 2.5.1.iib. Sensitivity and linearity testing using extraction from mobile phase. (n = 3)

The figures above indicate that even at lower concentrations, the linearity is maintained, making this assay suitable for determining pharmacokinetic curves in patients. The purpose of extracting from mobile phase is to exclude any interference offered by extraneous plasma peaks during sensitivity analysis.

The assay was shown to be **linear**, coefficients of determination of 0.9911 and 0.9927 for isoniazid and *N*-acetylisoniazid respectively (Figures 2.5.1.iia.). Each point was determined using a set of three samples, constructing a curve from 6

concentration points, including zero, over the concentration range 10 μg/ml to 600 ng/ml for isoniazid and 300 ng/ml for *N*-acetylisoniazid.

The plasma **detection limits** (figure 2.5.1.iia.) were easily distinguished, with *N*-acetylisoniazid being more distinguishable than isoniazid at lower concentrations, due to the absence of plasma peaks at the later retention time. As long as the peak was clearly visible and successfully integrated, it was determined successfully detected. A lower limit of 300 ng/ml was observed for isoniazid and 150 ng/ml for its metabolite using plasma extractions. Excellent linearity was observed even at these low concentrations (i.e. r^2 above 0.99).

2.5.2.iii ***Temperature stability*** was deduced using a set of experiments designed to decide upon optimal storage conditions (figures 2.5.1.iia. and 2.5.1.iib.) for patient plasma samples. A stock plasma solution at 5 μg/ml of both analytes was prepared and aliquotted to minimise damage by the freeze-thaw process, and stored at various temperatures for up to 72 hours. Room temperature (±21°C), 4°C, -20°C and -80°C were selected. Each day, three aliquots were removed, extracted and analysed using the predetermined assay method. Recoveries were calculated and any deviations from 5 μg/ml were noted. Results are shown in figure 2.5.1.iiia.

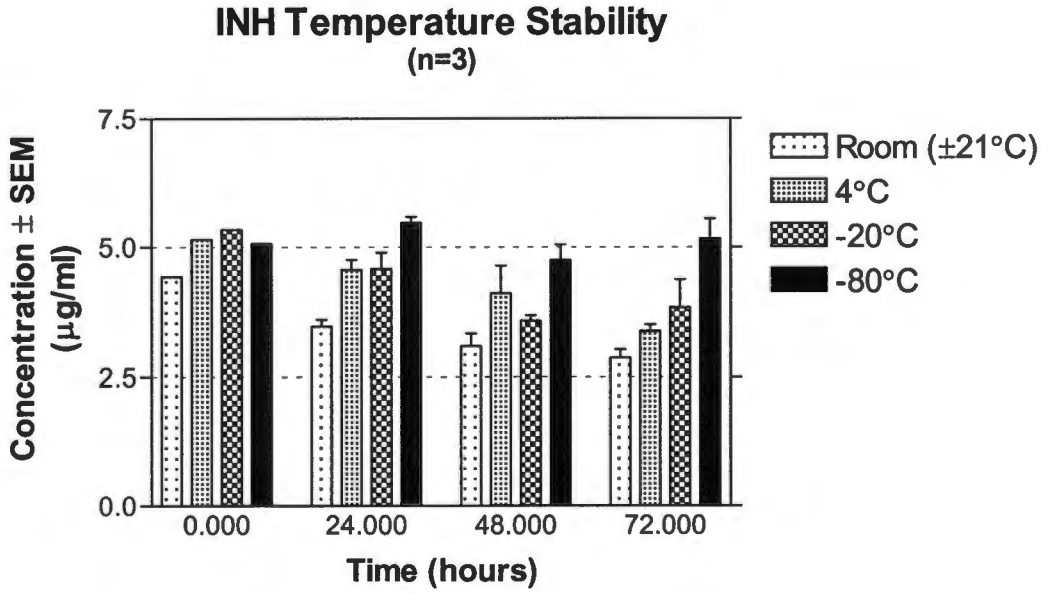


Figure 2.5.1.iiia. Isoniazid temperature stability. (n = 3)

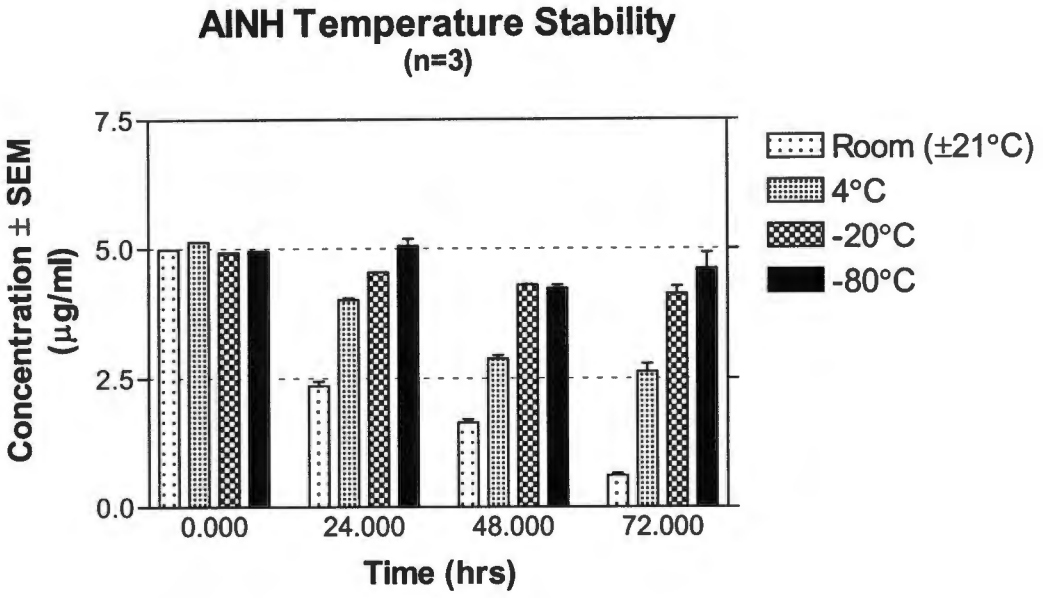


Figure 2.5.1.iiib. *N*-acetylisoniazid temperature stability. (n = 3)

Both isoniazid and *N*-acetylisoniazid are stable at -80°C, without adding any stabilising agents to the plasma. The stability over three days was acceptable without the addition of any stabilising agents. The other temperatures indicate marked degradation in both analytes.

Up to 72 hours, the stability of isoniazid at -80°C was adequate. The general stability of *N*-acetylisoniazid was considerably reduced at temperatures above -80°C, compared to that of isoniazid. This was most likely because the standards were supplied by different sources. From this set of experiments, it is clear that the optimal storage option would be at -80°C, with no additives. Some researchers add chemicals to the plasma samples to promote preservation, such as a crystal of thymol[17] or ascorbic acid[45], but these samples were seen to be stable without any such addition.

2.5.1.iv. To determine ***storage stability*** pooled stocks of 10 µg/ml (isoniazid and *N*-acetylisoniazid) plasma samples were aliquotted and stored at -80°C for up to 8 days. Each day, a set of four was removed, extracted and analysed. Recoveries were calculated and signs of deterioration were noted.

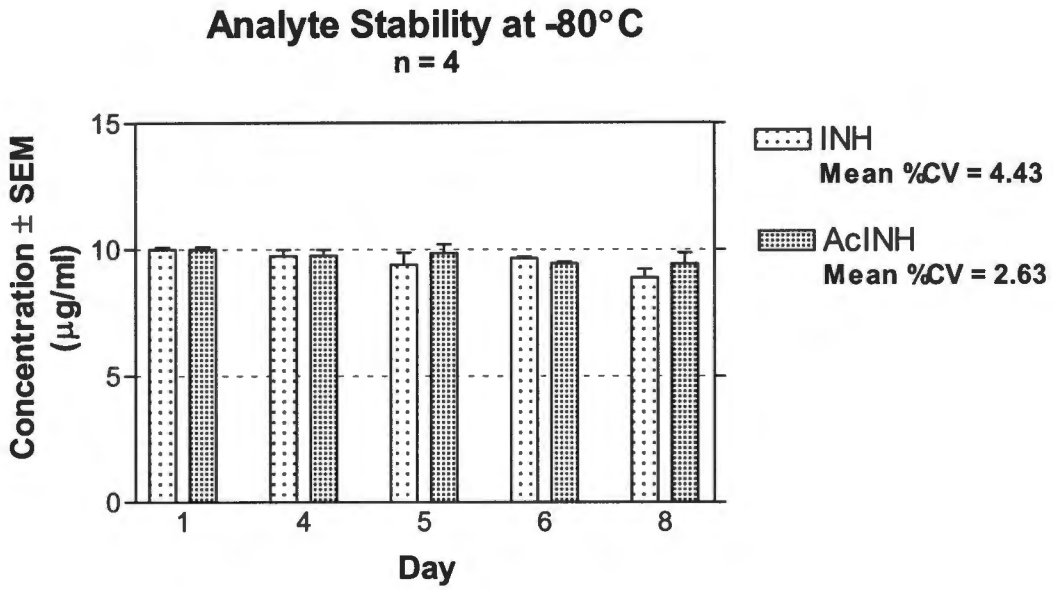


Figure 2.5.1.iv. Analyte stability at -80°C. (n = 4)

The mean coefficients of variation were calculated as 4.43% for isoniazid and 2.63% for *N*-acetylisoniazid. Since this experiment began with one pooled solution of spiked plasma, the only factors contributing to variation could be the storage temperature, or daily variation. The results show clearly that both analytes are stable in plasma at -80°C for at least 8 days.

2.5.1.v. ***Precision*** was estimated using a similar set of experiments. Coefficients of variation (%) were monitored throughout the validation series of experiments. **Reproducibility** may also be established by considering the coefficients of variation. Care was taken in monitoring these during every test performed. It was noted that these remained under 10% during each experiment, determining the assay in terms of being reproducible.

2.5.1.vi. ***Specificity*** was determined using a number of other drugs, known to be common concomitant medications in TB patients. This was done knowing that the population to be sampled for the phenotypic data consisted of sick TB patients, many on different concomitant medications. The drugs tested were kanamycin (Sigma), amikacin (Sigma), thioacetazone (Sigma), ethionamide (Sigma), pyrazinamide (Hoechst Marion Roussel), rifampicin (Hoechst Marion Roussel) and para-aminosalicylic acid (Sigma). A set of 10 µg/ml plasma samples (containing both isoniazid and *N*-acetylisoniazid) were prepared. To each sample, an aliquot of each drug was added to a final concentration of 10 µg/ml. These were then analysed and

run on the HPLC with a corresponding 10 μg/ml standard of each drug, separately. Any change in recovery was noted, as well as the appearance of any interfering peaks.

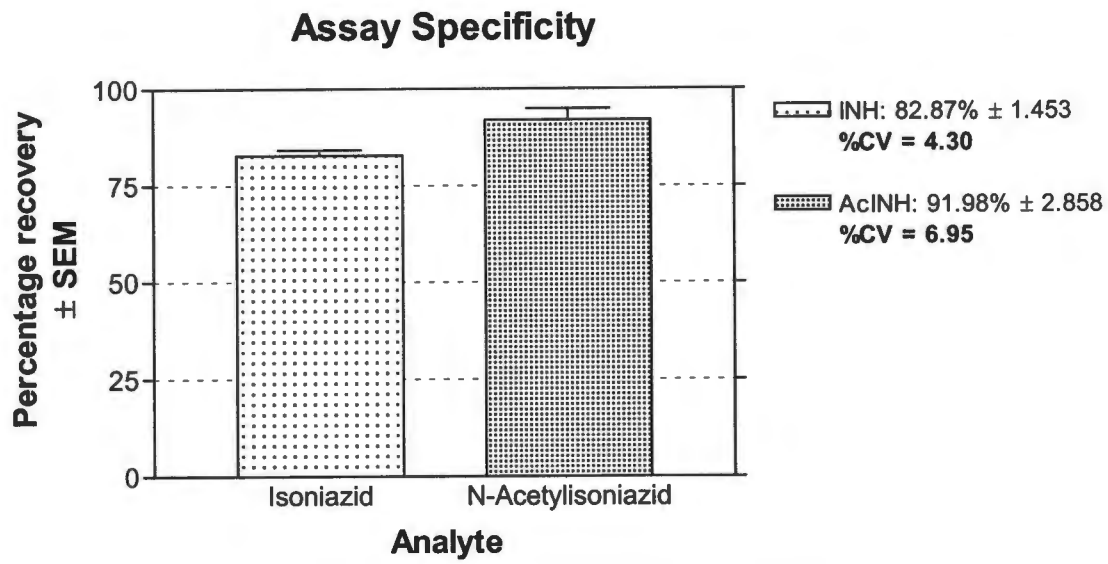


Figure 2.5.1.via. Specificity testing and the effects on recovery. (n = 6)

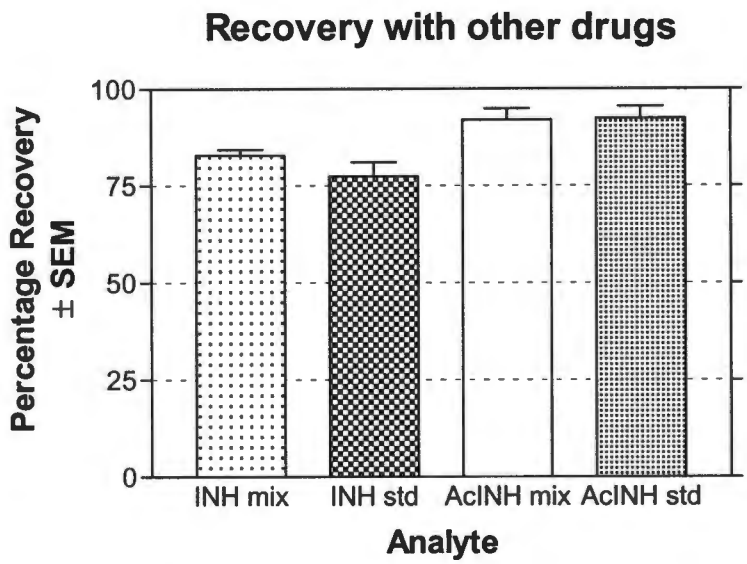


Figure 2.5.1.vib. Isoniazid and *N*-acetylisoniazid recovered from plasma spiked with other drugs, expressed as a percentage of the standard run in mobile phase. (n = 6)
(std = alone; mix = with other drugs)

The results of the **assay specificity** (figures 2.5.1.via. and 2.5.1.vib.) tests were conclusive. It was clear that not one of the other drugs, all of which are commonly given as concomitant medication to tuberculosis patients, produced an interfering

peak or inhibited drug recovery. The only drug that produced a small peak upon HPLC analysis was thioacetazone, but this was as a standard in mobile phase at 3.48 minutes. Once extracted, this peak was not evident. The recovery was highly reproducible compared to the previous recovery determination. Mann Whitney tests (two-tailed at 95% CI) showed no significant difference between the recoveries obtained for the mix (with additional drugs) and standard (isoniazid and *N*-acetylisoniazid alone) sets.

2.5.1.vii. ***Recovery*** was also determined experimentally prior to patient sample analysis. This was done using standards in mobile phase and spiked plasma samples. Each plasma sample was spiked with 10 μg/ml isoniazid and *N*-acetylisoniazid, extracted and run with a corresponding standard in mobile phase. After correcting for the dilution factor of 2, implemented during the extraction process, the recovery was calculated. This was repeated using 5 μg/ml and 2.5 μg/ml.

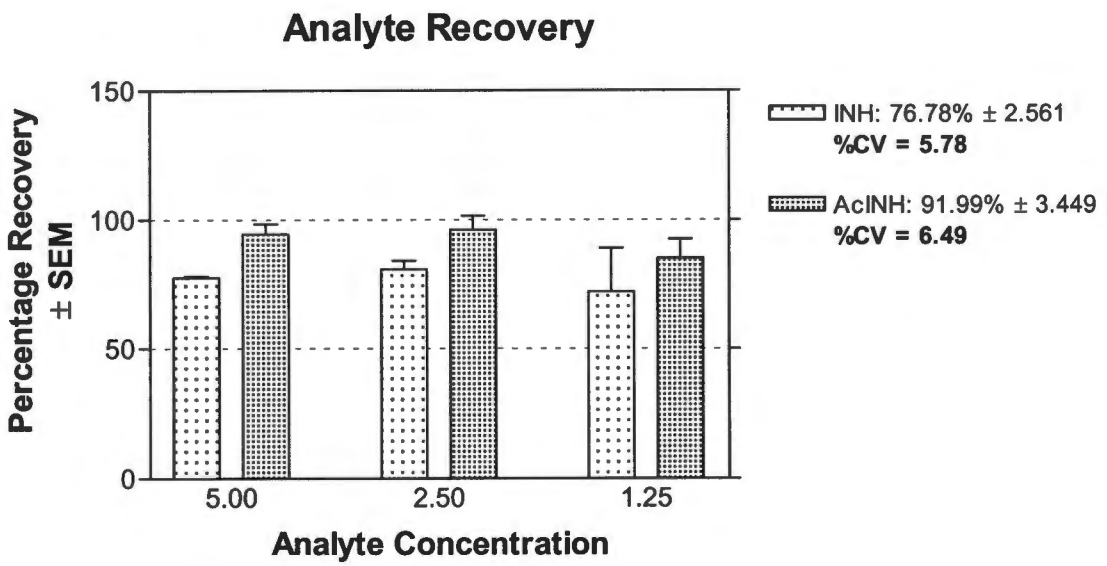


Figure 2.5.1.vii. Recovery analysis. (n = 4)

Analyte recovery (figure 2.5.1.vii.) is a good indication of assay quality. Certain drugs are known to give poor recoveries, but as long as the reproducibility is of a high standard, the assay is useful. In the method by Smith and Van Dyk *et al.*,[42] the recovery of isoniazid (>70%) may be viewed as being low, but the results are highly reproducible and thus give accurate estimations of drug levels. The results show that

the recoveries, apart from being acceptable (76.78% for isoniazid and 91.99% for *N*-acetylisoniazid), were also shown to be consistent over the concentration range 5 μg/ml to 1.25 μg/ml, with coefficients of variation of 5.78% and 6.49% for isoniazid and *N*-acetylisoniazid respectively. It may be noted that recoveries become less reproducible at lower concentrations (i.e. 1.25 μg/ml; figure 2.3.1.vii), yielding larger error bars, but are still within the limits of acceptability. The curves set up to determine linearity (figures 2.5.1.iia. and 2.5.1.iib.) did indicate linearity at lower concentrations, with acceptable coefficients of determination.

2.5.2. FINAL CONCLUSIONS

Thus, from the experiments above, this assay may be deemed successful and useful in terms of its reproducibility, specificity, recovery, sensitivity, linearity and stability. The assay is acceptable in terms of the coefficients of determination (all above 0.990) and coefficients of variation (all below 10%). The recoveries are acceptable because they are consistent, all achieving yields of above 70%.

In terms of the objectives prior to setting up the assay, those being primarily time and quality, these have been successfully achieved for the purposes of determining pharmacokinetic data for 100+ tuberculosis patients.

CHAPTER THREE

PHARMACOKINETICS AND ACETYLATOR STATUS

3.1. INTRODUCTION

Isoniazid (isonicotinic acid) is bactericidal for intra- and extra-cellular mycobacteria as well as mycobacteria in closed caseous lesions. The mechanism of action involves the inhibition of mycolic acid synthesis in the mycobacterial cell wall.[46] It is administered to treat tuberculosis, as well as preventative therapy.

The pharmacokinetics of isoniazid are well known. Isoniazid may be administered orally or intravenously. When administered orally, it is absorbed rapidly and completely within 2 hours of administration.[15, 47] Food and antacids are known to prolong absorption. Bioavailability is significantly reduced by first-pass metabolism. Maximum concentrations reach an average of between 3-5 μg/ml at approximately 1-2 hours.[14] Isoniazid is a polar drug, the volume of distribution being comparable to that of total body water in all tissues and fluids including CSF, and falling in the range of 0.6-0.7 L/kg.[8] The half life of this drug differs between individuals according to acetylator status and may range from 0.5-1.6 hours in rapid acetylators and 2-5 hours in slow acetylators. Less than 10% of the drug is protein bound. [14, 15]

The rates at which individuals convert isoniazid to metabolites devoid of any anti-tubercular activity differ greatly between individuals, although they do remain constant within that individual.[15] The determining factor in isoniazid the rate of elimination of isoniazid is the rate of acetylation performed by the liver.[48] The efficacy of isoniazid and the incidence of the usual forms of isoniazid-induced toxicity are directly related to the intensity and duration of exposure to the drug.[15] Differing rates of acetylation account for the vast range observed in the half-life of isoniazid in individuals, resulting in different acetylator phenotypes.[49] The enzyme responsible for this acetylation is *N*-acetyltransferase 2 (NAT2). The rate at which isoniazid is metabolised is not inducible and is inherited in a true Mendelian fashion so that individuals either display a slow (homozygous slow or mutant), intermediate (heterozygous) or rapid (homozygous rapid or wild type) phenotype.[50,13]

N-acetylation is the primary metabolic route taken by certain drugs such as arylamines and hydrazines, including isoniazid. These drugs have been observed to

undergo N-acetylation at remarkably different rates *in vitro* as well as *in vivo*.[51] As certain other drugs belonging to this category have not displayed such a variance in metabolic rate, it was thought that there existed two types of N-acetyltransferase enzyme, both being responsible for N-acetylation, but only one of them showing such a distinct polymorphism.

In the case of rapid and slow acetylators, it was hypothesised that either:

- Each acetylator enzyme type possessed distinctly different **physicochemical** and **kinetic** properties, or
- The two identical rate-limiting enzymes were present in different **amounts** due to structural gene differences.[51]

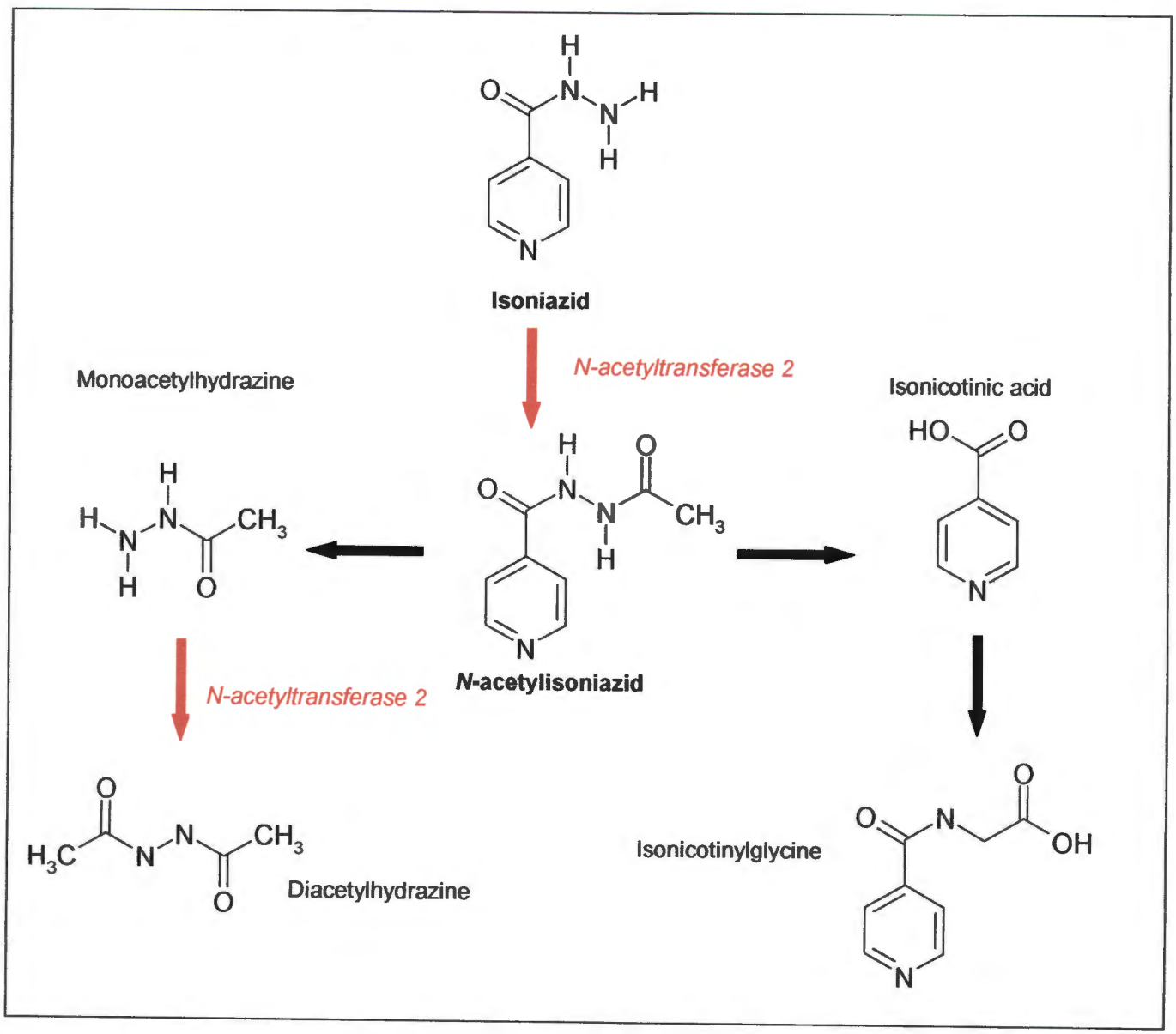


Figure 3.1a. The metabolism of isoniazid (adapted from Ellard and Gammon)[49]

The other drugs shown not to be influenced by acetylator status, are shown to be metabolised by a seemingly invariant enzyme termed **monomorphic** *N*-acetyltransferase.[51] It has more recently been elucidated that this seemingly invariant enzyme is also polymorphically expressed.[51]

The acetylation polymorphism of isoniazid is probably the most documented and well-described metabolic polymorphism in medicine. Price-Evans described it in 1960.[13] A study of 20 families, including 10 pairs of twins, was performed and the results showed conclusively that the phenomenon was evidence of a genetic polymorphism. Upon close examination of the twin data, it was concluded that the polymorphism was controlled by two segregating alleles. It was described as being bimodal, although Sunahara followed closely with the trimodal theory[11] which has been supported by more recent publications.[28,37,52] Today, the distribution is still considered to be trimodal, especially when dealing with genetic work, but the distinction between intermediate and rapid acetylators is still phenotypically tentative.[52]

Non-compartmental analysis has been utilised by researchers to calculate the pharmacokinetics of isoniazid.[49] First order rate constants are calculated to describe the rate of acetylation in individuals (i.e. the rate of conversion of isoniazid to *N*-acetylisoniazid). This rate constant has been proven to differ considerably between those classified as rapid acetylators and those classified as slow acetylators. In an experiment performed by Ellard and Gammon,[49] it was shown that the rapid acetylator converted isoniazid to *N*-acetylisoniazid 3-4 times faster than the slow acetylator. It was then shown that this rate was comparable in each individual when acetylating monoacetylhydrazine, indicating that the enzyme responsible for converting isoniazid to *N*-acetylisoniazid was the same as that responsible for acetylating monoacetylhydrazine.

Once isoniazid has been acetylated to form *N*-acetylisoniazid, it is biotransformed into isonicotinic acid and monoacetylhydrazine. Isonicotinic acid is then conjugated with glycine and the resulting product (isonicotinyl glycine) is excreted via the kidneys.[49]

Studies have shown that isoniazid possesses the greatest early bactericidal activity of all known anti-tuberculosis agents. It was shown that the ratio of 300 mg isoniazid to the lowest dose producing a detectable EBA (18.75 mg), was observed to be 16.[7] By comparison, the ratio of a 600 mg dose of rifampicin to the lowest dose resulting

in a detectable EBA, was only 4.[7] This indicates the effectiveness of this relatively inexpensive drug.

Various techniques have been implemented to ascertain individual phenotypes. Drugs such as isoniazid,[21, 28, 53, 54, 55] dapsone[27], caffeine,[25, 26, 56] sulphamethazine[50] and sulphadimidine,[23] known to be acetylated polymorphically, have been used. Plasma levels are favoured over urinary levels, because urine collection is less reliable than timed blood sampling.[24] Markers such as isoniazid half-life, the level of isoniazid at three hours and the ratio of *N*-acetylisoniazid and isoniazid plasma levels at three hours[28] have been some of the isoniazid markers used to classify patients as rapid, intermediate or slow acetylators.

The influence of race or ethnicity of the individual on drug pharmacokinetics has been a topic of research for decades. Many studies have been performed to assess the differences between races on different drugs. The majority of recent studies have been performed comparing Asian (often Chinese) to Caucasian populations with limited publications comparing Caucasians with Africans (or African Americans).[57] The coloured is unique to the Western Cape in terms of ethnicity. Nowhere in the world, besides South Africa, is there a comparable population. The incidence of tuberculosis is exceptionally high in the Western Cape,[107] historically deprived of good education and amongst whom poverty is rife. Many rely upon manual labour for their livelihood, such that becoming incapacitated seriously threatens their ability to sustain themselves and their dependants.

In this report, the pharmacokinetics of isoniazid in patients admitted to Brewelskloof Hospital in Worcester in the Western Cape will be described. Using the pharmacokinetic data, it will be determined whether the distribution of acetylator status is indeed trimodal in this study population, as has been found in a prior study in the Western Cape.[37]

3.2. MATERIALS AND METHODS

This study was passed by the ethics department at the University of Cape Town as well as the ethics committee set up at Brewelskloof TB hospital. It was carried out according to the study protocol for this project approved by the Medicines Control Council of South Africa and the Ethics Committee at the University of Cape Town. Each patient was required to sign an informed consent form prior to study recruitment (See Appendix I). Individual patient details are attached as Appendix V.

Patient recruitment Patients admitted to Brewelskloof Hospital were recruited for the study the day prior to the study. Written, informed consent was obtained from each individual. Patients were recruited and sampled over an eight-month period. One hundred and fourteen patients successfully completed this study, 63 female and 51 male. All patients had been admitted to Brewelskloof Hospital two months prior to recruitment, and had been receiving standard therapy for 8 to 10 weeks. Concomitant therapy was not stopped for the purposes of this study. The standard therapy used at Brewelskloof TB Hospital consisted of rifampicin, isoniazid and pyrazinamide, with the addition of streptomycin and/or ethambutol if deemed necessary by the clinician responsible.

Inclusion criteria:

- 18 years old
- Pulmonary tuberculosis: diagnosed by positive sputum or sputum culture
- Patient with tuberculosis on treatment containing isoniazid

Exclusion criteria:

- critically ill
- Contraindication to multiple blood sampling eg. mental confusion/poor venous access

Study procedure The patients were fasted overnight from 24h00 until 10h00 the next morning.[58] The zero hour blood sample was drawn within 30 minutes prior to medication administration. The medication was administered from 8h00, with 200 ml water. A light breakfast was allowed 2 hours after drug administration, providing time for adequate absorption. Following this, food and fluid intake was unrestricted.

Patient sample collection Blood samples were collected from each patient over an eight hour period. Specific time points included 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 and 8.0 hours after drug administration. Each time, venous blood was drawn from an indwelling cannula into gel lithium heparin-coated 4 ml glass tubes. These were stored on ice until being centrifuged, within 10 minutes, at 3 000 rpm for ten minutes. The plasma was then decanted into clean, labelled polypropylene tubes and stored on dry ice. Upon arrival back in Cape

Town, these were transferred to a -80°C freezer. Following the 6-hour blood sampling, blood was drawn into EDTA tubes for genotypic analysis.

Sample analysis Each sample was analysed for isoniazid and *N*-acetylisoniazid content by the method described in the previous section. The samples were taken out of -80°C storage and allowed to thaw at room temperature. Following this, each tube was vortexed and spun down at 3 000 rpm for 5 minutes to remove any unwanted particulate matter, which would clog the solid phase extraction columns. Each sample was extracted and analysed according to the newly developed method discussed earlier. A new set of extraction columns was used for every twenty-five to thirty samples.

Analyte recovery Each extraction set was monitored for consistent recovery by using spiked controls. Each extraction was accompanied by a 10 μg/ml spiked control of isoniazid and *N*-acetylisoniazid to monitor that specific extraction. These spiked controls were also used to convert the HPLC peak area traces to concentration values. Standard curves were run with each set of analyses, to verify the assay linearity and conversion factors throughout sample analysis.

Patient profiles A pharmacokinetic profile of isoniazid and *N*-acetylisoniazid was constructed for each patient using GraphPad Prism (v 2.01, 1996). All subsequent graphs were plotted using this software package.

Pharmacokinetic data The software package WinNonLin Standard Edition (v 1.5) was used to calculate the non-compartmental pharmacokinetic parameters for each patient. Parameters calculated included:

1. **Cmax** - the maximum concentration reached (achieved via inspection of the pharmacokinetic time curve)
2. **Tmax** - the time at which this observed concentration is reached (achieved via inspection of the pharmacokinetic time curve)
3. **Thalf** - the apparent terminal half-life of the analyte (*NOTE: the WinNonlin algorithm was used for the decision of the number of points to include in the calculation of half-life.)*

The terminal half life of a drug is estimated when estimating λ_z. λ_z may be defined as the first order rate constant associated with the terminal (log-linear) portion of the curve. It is estimated by plotting a linear regression of time vs. natural log concentration. Lambda z = -1 * slope of regression. The estimated lambda z value is always associated with a coefficient of determination (R^2), which should tend to 1.

The terminal half life is calculated using the equation: $\mathbf{t_{1/2} = \ln (2) / \lambda_z}$

The goodness of fit calculated for this terminal elimination phase curve is given as Rsq.

4. **AUCall** - the total area under the curve from the time of dosing to the time of the last observation (referred to as **AUCT** for the purposes of this work).

AUClast – the total area under the curve from the time of dosing to the time of the last measurable concentration. This differs from AUCall because it excludes the use of any points, which produced concentrations of zero.

Two methods are available to calculate area under the curve using this package. When the Linear/Log Trapezoidal calculation method is implemented, the Linear Trapezoidal rule is used up to Cmax. The Logarithmic Trapezoidal Rule is used after Cmax has been reached. If there are any concentrations of zero after Cmax, the linear trapezoidal rule is used for intervals that contain the zero concentration. If Cmax is not unique, then the first occurrence of Cmax and its corresponding Cmax is used.

Linear Trapezoidal Rule: $AUC \int_{t1}^{t2} = \delta t * \frac{C1 + C2}{2}$

Logarithmic Trapezoidal Rule: $AUC \int_{t1}^{t2} = \delta t * \frac{C2 - C1}{\ln \frac{C2}{C1}}$

where δt is $(t_2 - t_1)$.

5. **AUCINF** - the area under the curve from the time of dosing extrapolated to infinity, if the last measured concentration was above zero.

This parameter is calculated using the equation: **$AUCINF = AUClast + Clast / \lambda_z$**

Rate constant calculation was performed using the equation: **$\ln C_t = \ln C_0 - kt$**
Natural logarithms of each point subsequent to that of Cmax, up to 8 hours, were taken. From the above equation, the elimination rate constant is the slope of the regression line multiplied by -1. These were then compared to the lambda z values obtained using WinNonLin, calculating the half-life. This was done because it provided a good estimation of the validity of the use of program-selected lambda z values to calculate half-life. WinNonlin calculates lambda z in the same manner as the rate constant is calculated manually, deciding upon the validity of including each point.

Statistical Analysis was performed using non-parametric statistics on GraphPad Prism (v. 2.01). Correlations were assessed using the Spearman r test, and statistical differences between groups using the Mann Whitney test.

3.3. RESULTS OF SAMPLE ANALYSIS

The study began with the analysis of each plasma sample from each patient. These were analysed according to the method described in Chapter 2, and pharmacokinetic curves for isoniazid and *N*-acetylisoniazid were constructed. These are shown in Appendix II (Figures IIa – IIp).

The entire sampling population was assayed using HPLC, in sets to facilitate optimum column usage, and the composite profiles obtained for each drug in each set are shown in figures 3.3.1a and b.

Controls were used throughout sample analysis in order to ensure assay integrity. The results of each of the sets of controls are presented in figure 3.3.2. Care was taken to minimise the error bars during analysis.

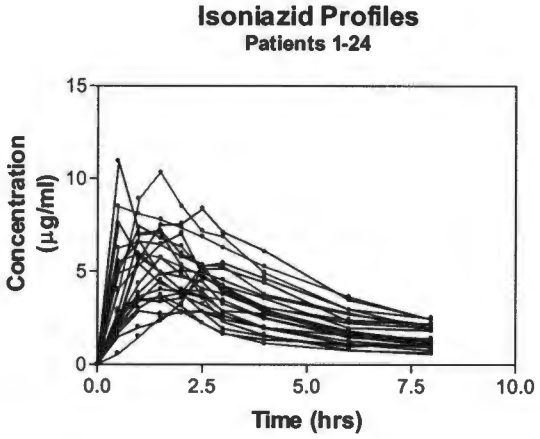
The data obtained using the previously discussed assay is represented in the above set of graphs. Each graph represents an individual in the study, showing the pharmacokinetic profile of isoniazid and *N*-acetylisoniazid. It is evident from these graphs that there is indeed a wide range of acetylation capacity within this study population. A previous study performed by Peloquin and Jaresko *et al.*[59] showed ranges using a 250 mg dose of isoniazid, expected for the major pharmacokinetic parameters discussed here.

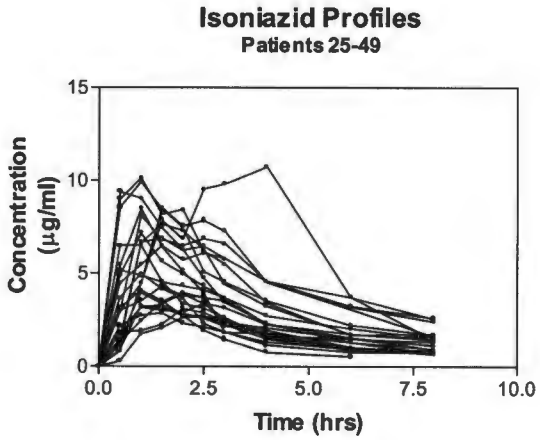
Table 3.3.1. Observed ranges of three major pharmacokinetic parameters[59]

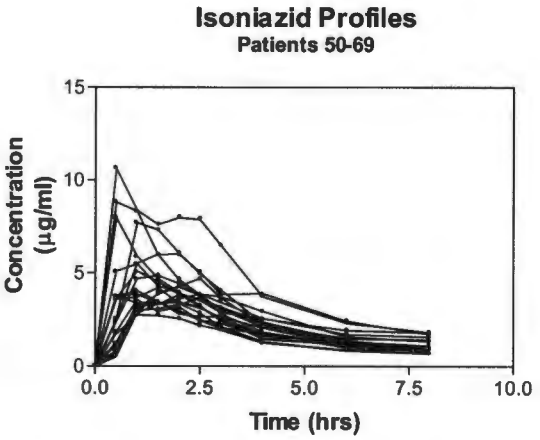
Parameter	*Range*	
	Slow Acetylators	*Rapid Acetylators*
Cmax (µg/ml)	2.17 – 5.11	1.04 – 2.96
Tmax (h)	0.50 – 3.00	0.50 – 1.00
AUCINF	13.10 – 26.39	2.96 – 6.95

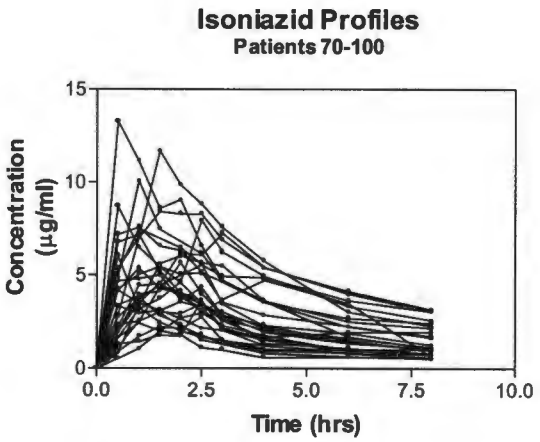
Although the majority of subjects display adequate levels with regard to therapeutic ranges, the variability is distinct, with eight subjects not reaching the lower limit of the therapeutic range (3 μg/ml). Certain Cmax values for isoniazid show that the levels obtained for a number of individuals are elevated, many reaching above 10μg/ml. The metabolite levels range from far below that of the parent drug to well above the maximum concentration reached by isoniazid.

Rapid absorption of the drug is evident, most concentrations peaking before two hours. It is also clear that the time period over which each individual was studied was not long enough to provide an accurate estimation of terminal half-life of isoniazid as certain individuals (including 81, 90, 92, 93 and 102) did not have time to achieve very low concentrations of isoniazid.









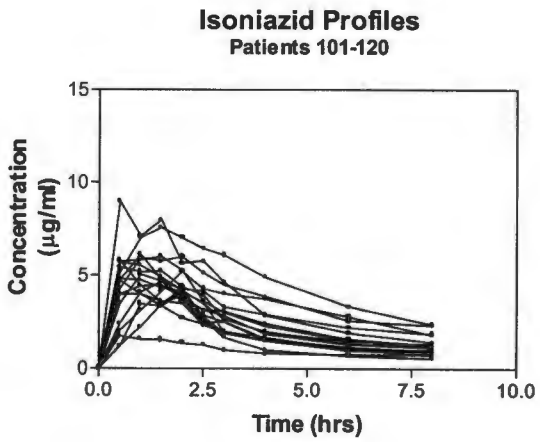


Figure 3.3.1a. Isoniazid profiles of all patients in composite form. The graphs are displayed in the sets in which they were analysed. In certain cases, it is evident that there are distinct differences in the profiles of isoniazid between individuals, even when they are receiving equivalent dosing according to body weight.

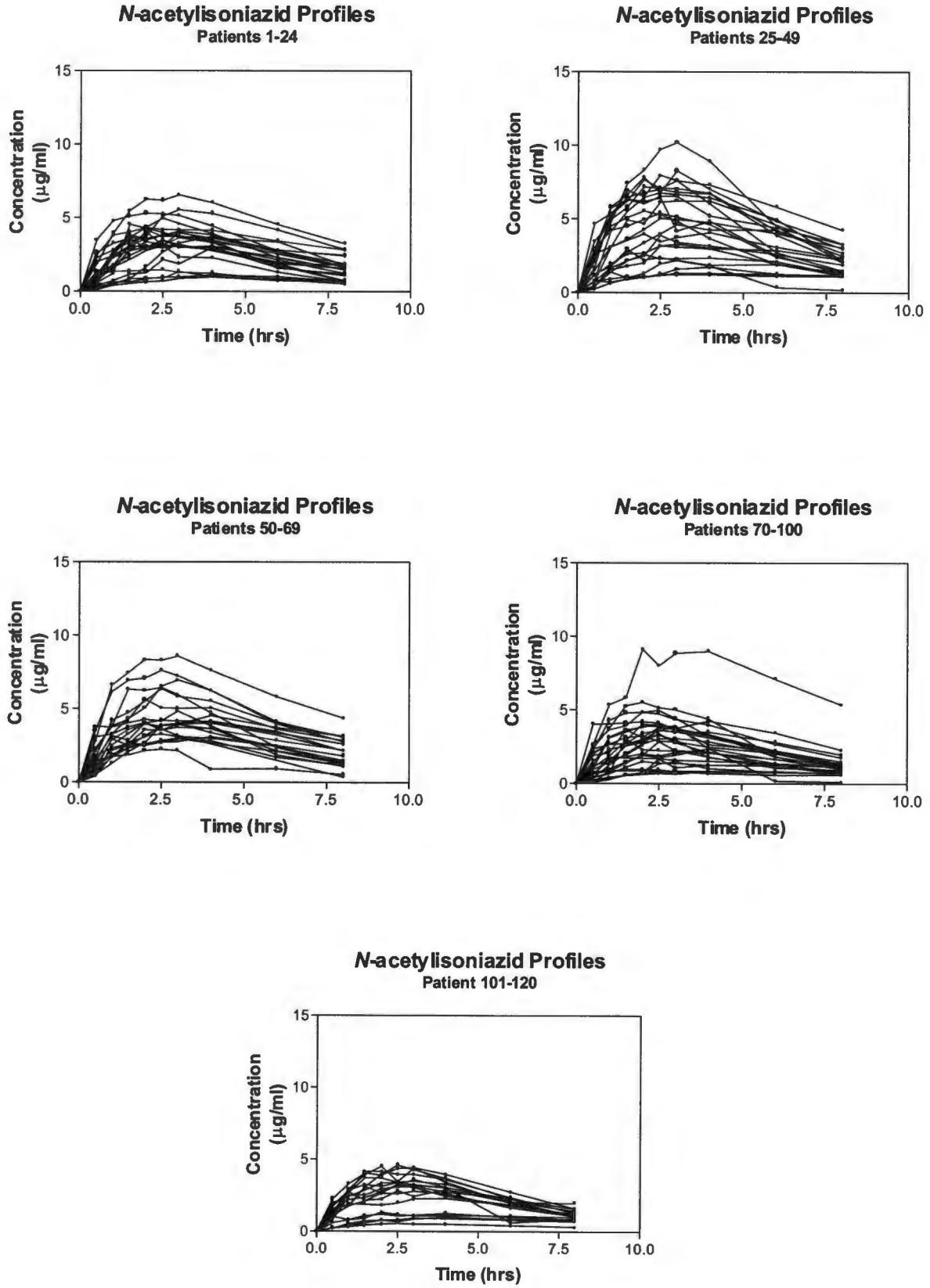
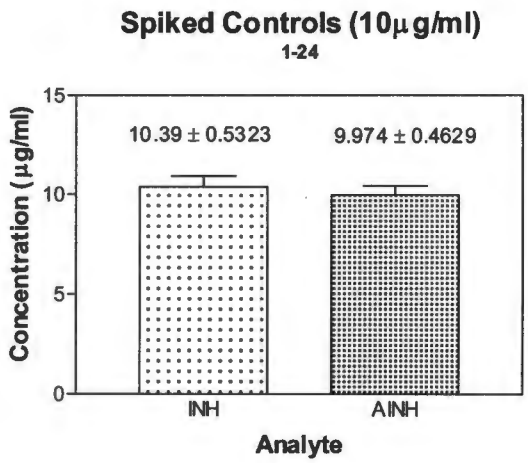
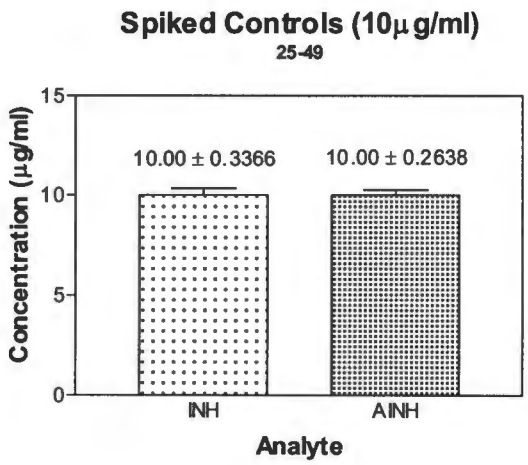
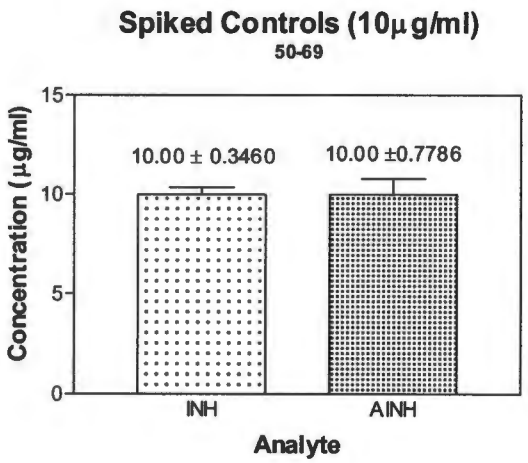
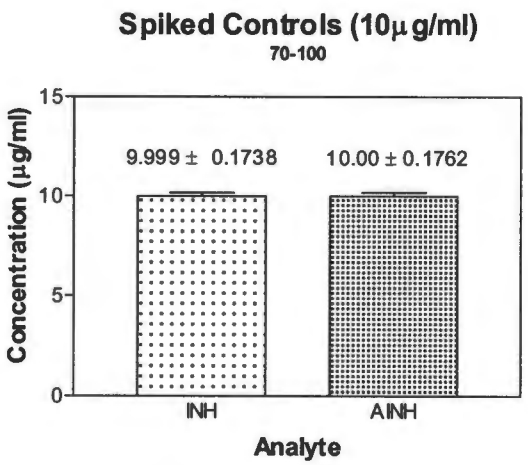


Figure 3.3.1b. *N*-acetylisoniazid profiles of all patients in composite form. Again, the graphs are arranged in sets according to their analysis. The separation of individuals into phenotypic acetylator groups is again fairly evident, since this metabolite is the product of the phenotypic polymorphism.









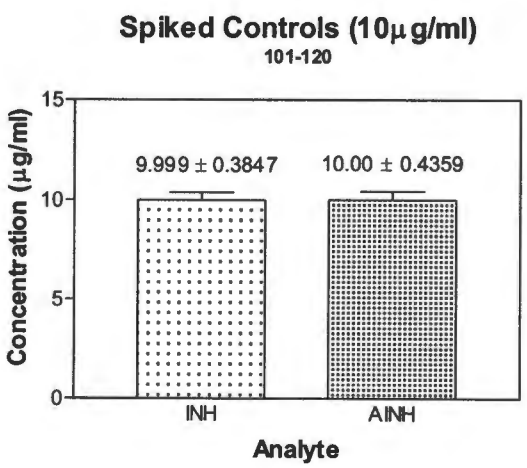


Figure 3.3.2. The spiked controls ran during each set of analysis are represented here. The ratio of each set was used to convert the peak areas in that set into concentration.

As can be seen from the graphs, the controls remained consistent with their spiked concentrations. The standard errors of the mean were minimal. The conversion factors calculated from that specific set of controls were used to calculate the apparent concentrations of the controls following analysis, and subsequently, those patients' samples analysed in that set.

All the samples obtained from each subject enrolled in the study were analysed successfully. The controls produced excellent estimations of concentration, with minimum variation and error. The concentration data was analysed and used to assign acetylation status to each individual.

3.4. ANALYSIS OF RESULTS

The analysis of plasma samples was followed by interpretation of these values. In order to be able to do this, three areas had to be dealt with. They were:

i. Frequency distributions:	Determining the optimal method of phenotypic classification.
ii. Pharmacokinetic Trimodality:	Determination and analysis of pharmacokinetic parameters according to the phenotypic classifications made using frequency distributions.
iii. Rate constant Evaluation:	Determination of the elimination rate constant of isoniazid and the rate of formation of *N*-acetylisoniazid.

3.4.1. Frequency Distributions: Phenotype Criteria

Various means have been used to ascertain the acetylator phenotype in humans. Using isoniazid, half-lives have been assessed, as well as the plasma level at three hours.[28] The ratio of AcINH:INH at three hours post dose was examined first. (See figure 3.4.1a.)

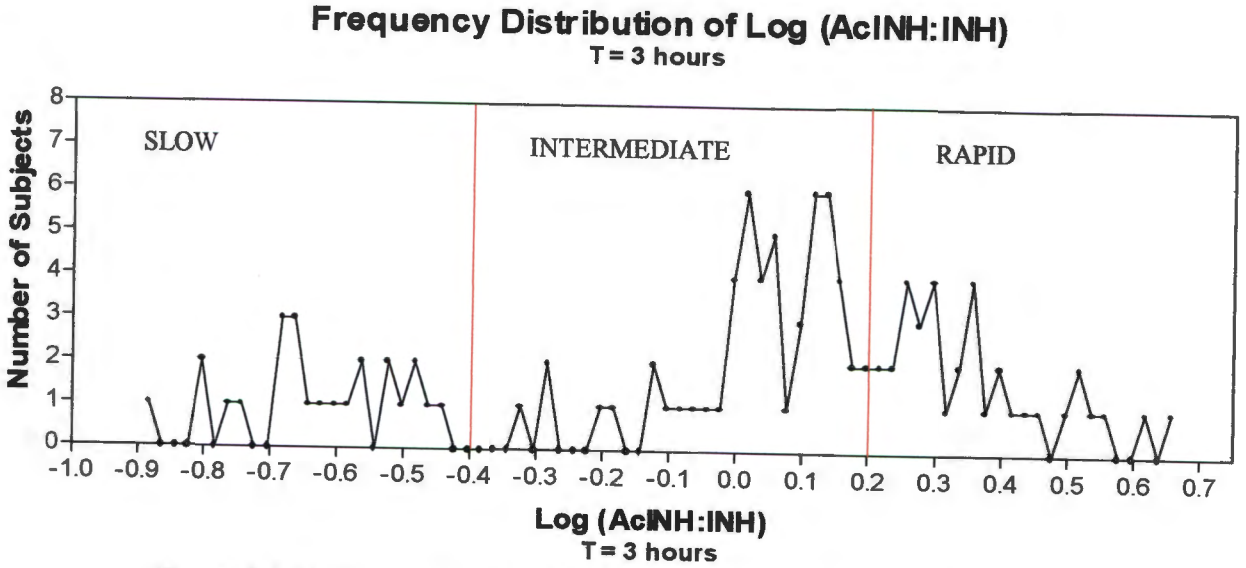


Figure 3.4.1a. Frequency Distribution of the logarithm of the ratio of isoniazid to *N*-acetylisoniazid at three hours.

The above distribution suggests that:

1. the distribution is trimodal,
2. the separation of rapid and intermediate acetylators is not discrete, whilst that of slow and intermediate is, and
3. the antimode separating slow and intermediate acetylators is discrete and may be estimated as being -0.4. The antimode between rapid and intermediate acetylators is not as discrete, but may be estimated as being 0.2.

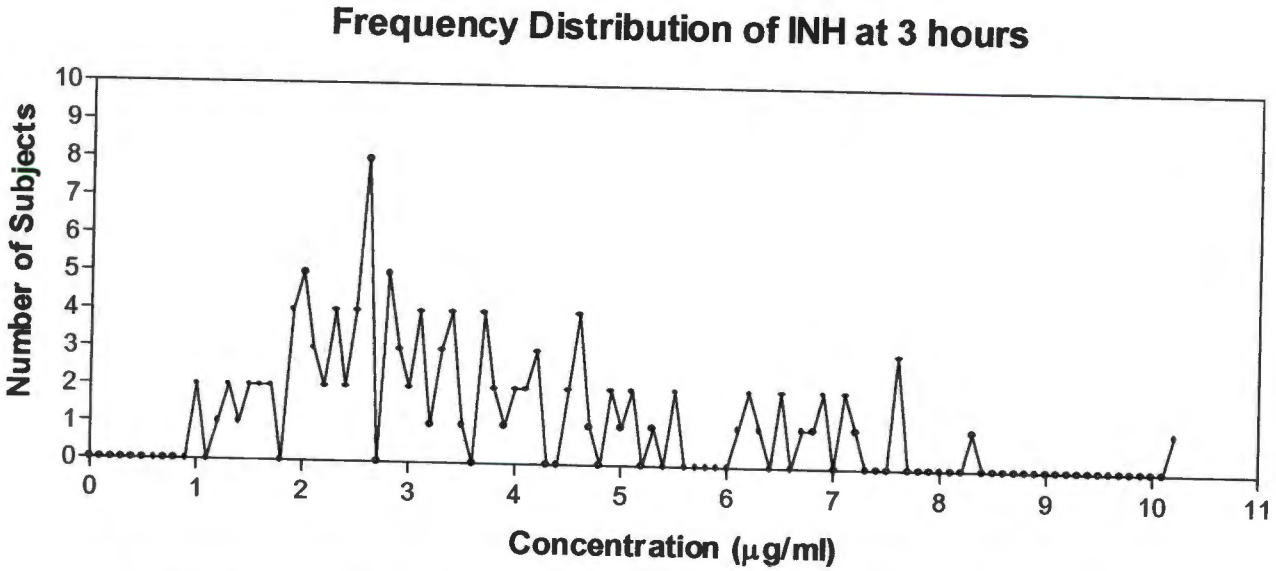


Figure 3.4.1b. The distribution of isoniazid concentration at three hours post dose.

A trimodal distribution is not as easily seen using this marker. This marker fails to independently provide an adequate means of determining acetylator phenotype. The distribution of isoniazid concentration at three hours post dose illustrates a bimodal distribution, the antimode being under 6 μg/ml.

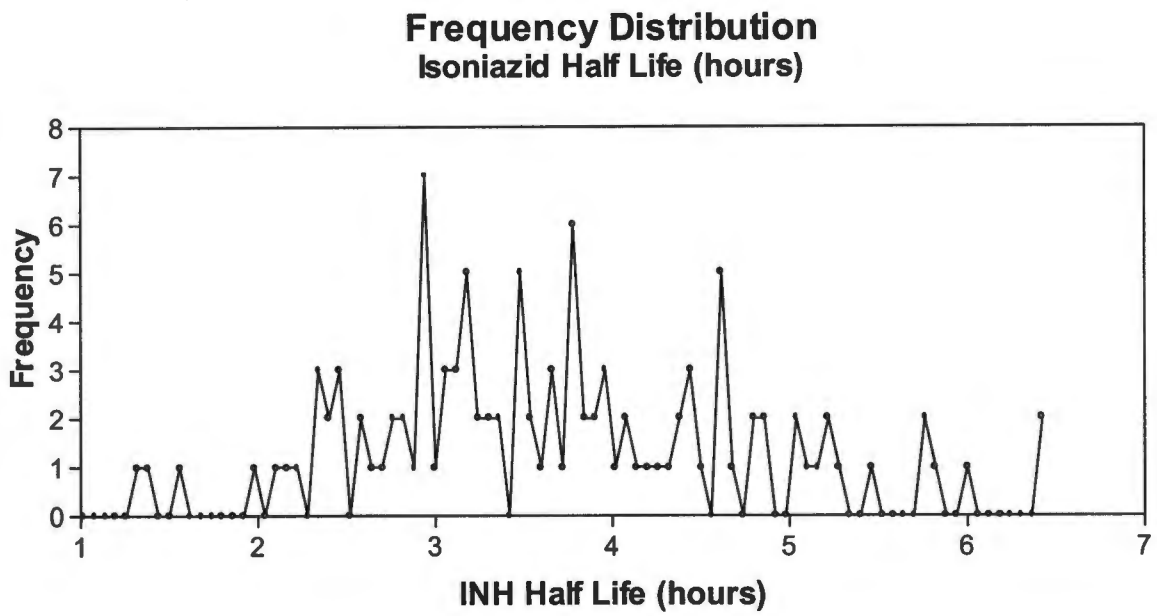


Figure 3.4.1c. Distribution of acetylation using the half-life of isoniazid as a marker.

The distinction between the different classes of acetylators is not easily seen when using the half-life of isoniazid as a phenotypic marker for acetylator status (figure 3.4.1c.). The slower the acetylator, the longer the half-life of isoniazid should be. The terminal elimination phase was not adequately represented in some patients as sampling only occurred up to eight hours post drug administration. Possibly, the sampling period could have been extended in order to provide better half-life estimation, remembering that the determination of half-life requires at least three points on the terminal elimination curve.

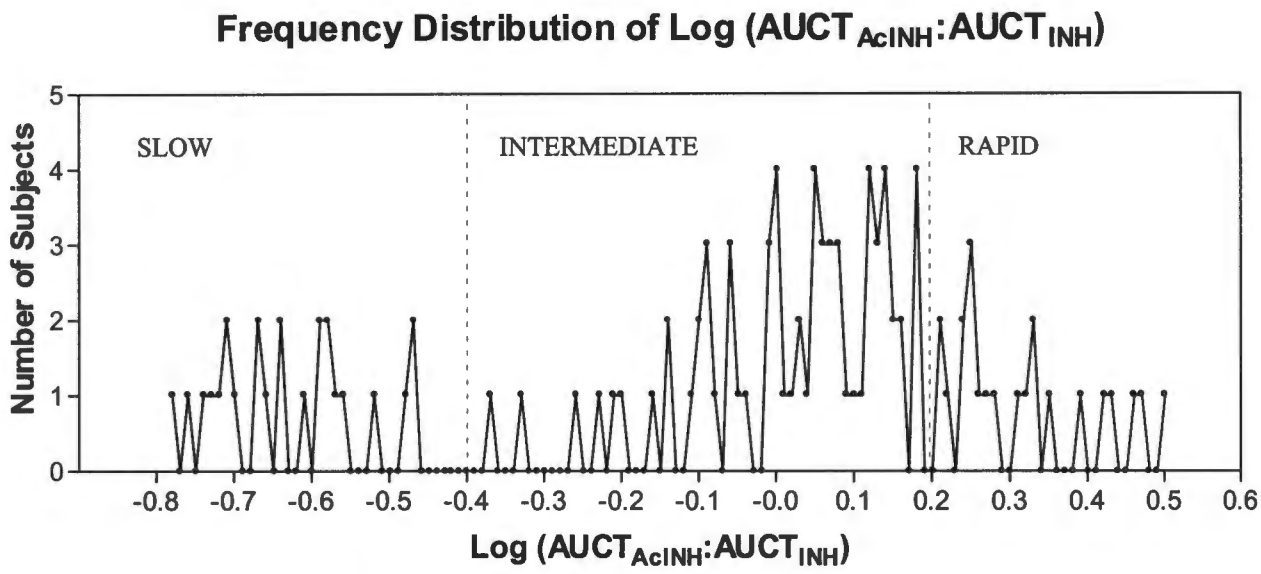


Figure 3.4.1d. Distribution of acetylator status using the calculated AUCT values to determine phenotype.

An apparent trimodal distribution is evident in this figure, pointing towards the influence of acetylator status upon the attained area under the curve for isoniazid.

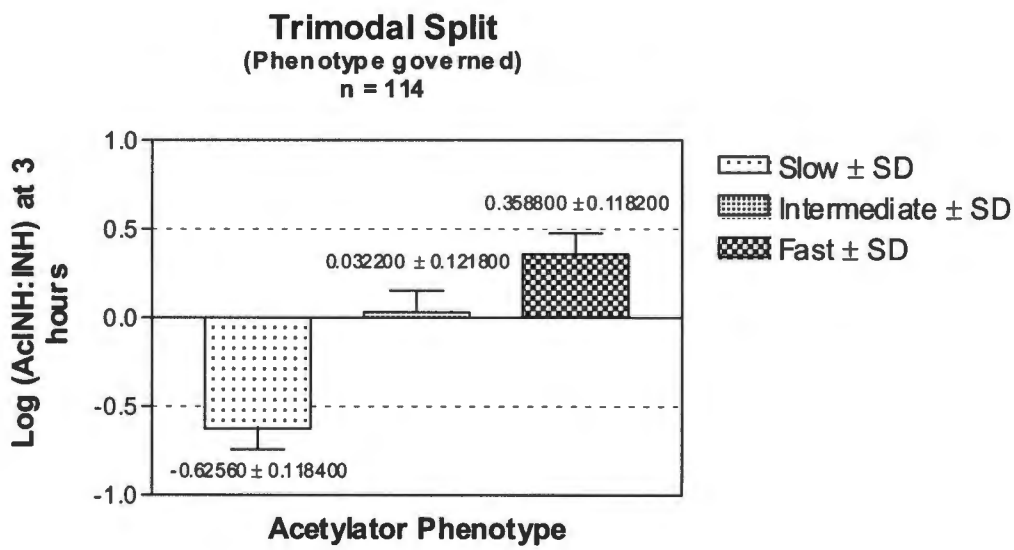


Figure 3.4.1e. The mean phenotypic split of log (*N*-acetylisoniazid : isoniazid) at three hours.

The distribution of Log (AcINH:INH) at three hours gave the best estimation of trimodal distribution, and the criteria for phenotypic classification. Performing non-parametric Mann Whitney statistical tests on these three subgroups, rapid, intermediate and slow, each group was determined to be **highly significantly**

different at a 95% confidence interval, using a two-tailed test ($p < 0.0001$). This classification criterion divided the population into **36 rapid acetylators**, **54 intermediate acetylators** and **24 slow acetylators**.

Table 3.4.1i. Correlation between the use of different phenotypic markers for determining acetylator status (using a two-tailed Spearman Correlation at 95% CI)

MARKER CORRELATION	*Spearman r value*	*P value*
Log (AcINH:INH) at 3 hours vs. Isoniazid at 3 hours	-0.2841	0.0022
Log (AcINH:INH) at 3 hours vs. Isoniazid half-life	-0.1043	0.2694
Isoniazid half-life vs. Isoniazid at 3 hours	-0.2209	0.0182

Thus, the method chosen for phenotyping and classifying individuals into acetylator status subgroups, was the log (AcINH:INH) value at three hours.

Table 3.4.1ii. The degree of difference between acetylation in the three phenotypes (± SEM).

	Slow Acetylators	Intermediate Acetylators	Rapid Acetylators
Mean Ratio at 3 hours Log (AcINH:INH)	0.2451 ± 0.06404	1.118 ± 0.2703	2.280 ± 0.7773
vs. Slow	-	4.561 times	9.302 times
vs. Intermediate	0.2192 times	-	2.039 times
vs. Rapid	0.1075 times	0.4904 times	-

All comparisons are **highly significantly different**, at a 95% CI, using a two-tailed Mann Whitney test.

3.4.2. Pharmacokinetic Trimodality

The resulting individual values obtained for Cmax, Tmax, Thalf, AUCT and AUCINF were analysed by grouping individuals according to the criteria decided upon using the frequency distribution of Log (AcINH:INH). All statistical testing was performed using two-tailed nonparametric Mann Whitney tests, at a 95% CI. (* indicates the degree of statistical significance.)

Groups	*Sig. Difference?*
Rapid vs. Slow	Yes; p < 0.0001
Intermed vs. Slow	Yes; p = 0.0009
Intermed vs. Rapid	No; p = 0.1329

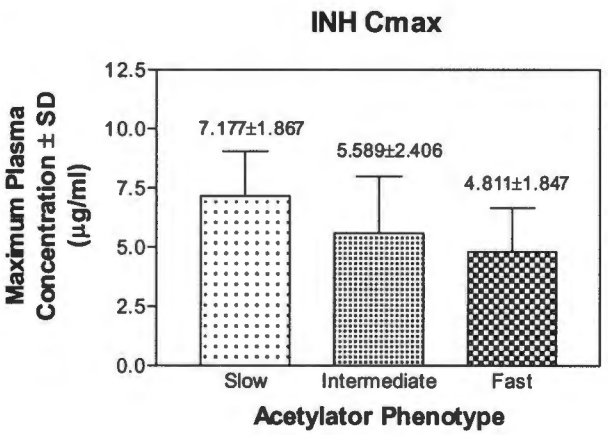


Figure 3.4.2a. Trimodal comparison of maximum plasma concentration reached (± SD).

The non-discrete separation between the intermediate and rapid acetylators, as displayed in the frequency distribution of concentration (figure 3.4.1b), is evident here.

Eight subjects were found to display maximum concentrations of isoniazid, below the suggested therapeutic range (i.e. below 3 µg/ml). Five of these subjects were rapid acetylators and 3 were intermediate acetylators. If one could assume that the acetylator status of the individual was the only factor affecting the Cmax value, then the relative risk of rapid acetylators to fall below the therapeutic range, compared to intermediate acetylators would be **2.500** compared to intermediate acetylators. It is not possible to compare this incidence to that of slow acetylators, as there were no subjects, regarded as slow acetylators, who failed to achieve at least minimum therapeutic levels.

Groups	Sig. Difference?
Rapid vs. Slow	Yes; p = 0.0204
Intermed vs. Slow	No; p = 0.2362
Intermed vs. Rapid	No; p = 0.1630

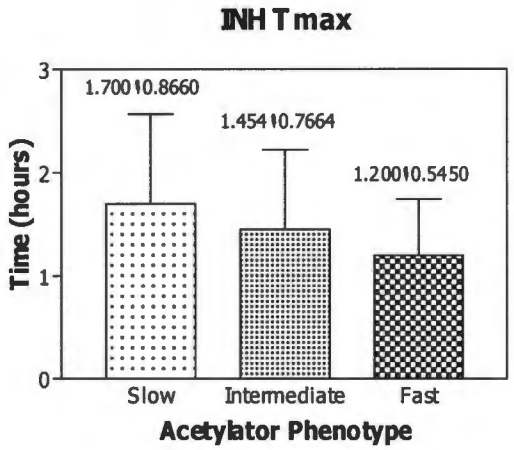


Figure 3.4.2b. Analysis of the time of maximum plasma concentration (± SD).

The only groups displaying any significant difference, were the rapid and the slow acetylators. There was a large degree of variation within each group, demonstrated by the large standard deviation.

Groups	Sig. Difference?
Rapid vs. Slow	Yes; p = 0.0394
Intermed vs. Slow	Yes; p = 0.0203
Intermed vs. Rapid	No; p = 0.8991

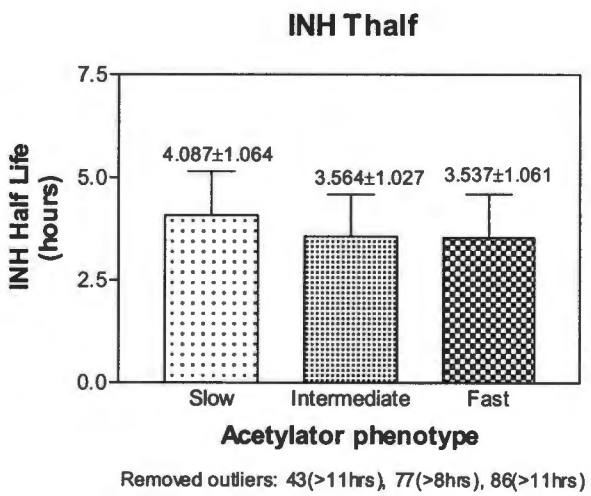


Figure 3.4.2c. Trimodal comparison of the mean half-lives (± SD) displayed by each acetylator group.

This finding is evidence of the non-discrete separation of the intermediate and rapid acetylators. It shows that these two groups are difficult to distinguish between. The separation of slow acetylators into a discrete group is apparent.

Groups	Sig. Difference?
Rapid vs. Slow	Yes; p < 0.0001
Intermed vs. Slow	Yes; p < 0.0001
Intermed vs. Rapid	Yes, p = 0.0003

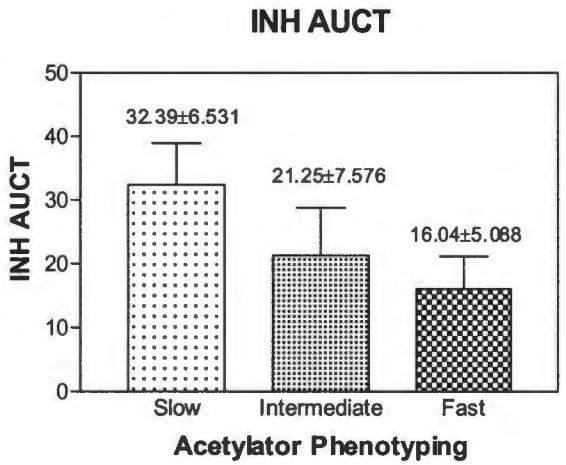


Figure 3.4.2d. A comparison of the differences between the acetylator groups in terms of total area under the curve (± SD) (i.e. to the last observation).

It is clear that acetylator status affects this pharmacokinetic parameter quite considerably, reducing the exposure of rapid acetylators to isoniazid by half.

Groups	Sig. Difference?
Rapid vs. Slow	Yes; p < 0.0001
Intermed vs. Slow	Yes; p < 0.0001
Intermed vs. Rapid	Yes; p = 0.0014

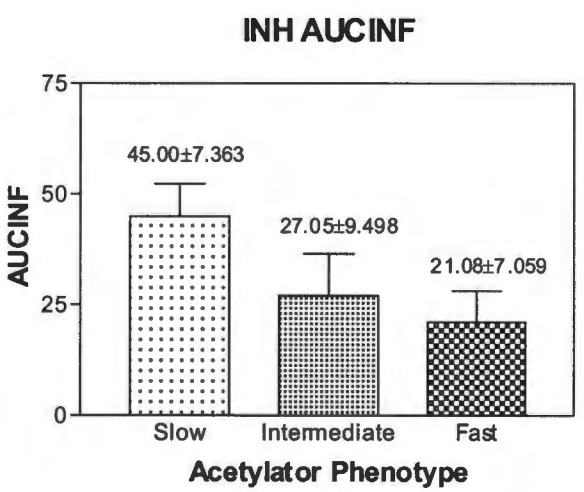


Figure 3.4.2e. Statistical comparison of the acetylator phenotypes in terms of the area under the curve, extrapolated to infinity (± SD).

The enzymatic polymorphism has a distinct effect on the values generated for this parameter, as it had with the total area under the curve.

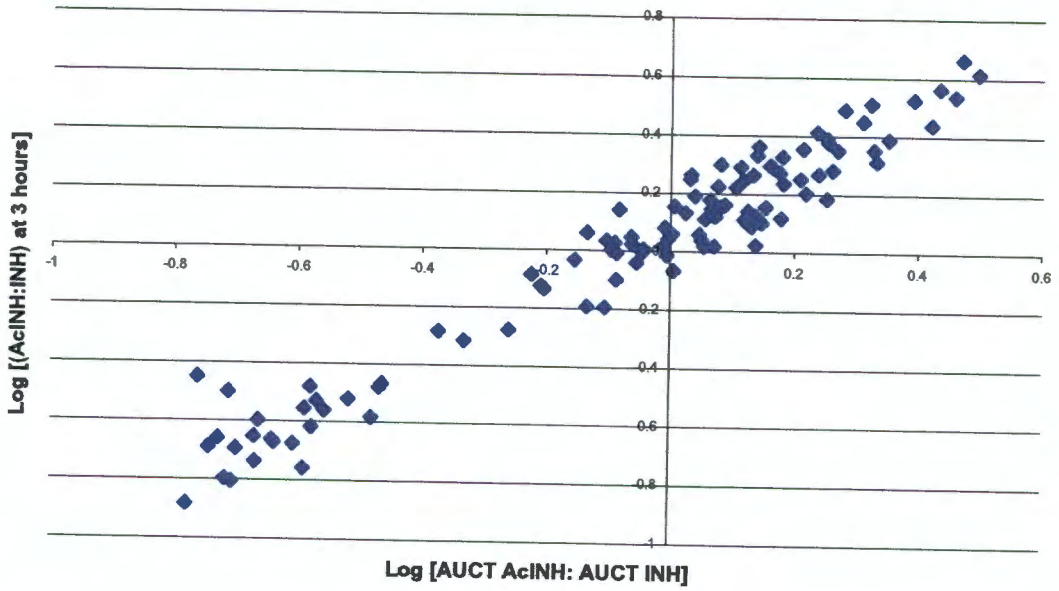


Figure 3.4.2f. The correlation between the use of Log (AcINH:INH) and the use of AUCT (AcINH):AUCT (INH) as markers for acetylator status.

This figure illustrates the significant correlation between the use of the [AcINH:INH] ratio at 3 hours as a marker for acetylator status; and the use of the ratio of [AUCT for *N*-acetylisoniazid : AUCT for isoniazid], emphasising the effect of acetylator status on the area under the curve. Statistically, the correlation is significant, producing a Spearman r-value of 0.9367 ($p<0.0001$). By comparing this figure with figure 3.4.1d (Page 57), acetylator status phenotype may be determined by either using the ratio of a single point on each curve, or by the ratio of the areas under both curves, the curves being that of metabolite and drug.

Table 3.4.2i. The mean observed pharmacokinetics of isoniazid for each acetylator phenotype (±SEM)

	Slow	*Intermediate*	*Rapid*
Cmax (µg/ml)	7.177* ± 1.867	5.589 ± 2.406	4.811 ± 1.847
Tmax (h)	1.700 ± 0.866	1.454 ± 0.7664	1.200 ± 0.5450
Thalf (h)	4.087* ± 1.064	3.564 ± 1.027	3.537 ± 1.061
AUCT (µg.h/ml)	32.39* ± 6.531	21.25* ± 7.756	16.04* ± 5.088
AUCINF (µg.h/ml)	45.00* ± 7.363	27.05* ± 9.498	21.08* ± 7.059

The pharmacokinetic parameter means do indicate a marked difference between all three phenotypes, but the trimodal theory still remains tentative in certain areas, such as Cmax and Thalf. It is difficult to ascertain the extent to which these figures agree previously determined values, as little pharmacokinetic modelling has been performed using three acetylator groups. Compared to the values from Table 3.3.1 (page 49) the figures above seem raised, although the first study did use a 250 mg dose instead of 300 mg.

3.4.3. Rate Constant Evaluation

In order to obtain an overall view of the interindividual difference in isoniazid plasma levels, composite graphs of each acetylator phenotype were plotted. All the calculated rate constants may be found in Appendix IV.

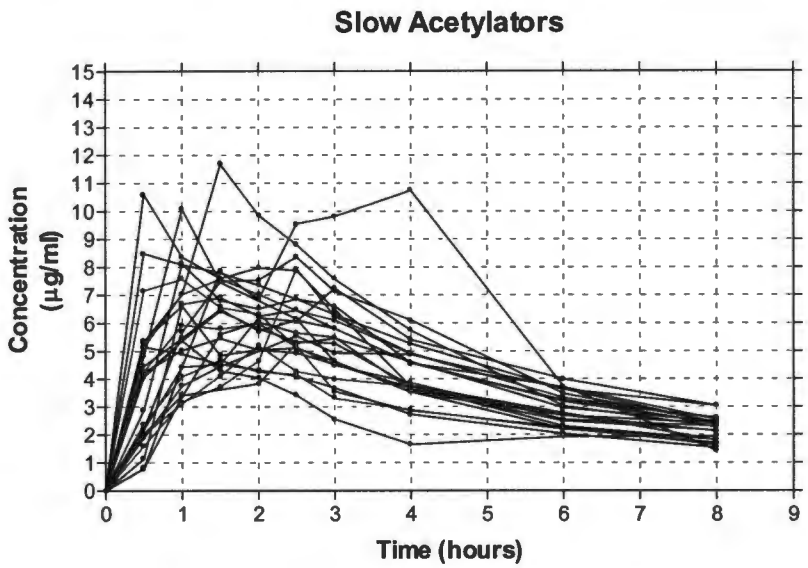


Figure 3.4.3a. Composite plot of the phenotypically slow acetylators. The solid lines indicate the therapeutic range of isoniazid.

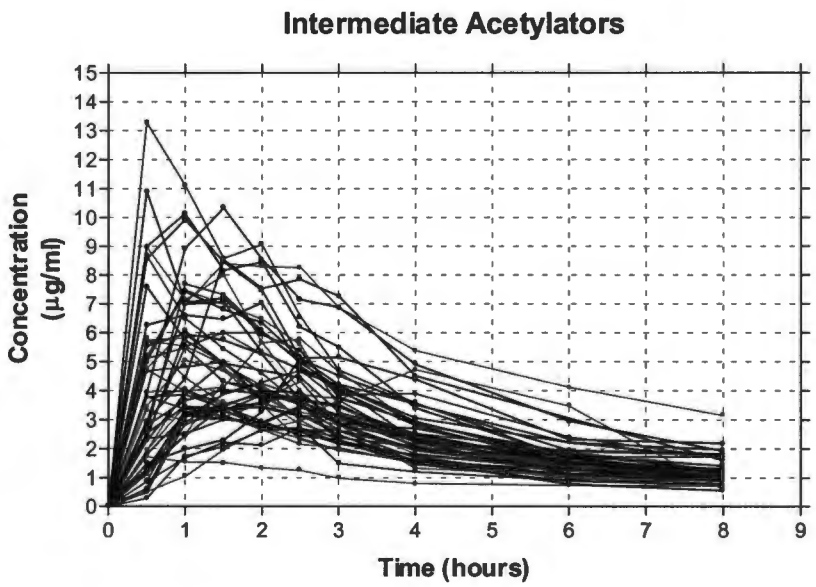


Figure 3.4.3b. Composite plot of the phenotypically intermediate acetylators. The solid lines indicate the therapeutic range of isoniazid.

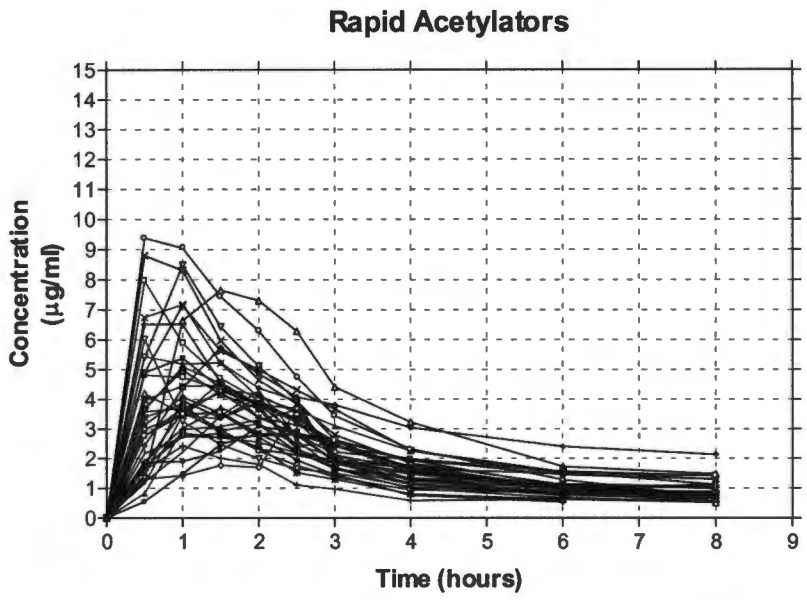


Figure 3.4.3c. Composite plot of the phenotypically rapid acetylators. The solid lines indicate the therapeutic range of isoniazid.

There is the marked amount of variation within each phenotype, and not just between the groups.

A. The Observed Rate of Elimination of Isoniazid

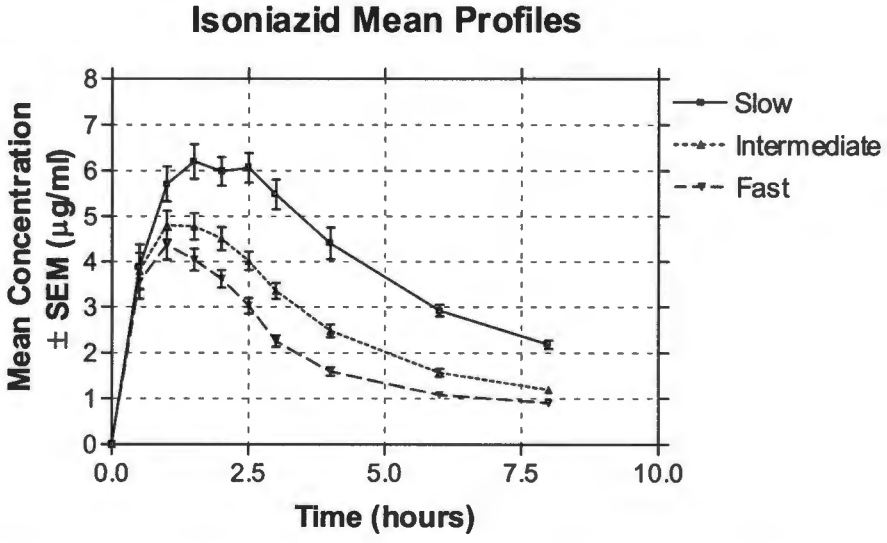


Figure 3.4.3d. A graphical representation of the mean pharmacokinetic profiles of isoniazid in each acetylator phenotype.

The profiles of each individual in a specific phenotypic group were averaged and mean profiles compiled. Each point is associated with a standard error. It is evident that the mean profiles are very different, and this supports the calculation of individual rate constants for isoniazid.

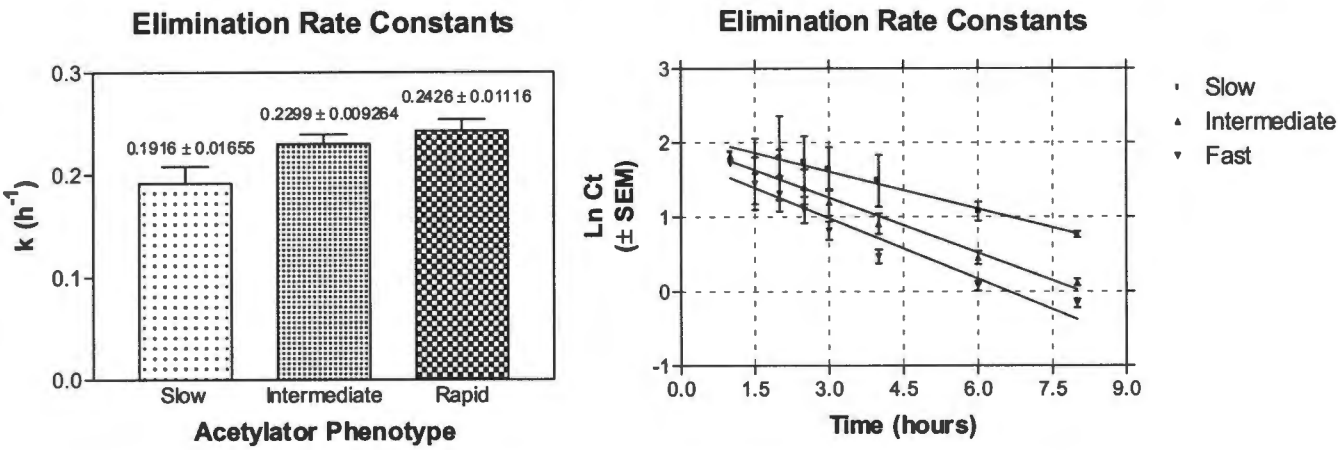


Figure 3.4.3e. The calculation and trimodal split of mean elimination rate constants of isoniazid phenotype subgroups.

Natural logarithms were taken of each mean concentration point, and linear regression was performed in order to calculate the mean rate constant. The coefficients of determination for the resulting regression lines were 0.9890, 0.9852 and 0.9403 for slow, intermediate and rapid acetylators respectively.

Using a two-tailed non-parametric Mann Whitney test, significant difference was found in slow vs. rapid, and slow vs. intermediate acetylators, but not between intermediate and rapid acetylators, thus reflecting the previous findings using isoniazid half-life.

From the data above, the alleles coding for acetylator status appear to be co-dominant. By averaging the mean k values for slow and rapid acetylators, it is possible to estimate the mean k value for intermediate acetylators within 6% of the true mean (0.2171 vs. 0.2299).

This also served to indicate the validity of the choice of lambda z by WinNonlin for use in calculating the half life of isoniazid. By specifically selecting the points used to calculate the rate constant, calculating the rate constant and comparing it with the lambda z value estimated by WinNonlin, the non-parametric Spearman r correlation was significant, producing a p value of <0.0001.

B. The Observed Rate of Formation of N-acetylisoniazid

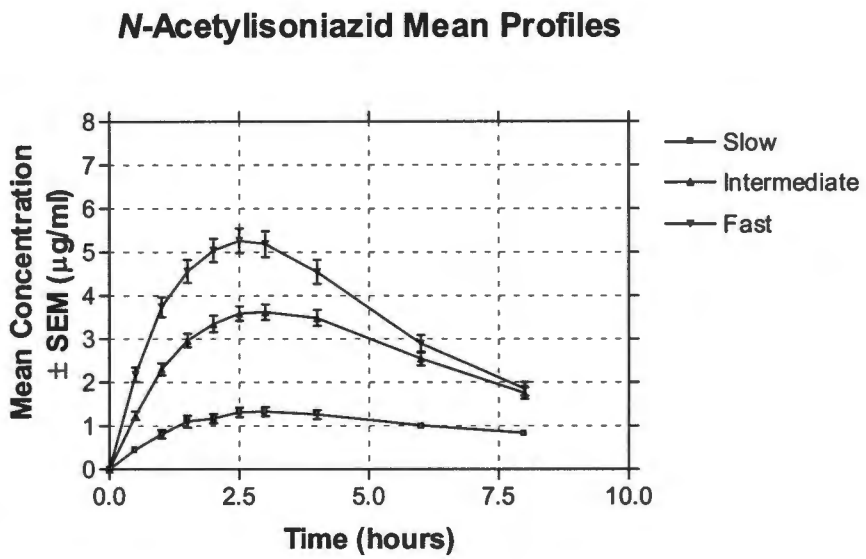


Figure 3.4.3f. The mean pharmacokinetic profiles of *N*-acetylisoniazid phenotypically split into a trimodal distribution.

These profiles were then used to calculate the apparent rate of formation of *N*-acetylisoniazid, by plotting the regression of all the points leading to the Cmax. Individual rates of formation were calculated, and then the mean determined as a phenotypic characteristic, in order to judge whether the groups are significantly different in terms of rate constants, governed by the enzyme, *N*-acetyltransferase.

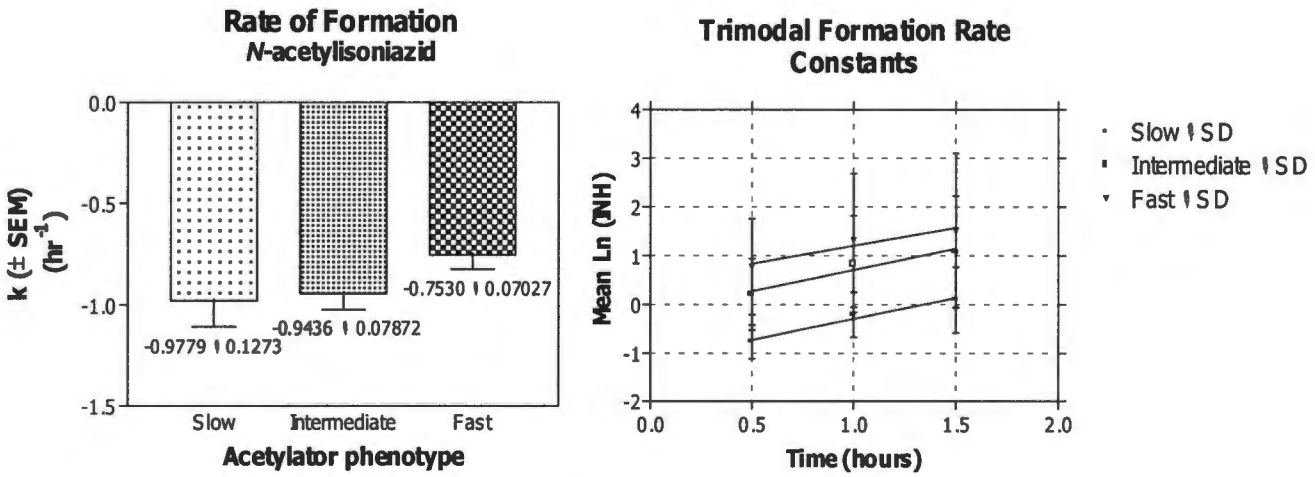


Figure 3.4.3g. The calculation of the rates of formation of *N*-acetylisoniazid in each phenotype.

R^2 values for each regression line were 0.9715, 0.9443 and 0.9329 for slow, intermediate and rapid acetylators respectively. The mean rate of formation for each group is **highly significantly different** (***) from those of the other groups, using the two-tailed Mann Whitney test at a 95% CI.

The correlation between the rate of disappearance of isoniazid and the rate of formation of *N*-acetylisoniazid was found to be **insignificant**, using the Spearman correlation (non-parametric) at a 95% CI, with the Spearman r = 0.01944, and the P value of 0.8373.

3.5. DISCUSSION AND CONCLUSIONS

The distribution of acetylator status in most populations has long been assumed to be bimodal.[17, 19, 20, 20, 22, 31, 33, 34, 35, 60, 61] Even with the advent of genotypic methods to assess the genotype coding for acetylator status there was still this assumption, although there was evidence of a separation between heterozygous fast and homozygous fast acetylators.[32, 50]

However, over the years, there has been an accumulation of evidence to show that the elimination of isoniazid is trimodally distributed.[11, 28, 52, 37, 62] This study has confirmed this, demonstrating significant differences between the three acetylator phenotypes.

The acetylation polymorphism is overtly evident in the individual patient profiles (Appendix II). Comparing the graphs of parent drug to metabolite provides insight into the apparent phenotype of the subject. The implementation of markers to gauge acetylation rates proved that the only reliable markers were

(a) the logarithm of the *ratio of N-acetylisoniazid to isoniazid at three hours*

(b) and the logarithm of the *ratio of the areas under the curve* for *N*-acetylisoniazid to isoniazid.(See figures 3.4.1a-d).

Thus the determination of acetylator status via ratio analysis is the most definitive, the ratio analyses correlating highly (Figure 3.4.2f.).

Numerous other publications have used the 3-hour level indicator to assess acetylator status, producing very distinct results. The distribution in this study was trimodal, with the antimodes clearly visible at **-0.4** and **0.2**, using a standard dosage of 300 mg isoniazid (Figure 3.4.1a.). These antimodes also apply to the use of the

AUCT ratios (Figure 3.4.1d.). Phenotypically assigning each subject an acetylator phenotype using the 3-hour level model worked well and provided an excellent starting point for pharmacokinetic analysis. Ease and practicality of sampling influenced selection of this marker.

Statistical analysis of the log (AcINH:INH) ratios for each group did show significant differences between all three groups, at a 95% CI (figure 3.4.1e). Other indicators used (Cmax and isoniazid half-life) did not provide such a definite phenotypic distinction, but the trend did tend towards trimodality, with notable separation of the slow phenotype from the remaining subjects. The separation between intermediate and rapid acetylators is not as discrete, regardless of the marker implemented, although the use of the area under the curve does provide better insight into the acetylator status of the population than most other parameters.

In previous publications, the difference in the rate of acetylation has always been between rapid and slow acetylators, and ranges from a 4- to 6-fold difference.[14, 15, 49] Using the ratio of *N*-acetylisoniazid to isoniazid plasma levels at three hours, this trimodal distribution proposes that rapid acetylator display acetylation rates over nine times that of slow acetylators, and double that of intermediate acetylators (See table 3.4.1ii). Bearing this in mind, it is evident why there should be such a difference between pharmacokinetic parameters for the three groups.

The absence of absolute correlation between the three indicators used suggests that they are not interchangeable (Table 3.4.1i). The best correlation was between the ratio of metabolite to drug at 3 hours and the plasma level of isoniazid at three hours. This was expected, as the two markers are related, both involving the use of the concentration of isoniazid at three hours. The correlation between isoniazid half-life and isoniazid at three hours suggests that these markers are still useful. It may be argued that the correlation between half-life and the ratio was insignificant due to the error in half-life estimation because of the reduced sampling period. Sampling up to eight hours provides useful information for most pharmacokinetic parameters, but the best estimation of half-life would have to include later points. The elimination rate constants concur with this finding.

The range of maximum concentrations reached (**Cmax**) is broad even within various acetylator groups, shown by the large standard deviations (figure 3.4.2a). It is evident that the study design allowed for a good estimation of Cmax, with numerous sampling points within the first three hours following drug administration. Acetylator

status definitely affects Cmax, showing statistical significance between rapid vs. slow and intermediate vs. slow, but again alludes to the fact that the distribution is bimodal in terms of maximum concentration reached, by failing to draw significance between rapid and intermediate acetylator status.

Penetration of isoniazid into lesions and sites of infection is critical to its activity during drug therapy. The aim of drug therapy would be to intensify drug exposure, maximising the maximal concentration reached, such that distribution around the body is optimal and allows maximal penetration into lesions and cysts.[29, 63]

Even though evidence has established that acetylator status does not affect outcome during the daily treatment,[55] according to the estimated therapeutic range of isoniazid (3-6 μg/ml [64]), 8 out of 114 subjects failed to achieve this plasma concentration. Of these, 5 were rapid acetylators, translating into a relative risk of 2.5 that rapid acetylator will be sub-therapeutic compared to intermediate acetylators. This means that according to the information obtained from this population sampling, rapid acetylators are 2.5 times more likely not to achieve a minimum concentration of 3 μg/ml. Whether this may be translated into a compromised outcome, depends on further kinetic studies, relating these concentrations to clinical outcomes. There were no instances where a slow acetylator peaked below 3 μg/ml.

The determination of the time at which the maximum plasma concentration was reached (**Tmax**) was performed by inspection of each pharmacokinetic profile. The resulting values were consistent with literature values,[15, 47] the means for rapid, intermediate and slow being 1.2 h, 1.45 h and 1.7 h respectively (Figure 3.4.2b). There was a significant difference ($p = 0.0204$) between the Tmax values of the rapid and the slow acetylator groups. This factor is strongly confounded by absorption, a factor inherently variable and independent of acetylator status.

The distribution of **half-life** is evidently bimodal in this population, as has been found previously.[59] Although sampling interval was short, the estimates of goodness of fit (Rsq) during the determination of half-life are excellent (Appendix III) and in previous studies sampling was extended to 36 hours with still no evidence of trimodality.[59] The frequency distribution of half-life (figure 3.4.2c.) also did not discriminate between rapid and intermediate acetylators. It was clear though, that there is a discrete isolation of the slow acetylators, each t-test showing significant difference.

Isoniazid targets the mycobacterial cell wall.[65] Thus, optimisation of drug therapy by maximising the amount of time the drug level exceeds the minimum inhibitory concentration (MIC) follows.[47] In terms of pharmacokinetics, the most important indicator of drug efficacy should be the total area under the curve (**AUCT**), with special reference to the time that drug concentration exceeds the MIC.[47] In rapid acetylators, this may become a problem if the patient is exceptionally fast and does not achieve adequate absorption. Significant differences exist between all three of the acetylator subgroups, implying that this parameter is largely influenced by acetylator status (Figure 3.4.2d). The fact that the slow acetylators showed an AUCT double that of rapid acetylators means that the exposure of the mycobacteria to isoniazid is halved in rapid acetylators. In the absence of regular dosing, 5% of rapid acetylators are known to fail in treatment or at least fair considerably worse than slower acetylators.[66]

The acetylator phenotype is of considerable significance during once-weekly therapy, but it may also be of significance during twice weekly treatment, when the companion drug is relatively weak.[55] This is emphasised when extrapolating the area under the curve to infinity (**AUCINF**). The trend between acetylators is the same here, the rapid acetylators (21.08 μg.h/ml ± 7.059) still receiving less than half the exposure of slow acetylators (45.00 μg.h/ml ± 7.363) to isoniazid (figure 3.4.2e.). Each group was significantly different ($p < 0.0001$), implying that there is a significant difference in drug exposure experienced by each of the phenotypes.

Taking this observation into account, the use of total area under the curve for determining acetylator phenotype is a viable option, and this, including significant correlation between the two factors, has been shown incontrovertibly by figure 3.4.2f. The mirroring of the scatter of Log (AcINH:INH) at three hours is obvious.

The significant reduction of the AUC in rapid acetylators would surely affect drug exposure during once-weekly intermittent therapy, where the action of isoniazid is critically complementary to rifampicin, bactericidal activity and resistance prevention.[63]

The **elimination** rate constant must be clarified in terms of it being the portion of the pharmacokinetic profiles (2.5 to 6 hours) indicating the elimination phase, specific to each patient profile, related to the disappearance of isoniazid from the blood plasma.

The disappearance of isoniazid in this aspect was observed to be bimodal, as expected after viewing the results from the half-life calculations. Slow acetylators were discretely separated from intermediate and rapid acetylators. Slow, intermediate and rapid acetylators were statistically significantly different from each other, calculating linear regressions for each group. Thus the individual rate constants in each group did produce significant difference for acetylator phenotype (See figures 3.4.3d. and 3.4.3e.).

In terms of **gene penetrance**, it is evident that the alleles coding for slow or rapid acetylation are codominant. It is possible to estimate (within 6%; 0.2171 vs. 0.2299) the elimination rate constant for the intermediate acetylators by averaging those obtained for the rapid and slow groups.

N-acetylisoniazid may be the major metabolite of isoniazid, but it is not the only product. Isoniazid is also broken down into α-oxoacids, namely pyruvic hydrazone, α-ketoglutaric hydrazone and direct hydrolysis to isonicotinic acid and hydrazine (See figure 3.1a).[49] Thus this terminal rate constant cannot be assumed to reflect the absolute activity of *N*-acetyltransferase 2, but it may be indicative of it, depending upon the nature of hydrazone formation and the rate of renal excretion of unchanged isoniazid. Taking all these confounding factors into consideration, the rate of disappearance of isoniazid in an individual would not be an ideal method of determining the acetylator phenotype of that individual.

The only means of forming *N*-acetylisoniazid is via the enzymatic action of *N*-acetyltransferase 2. So it would be logical to deduce that the **rate of formation** of *N*-acetylisoniazid would correlate more directly with the activity of the enzyme. Allocating individuals acetylator status determined by the log (AcINH:INH) ratio at three hours, and subsequently analysing the differences seen in the rates of metabolite formation, shows a distinct trimodality. The mean rates of formation are highly significantly different from each other ($p < 0.0001$) (figures 3.4.3f. and 3.4.3g.).

The **correlation** (figure 3.4.3h) between the rate of disappearance of isoniazid and the rate of formation of *N*-acetylisoniazid was found to be insignificant. An increased rate of isoniazid disappearance did not necessarily translate into a high rate of *N*-acetylisoniazid formation. Providing a more complete graph of the metabolite in the early phase following drug administration might strengthen the rate calculation. Confounding factors may be the renal elimination of unchanged isoniazid, owing to unregulated fluid intake, and the hydrolysis of isoniazid into other products.

Thus the conclusions drawn from this investigation are that acetylator status is trimodal in this population. This is best determined using a frequency distribution of log (AcINH:INH) at three hours (see Chapter 4 for validation of this) for ease, and substantiated by the distribution of the ratio of AUCT AcINH:AUCT INH.

3.5. FINAL CONCLUSIONS

This population has been shown to be demonstrative of a previous report regarding the trimodality of isoniazid.[37] The logarithm of (AcINH:INH) at three hours has been determined to be an accurate marker for phenotype determination, displaying a trimodal distribution. This was substantiated by using the logarithm of ($AUCT_{AcINH}$:$AUCT_{INH}$), both markers correlating well with each other. Trimodal analysis the resulting pharmacokinetics revealed significant separation of the slow acetylators from the intermediate and rapid acetylators in all cases. Cmax and half-life was bimodally distributed, failing to draw significance between the intermediate and the rapid acetylators. The area under the curve was observed to be trimodal, significant statistical difference existing between all three acetylator phenotypes. Of concern is the significant reduction in AUC displayed by rapid acetylators, as well as the elevated relative risk of rapid acetylators to achieve sub-therapeutic levels of isoniazid. In other drugs, this would not always attract heightened concern because many metabolites are still active against the targeted organism. The fact that the metabolites of isoniazid are totally inactive makes this reduction therapeutically significant.

Examination of the elimination rate constants for isoniazid confirm earlier suggestions[11,67] that the alleles are codominant, the predicted mean rate constant for the intermediate group being within 7% of the calculated mean.

CHAPTER FOUR

THE GENETICS OF *N*-ACETYLTRANSFERASE 2

4.1. INTRODUCTION

N-acetylation may be defined as the conjugation, catalysed by acetyl CoA-dependent arylamine acetyltransferase (NAT) enzymes, of primary amino and hydroxylamino groups with acetate. The is a certain amount of toxicity connected to this group of enzymes, due to the fact that they catalyse the conjugation of many xenobiotics and procarcinogens.[68] Shortly after the demonstration of the activity of isoniazid against tuberculosis in humans[1], it was observed that there existed a distinct **polymorphism** in the metabolism of this drug.[10, 69, 70]

Prior to the advent of molecular genetics, **family studies** were undertaken to understand the nature of the polymorphism.[13] It was noted that a slow acetylator seemed to possess autosomal homozygous recessive alleles. This confirmed the earlier work of Knight and Selin *et al.*[70] Furthermore, it was concluded that there existed a dosage effect, whereby the heterozygous inactivators displayed a considerably higher plasma isoniazid concentration than homozygous dominant individuals. From the above facts, it was presumed that "the allele controlling the dominant character gives rise to a process, which speeds the inactivation of isoniazid"[13] and that the process was most likely enzyme-mediated. Subsequently, Jenne[71] published the results of a partial purification of isoniazid transacetylase from human liver. He was unable to distinguish the heterozygotes in terms of *in vitro* analysis, and the lack of correlation between *in vitro* and *in vivo* results was explained by other physiological processes contributing to drug disappearance, such as hydrazone formation or continuous diffusion. But a broad range of acetylation activity was observed, most likely to be dependent upon the amount of enzyme present, and the activity was definitely distributed bimodally,[72] if not trimodally,[11, 73] with slow acetylators exhibiting significantly less activity than their rapid counterparts.[71]

Later, the impairments (or recessive traits) were pinpointed to a reduction in the rate of *N*-acetylation by a cytosolic CoASA:arylamine *N*-acetyltransferase enzyme in a two-step reaction involving the transfer of acetate from acetyl CoA to primary aromatic amino and hydrazino functional groups.[74]

N-acetyltransferase work achieved new heights when two distinct ***NAT* genes** – *NAT1* and *NAT2* - were cloned and the *NAT2* gene locus was determined to be the site of the human isoniazid acetylation polymorphism.[75] Alleleic variants are translated into different gene products resulting in differences in individuals with respect to the metabolism of xenobiotics.[68] The resulting protein isoforms exhibit overlapping degrees of selectivity, resulting in a large degree of versatility in different individuals.[68] Because this variation occurs within a single gene locus, it is termed monogenic and results in a range of acetylation activities from extensive to poor.[68] *N*-acetyltransferases catalyse a 2-step reaction involving the dissociation of the acetate group from Co-enzyme A, and the conjugation of this group to the primary amino or hydrazino functional group.[68] The use of molecular genetics was to shed more light on the issue surrounding the activity and structural heterogeneity of the acetylation phenomenon.

The allelic nature of *NAT2* was subsequently determined, and a number of **allelic variants** of *NAT2* have been identified; several of these alleles giving rise to the well-studied isoniazid acetylation polymorphism .Andres and Vogel *et al.* were the first to determine the amino acid composition of 6 tryptic peptides obtained from homogenised liver NAT from rapid acetylator rabbits, electrophoretically separated and sequenced.[76] These peptides made the construction of oligonucleotide screening probes possible. Using such probes, *NAT* cDNAs were isolated from Japanese subjects, obtained from libraries made with mRNA from two liver samples.[77] The *NAT* genes were identified at three distinct loci and named *NAT1* (supposedly monomorphic *NAT*), *NAT2* and *NATP* (a pseudogene). The sizes of these genes were determined by restriction enzyme digestion with EcoR1, yielding fragments of 1.3, 1.9 and 4.7 kb respectively.[75]

The *NAT2* gene contains an open reading frame of 870 bp, encoding a protein of 33.5 kDa. The coding exon for this gene is intronless. The coding region of *NAT2* presents with 87% nucleotide homology to *NAT1*, translating into 80% amino acid homology.[77,75]

In this study, the nomenclature of Hickman and Sim[50] has been adopted. By convention the wild type allele (FF) is considered to have no (known) mutations affecting the activity of the translated protein. Functional work thus far has suggested that mutant alleles are the result of a combination of between one and three nucleotide substitutions, occurring at positions 191, 282, 341, 481, 590, 803 and 857. Position 341 is the only mutation undetected by restriction enzyme digestion. It is

known that only mutations at positions 191, 341, 590 and 857 give rise to amino acid changes that in turn result in defective protein function.[68] The mutations are specific in the effect they have on the activity of the protein. It was found that nucleotide substitutions at positions 191 and 590 produced proteins with reduced stability and catalytic activity. Mutations at positions 341 alone, or coupled with positions 481, 803 or both, produced enzymes with significantly reduced catalytic activity only.[78]

In terms of **determining *NAT2* genotype**, the mutation at nucleotide 481 (C > T) results in the loss of the *Kpn*I restriction enzyme cutting site and has been named **S1** (or formally *NAT2*5A, 5B*). The S1 allele has been split up into three different alleles, namely S1a, S1b and S1c. The only S1 allele not picked up by the loss of the *Kpn*I site is S1c. It is evident via the addition of a *Dde*I cutting site. Additional mutations are also associated with the S1 allele, namely 341 (T > C) base change, resulting in a substitution of isoleucine[114] for threonine, and the silent 803 (A > G) base change, but it is the loss of the *Kpn*I cutting site, which has been used to differentiate S1 from other alleles. This mutation at position 341 is known to result in a decreased maximal velocity in enzyme activity, perhaps by hindering tertiary structure formation.[68, 78, 79]

The loss of the *Taq*I restriction enzyme site indicates the presence of the **S2** allele (*NAT2*6A*), resulting from the mutation at position 590 (G > A), accompanied by a silent one at 282 (C > T). The effect on the protein is a decreased stability, found by Blum and Grant *et al.*[75] using COS-1 cells, and confirmed by Hein and Ferguson *et al.*[78] when cloning into *E.coli*. This defect is brought about by the amino acid substitution of arginine[197] for glutamine.[68, 78, 79]

The **S3** (*NAT2*7B*) mutation is evident after digestion with *Bam*HI due to a point mutation at position 857 (G > A), causing the substitution of glycine[286] for glutamine. This mutation causes a decreased stability and increased affinity in the resultant enzyme. It would appear that the instability outweighs the increased affinity for the substrate, thereby reducing enzyme activity.[68] The coincident silent mutation at 282 (C > T) seems to have no (additional) effect.[68, 78]

The **M4** (NAT2*14) allele (referred to in this report as S4 for convenience) is absent in Caucasians and was only discovered later in 1993 by Bell *et al*, after obtaining discordance between genotype and phenotype.[80] This allele is the result of a point mutation at position 191 (G > A), altering codon 64 and replacing arginine[64] for glutamine. It has been proposed that this substitution could inhibit the formation of the acetylated enzyme intermediate in the acetylation process. Hein and Ferguson *et*

al. propose that since it occurs within the highly conserved Arg^{64} - Gly^{65} – Gly^{66} – Trp^{67} – Cys^{68} motif in the protein and the active site is known to hinge on the presence of Cys^{68}; the substitution of glutamine within this domain could hinder optimal binding.[78,81] Grant and Hughes *et al.* propose that this amino acid substitution offers no impaired substrate affinity but contributes to reduced stability.[68]

Upon studying alleles F (Allele 1), S2 (Allele 3) and S3 (Allele 2), Deguchi[52] found that the levels of mRNA did not differ significantly between alleles. This suggests that the differences observed in enzyme activity are determined by the amount of protein present, and that the amount of protein is dictated by the protein stability. This was shown in cultured CHO cells, where levels of allele 1-dictated *N*-acetyltransferase were high and allele 2- and allele 3-dictated *N*-acetyltransferase levels were low.[52] Grant and Eichelbaum *et al.* showed that the slow acetylator phenotype is due to the decreased level or absence of *N*-acetyltransferase in the human liver.[82]

Table 4.1a.* Frequencies (%) of NAT2 acetylator alleles in different ethnic groups[30]

Ethnic Group	*No. of genes analysed*	*Specific Frequencies*		*Slow Acetylator Allele Frequency (%)*			
		M1 (S1)	**M2 (S2)**	**M3 (S3)**	**M4 (S4)**	***Slow***	***Rapid***
Black, US	172	0.29	0.24	0.070	0.076	**0.68**	**0.32**
White, US	98	0.41	0.34	0.020	0	**0.76**	**0.24**
India	122	0.33	0.38	0.033	0	**0.74**	**0.26**
Hispanic I	166	0.23	0.17	0.17	0.006	**0.58**	**0.42**
Hispanic II	130	0.32	0.19	0.10	0.008	**0.62**	**0.38**
Filipino	200	0.065	0.36	0.18	0	**0.60**	**0.40**
Hong Kong	140	0.057	0.31	0.16	0	**0.52**	**0.48**
Japanese	158	0.019	0.23	0.11	0	**0.36**	**0.64**
Taiwanese	200	0.025	0.31	0.15	0	**0.48**	**0.52**
Korean	170	0.018	0.18	0.11	0.011	**0.32**	**0.68**
Samoan	50	0.040	0.34	0.020	0	**0.40**	**0.60**

*Adapted from Lin et al (1994)

Given the isoniazid acetylation polymorphism, population studies have been carried out to determine the epidemiology of *NAT2* in different ethnic groups (see table 4.1a.). Various authors have reported on numerous phenotypic studies. Lin and Han *et al.* have taken this epidemiology further, in that they have attempted to determine

the molecular epidemiology of these slow alleles and their ethnic distribution.[30, 83] From numerous population studies, they were able to determine the origins of mutation 857A (S3) to be Asian.

Bell and Taylor *et al.*[80] reported on the discovery of the M4 allele in an African-American population (referred to as S4 in this report). In 372 Caucasian individuals enrolled in his study, not a single M4 allele was found. This allele appears to be of African origin.[80] It has been known since the advent of phenotyping this trait,[12] that populations such as the Japanese exhibit a high proportion of rapid acetylators. A study in 51 Japanese individuals (1992) calculated the frequency of the rapid allele to be 72.5%,[62] compared to 71.05% found in 1961 by Sunahara and Urano *et al.*[11] From the table above, the prevalence of rapid alleles in eastern populations is evident, compared to the predominance of slow acetylator alleles in Caucasians.

The aim of this chapter is to offer further support for the validity of the assay developed to determine a patient's acetylator phenotype (using the method described in chapter one), as well as the markers used to determine phenotype (data presented in chapter two). Furthermore, this report will examine the *NAT2* genetics within this geographically unique population.

Being geographically, culturally and racially unique, this population, while the incidence of tuberculosis is high, remains relatively unstudied with regard to the isoniazid acetylation polymorphism. One study has been published, in which the trimodality of isoniazid elimination was conclusively reported in this population and allusions to the ethnic origins of this population made.[37] Codominance of the rapid and slow alleles has also been shown previously, as well as in chapter 2 of this report. Although often reported, trimodality is not an established fact in the western scientific community, with many researchers still referring to heterozygous rapid acetylators, eliminating the intermediate group completely.

In final support of the HPLC assay developed earlier, as well as the phenotypic data, this chapter aims to correlate the apparent phenotype with the determined genotype.

4.2. MATERIALS AND METHODS

Each patient donated a sample of blood, which was collected in EDTA-coated tubes. This was used to extract and purify genomic DNA, which was then used for the specific amplification of the *NAT2* gene sequences; the amplified DNA was then used to investigate the type of polymorphic N-acetyltransferase coded for in each patient.

4.2.1. DNA Extraction from Human Nucleated Cells in Whole Blood [84]

This process is essentially a salting out procedure.

1. Anticoagulated blood (7 ml) was poured into a 50 ml sterile tube. Approximately 30 to 35 ml of ice cold Cell Lysis Buffer was added to the tube, before shaking vigorously 15 to 20 times.
2. The tubes were centrifuged at 3000 rpm using a Sorvall RT6000 refrigerated centrifuge for 15 min at 4°C.
3. Nuclei Lysis Buffer (15 ml) was used to resuspend the pellet. The solution was then mixed gently and Proteinase K solution (10 mg/ml) (Roche) was added to a final concentration of 100 μg/ml.
4. The tubes were incubated overnight at 37°C. This time period may be reduced by increasing the concentration of Proteinase K added and incubated at 45°C until all particulate matter has dissolved.
5. After digestion was completed, 3-4 ml saturated NaCl (in excess of 6M) was added. The solution was mixed thoroughly, but gently.
6. Again the tubes were centrifuged at 3000 rpm for 20 min at 4°C.
7. The supernatant was transferred to 40 ml Absolute Ethanol (Merck), being careful to exclude all particulate matter from the protein pellet. If the Proteinase K digestion was successful, the resulting precipitate containing the DNA should be pure and white. Otherwise, it develops a brown hue indicative of residual protein.
8. The precipitate was allowed to float to the surface before being removed with a sealed Pasteur pipette. Autoclaved 1.5ml polypropylene tubes were used to store the DNA.
9. The DNA was washed with 500 μl 80% ethanol.
10. The ethanol was discarded and the DNA pellet dried at 37°C for 10 min, using a heating block (Techne Dri-block Model DB 3A, Techne). The dried DNA was reconstituted overnight in 1000 μl TE Buffer at 4°C.

Note: Certain samples gave DNA yields, which were too low to observe and collect visually. These tubes were mixed after adding the ethanol, and centrifuged at 3000 rpm for 10 min. This was done to pellet any DNA present. If visible after centrifugation, 500 μl TE was added and allowed to resuspend at 4°C.

Cell Lysis Buffer:	**Nuclei Lysis Buffer:**	**TE Buffer:**
320 mM Sucrose	400 mM NaCl	10 mM Tris, pH 7.5
1% Triton-X	10 mM Tris, pH 8.2	2 mM EDTA, pH 7.5
5 mM $MgCl_2$	2 mM EDTA, pH 8.2	
10 mM Tris, pH 7.6	1% SDS	

4.2.2. Determination of DNA Concentration [85]

Ten microlitre aliquots of each DNA solution were redissolved in 490 µl sterile water. This introduces a dilution factor of 2.5.

The optical density (OD) was read at two wavelengths to determine DNA content and purity using a spectrophotometer (Milton Roy Spectronic 1201). The initial reading at 260 nm allows the calculation of the DNA concentration. An OD reading of 1 unit represents either 50 µg/ml double stranded DNA, 40 µg/ml single stranded DNA or RNA, or 20 µg/ml single stranded oligonucleotides. The ratio of OD_{260}/OD_{280} gives an indication of the DNA purity. According to Sambrook and Fritsch *et al.* (1985)[85] pure DNA would give a ratio equal to 1.8, pure RNA, a ratio equal to 2 and any protein contamination, a ratio of less than 1.8. All calculations were carried out using the Beer Lambert Law:

$$A = \varepsilon cl$$

A = Absorbance at 260nm
ε = extinction coefficient
c = concentration
l = cuvette width (1cm)

given that 1 µg/ml solution of DNA gives an OD reading of 50.[85]

Using the above method, the concentration of DNA in each sample could be determined as well as the yield, knowing the volume of TE Buffer used to resuspend each sample.

4.2.3. Determining DNA Integrity using Gel Electrophoresis

A 0.7% agarose gel was prepared in order to check the purity of the DNA collected visually. Two hundred millilitres of 1 X TBE buffer (0.09M Tris-borate, 0.002M EDTA) was used to dissolve 1.4 g of FMC LE agarose (Seakem, FMC Bioproducts, Rockland, Maine, USA). The agarose-TBE suspension was heated to dissolve the powder, according to the manufacturer's instructions. The gel was run in 1X TBE buffer, using apparatus prepared in-house connected to a BioRAD Model 200/2.0 power supply. All samples were loaded in 1 µg aliquots, using the results from the spectrophotometry to calculate loading volumes. Promega's Blue/Orange 6X tracking dye was used to monitor the running of the gel. Two microlitres of the dye was

added to each DNA sample.

A DNA standard, in the form of 4 µl (1 µg) uncut lambda DNA (0.25 g/ml, BM) added to 2 µl tracking dye. The gel was run at 100 volts for approximately 60 min until the Bromophenol Blue dye front reached the end of the gel.

The bands of DNA were visualised by adding ethidium bromide to the staining buffer (1 X TBE) to a final concentration of 0.5 µg/ml. The gel was shaken in this solution for 10 min., and the DNA visualisation was achieved by ultraviolet transillumination.

5 X TBE Buffer (2000 ml)

108 g Tris Base (Saarchem)
55 g boris acid powder (Saarchem)
40 ml 0.5 M EDTA (pH 8)

4.2.4. Polymerase Chain Reaction (PCR)[50] (modified by Dept. Medical Biochem, US)

Genomic DNA from the whole blood was amplified using the PCR technique. A stock solution of the reaction mixture was made up consisting of the components listed below. Each 60 µl aliquot consisted of:

8 µl 2.5 mM PCR Nucleotide Mix (Bioline, London, UK)
10 µl 10 X PCR Buffer (GibcoBRL, Life Technologies)
4 µl 50 mM $MgCl_2$ (GibcoBRL, Life Technologies)
0.3 µl *Taq* Polymerase I (5 U/µl) (GibcoBRL, Life Technologies)
0.5 µl Nat-Hu 14 (Oligonucleotide primer) (Integrated DNA Technologies, Coralville, IA, USA)
0.5 µl Nat-Hu 16 (Oligonucleotide primer) (Integrated DNA Technologies, Coralville, IA, USA)
36.7µl Sterile Milli-Q water

The primers used in PCR:[50]

Nat-Hu14: 5'-GACATTGAAGCATATTTTGAAAG
Nat-Hu16: 5'-GATGAAAGTATTTGATGTTTAGG

The $MgCl_2$ and *Taq* Polymerase I aliquots were added last in order to prohibit non-specific activity by the polymerase. The DNA template aliquot (to be amplified) was made up to 40 µl using sterile water. By adding 60 µl of the PCR reaction mixture, the reaction volume totalled 100 µl. It was not necessary to lay mineral oil over the top of the solution in order to prevent evaporation and maintain the ion concentrations because the all the amplification reactions were carried out in a Perkin Elmer GeneAmp 2400 PCR system, which has an integrated heated lid, normalising the temperature environment.

The polymerase chain reaction consisted of an initial denaturation step of 94°C for 5 minutes, followed by 30 cycles of 94°C for 45 seconds (denaturation), 56°C for 50 seconds

(reannealing) and 72°C for 1:20 minutes (elongation). The process ended with a final elongation step of 15 minutes at 72°C. The samples were kept at -20°C until required for digestion.

A positive control sample – consisting of genomic DNA from an unrelated patient (supplied by CJ Werely, Dept. of Medical Biochemistry, Univ. of Stellenbosch) - was included in each amplification experiment/reaction. The negative control was made up of 40 µl sterile water and 60 µl reaction mix. Thus, if any component of the reaction mix were not functioning, the positive control would not amplify; similarly, any DNA contamination present in the 60µl reaction solution would show up in the negative control.

4.2.5. Determining PCR Quality

A 5 µl aliquot of each amplified sample tube/reaction was assayed on a 0.7% agarose gel in order to verify whether the PCR was successful. Again the running and staining buffers were 1X TBE. Intensities of the DNA bands were measured against a standard known to contain previously amplified NAT2 DNA (prepared by Cedric Werely, Dept. Medical Biochem, US). Later, pooled positive PCR controls were used, and served just as well. An aliquot of the 100bp DNA ladder (Promega) (5µl) served as molecular weight markers, each band increasing by 100bp in length, the 500bp fragment being twice as intense. Bromophenol Blue was added to each sample prior to running. Following the addition of ethidium bromide (0.5 µg/ml), the bands were visualised under UV, having run the gel at 100V until the Bromophenol Blue dye front to the end of the gel.

4.2.6. Restriction Enzyme Digestion[50] (Adapted by Dept. Medical Biochem, US)

Each amplified DNA sample was restricted with four different enzymes in order to determine the allelic genotype, as caused by the various point mutations, resulting in slow acetylator alleles. Each reaction required 15.7 µl DNA template, taken from the amplified aliquots. To this, 3.3 µl of enzyme reaction mixture was added, making up a total volume of 18 µl. This volume was split after initial digestion with the enzymes *Bam*HI, *Kpn*I and *Msp*I; an 8 µl aliquot was removed for an additional *Pst*I digestion. This double digestion protocol was developed by CJ Werely, Dept. of Medical Biochemistry, Univ. of Stellenbosch, and utilised because it gave a better resolution of the profile of bands generated by the above enzymes, thereby making the scoring of the allele profiles much easier and more reliable. The remainder of the initial sample digest (10µl) was analysed on a 2% Metaphor® agarose (FMC Bioproducts, Rockland, USA) gel. Each enzyme reaction mixture needed to be specific to the enzyme.

***Msp* I:**	1.8 µl 10X SuRE/Cut Buffer L (BM, Roche Molecular Biochemicals)
	1.5 µl *Msp* I (10U/µl; BM, Roche Molecular Biochemicals)
***Kpn* I:**	1.8 µl 10X SuRE/Cut Buffer L (Amersham, Pharmacia)
	1.5µl *Kpn* I (10U/µl; Amersham, Pharmacia)

***Bam* HI:** 1.8 µl 10X SuRE/Cut Buffer B (BM, Roche Molecular Biochemicals)
1.5 µl *Bam* HI (10U/µl; BM, Roche Molecular Biochemicals)

All the above reactions were incubated overnight at 37°C using a heating block set yo 37°C (Techne Dri-block Model DB 3A, Techne). *Pst*I digestions were also incubated overnight at 37°C. This was possible due to the fact that each of the enzymes had been tested for non-specific endonuclease activity over 24 hours.

The agarose gel was prepared using the rapid microwave instructions of the manufacturer. The agarose was slowly added to the buffer while swirling, in order to prevent the agarose from clumping. Having added all the agarose, the solution was dissolved using 100% power on the microwave for 35 seconds. Upon evidence of foaming, heating stopped until the bubbles subsided. This was carried out until no further foaming occurred, indicating total dissolution. The gel was poured, using a mini-gel apparatus and allowed to set at room temperature for 20 minutes. Thereafter the gel was chilled at 4°C for thirty before loading the gel and running it at room temperature. Ten microlitres of each digestion was loaded onto the gel, with 1 µl tracking dye, consisting of Bromophenol Blue and Orange G. The gel was run at 100V, until the Orange G dye front reached the end of the gel. Again, ethidium bromide was added to a final concentration of 0.5 µg/ml and allowed to stain for ten minutes. UV transillumination visualised the bands and the digests were scored.

***Pst* I:** 0.96 µl 10X SuRE/Cut Buffer H (Amersham Life Science)
0.8µl *Pst* I (15U/µl; Amersham Life Science)
0.24 µl sterile water

***Taq* I:** 1.8 µl 10X SuRE/Cut Buffer B (BM, Roche Molecular Biochemicals)
1.5 µl *Taq* I (10U/µl; BM, Roche Molecular Biochemicals)

This reaction was incubated at 65°C for 4 hours. Both of these digestions (the *Taq*I and *Pst*I series) were run on a 5% polyacrylamide gel.

4.2.7. Polyacrylamide Gel Electrophoresis (PAGE)

Two protocols were used to run 5% PAGE. The first set were prepared according to the following guidelines:

1.2 5ml 40% (w/v) Acrylamide/Bis Solution (19:1) (GibcoBRL Life Technologies)
125 µl 10% Ammonium persulphate solution (Ammonium peroxidisulphate – BDH)
2 ml 5X TBE
6.6125 ml Sterile water
12.5 µl TEMED (NNN'N'-Tetramethylethylenediamine – BDH)

The second set, not having the stock Acrylamide/Bis Solution available, was prepared according to the guidelines presented in Current Protocols in Molecular Biology 2; Greene Publishing Associates and Wiley Interscience; John Wiley and Sons (date unknown).

6.334 ml Sterile H_2O
2 ml 5 X TBE
1.67 ml 29:1 Acrylamide/Bisacrylamide mix
50 µl 10% Ammonium persulphate
5 µl TEMED

The Acrylamide/Bisacrylamide Mix was prepared by adding 29 g of acrylamide to 1 g of Bisacrylamide and dissolving in 100 ml water. This was then filtered through a 0.22 µm filter and stored in darkness at 4°C.

The gels were run using a Bio RAD Mini-Protean II Cell Gel Electrophoresis kit, connected to a Bio RAD Model 200/2.0 power supply. Four microlitres of the 100bp DNA ladder (Promega) was run alongside the samples, serving as a molecular weight marker. One microlitre of tracking dye was loaded to serve as a dye front. The gel was run in 1X TBE at 100V for 90 min using BioRad gel apparatus, until the Bromophenol Blue dye front had run off the gel.

Note: It was decided to load different digestion series onto the same gel, in order to compare band sizes between digests and samples of the same enzyme digestion.

4.2.8. Silver Staining [86] *Adapted for 10 X 6 cm gels*

The process of silver staining is preferred for DNA determination on polyacrylamide gels, because of its sensitivity.

1. Fix the DNA gel by washing with 50 ml 7.5% acetic acid at 37°C for 10 minutes. Rinse gel in water for 3 minutes at 37°C. Repeat this twice.
2. **Staining:**
   a) Prepare 0.1 % silver nitrate solution (m/v) by dissolving 0.05 g silver nitrate in 50 ml water. Heat solution to 37°C at the beginnig\ng of the gel electrophoresis. Five minutes before using, add 75 µl 3% formaldehyde (to a working concentration of 0.056%). This is adequate for a single 10 X 6 cm gel.
   b) Stain gel in this solution for 10 minutes.
   c) After staining, rinse gel very briefly in water at room temperature.
3. **Development:**
   1. Prepare a 3% (m/v) solution of sodium carbonate. Chill the solution at -20°C prior to gel electrophoresis. Thus the working temperature should be ± 8-10°C. Five minutes prior to use, add 75 µl formaldehyde solution and 10µl 40mM sodium

thiosulphate, yielding a working concentration of 8μm thiosulphate, to 50 ml sodium carbonate solution.

2. Stain the gel with the solution until the desired resolution is obtained. Depending upon the freshness of the silver nitrate, this should take 2-5 minutes. This should be very closely monitored.
3. When the desired resolution is obtained, add 50 ml chilled 7.5% acetic acid for 5 minutes and wash finally in water to terminate development. Optimally, there should be minimal background noise, and this may be aided by not adding the developing buffer directly onto the gel, and swirling immediately and well.

4. The gel may be dried or sealed in plastic to preserve and score the digests.

4.2.9. Digest Scoring Protocol

In order to score each subject according to the alleles present in their genome, it was necessary to set up a simple system for scoring these restriction enzyme digestions. Having decided to digest all the primary digestions, excluding that of *Taq*I, with *Pst*I for visualisation purposes, all the digestions were run on 5% polyacrylamide gels and silver stained. The banding patterns were easily distinguished and alleles were assigned to each individual. Figures 4.2.a, b, c and d indicate the expected banding patterns, using each restriction enzyme.

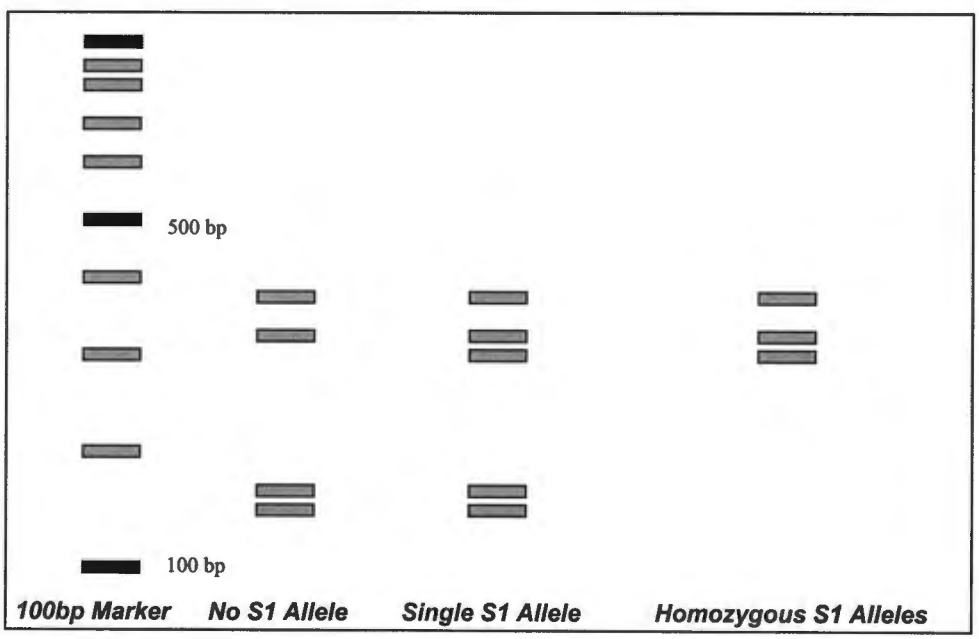


Figure 4.2.a. The specific banding patterns observed using *Kpn* I followed by *Pst* I.

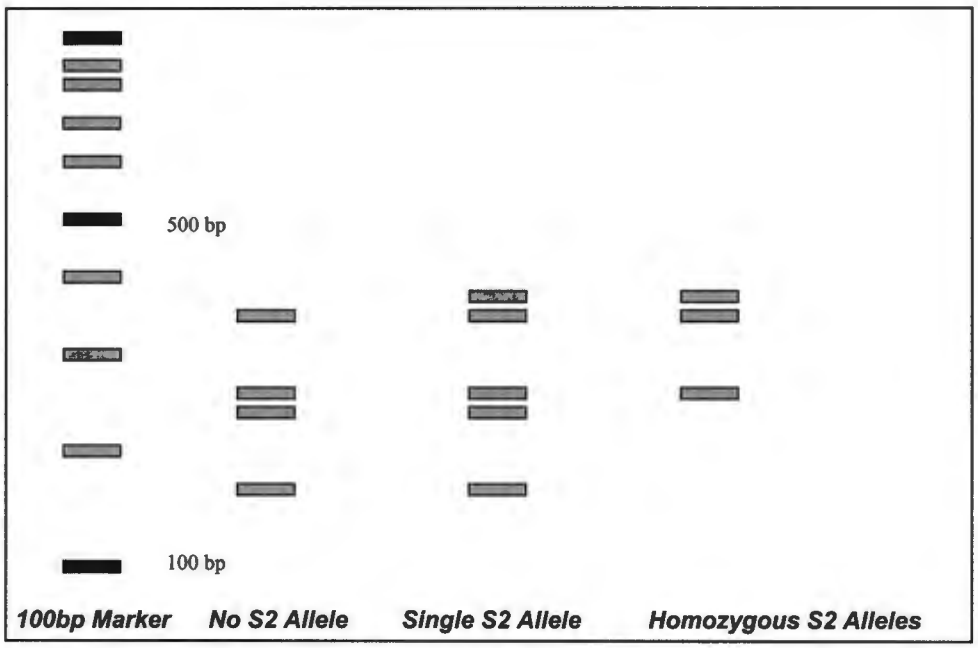


Figure 4.2.b. The expected banding patterns using the restriction enzyme *Taq* I.

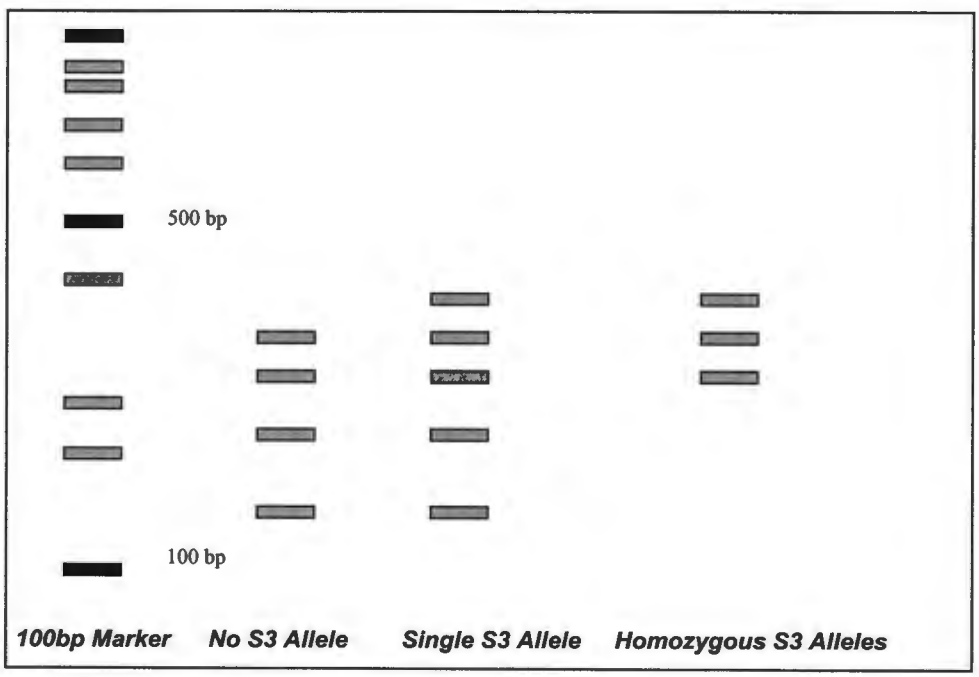


Figure 4.2.c. The banding pattern seen when *NAT2* is cut with *Bam* HI, followed by *Pst* I.

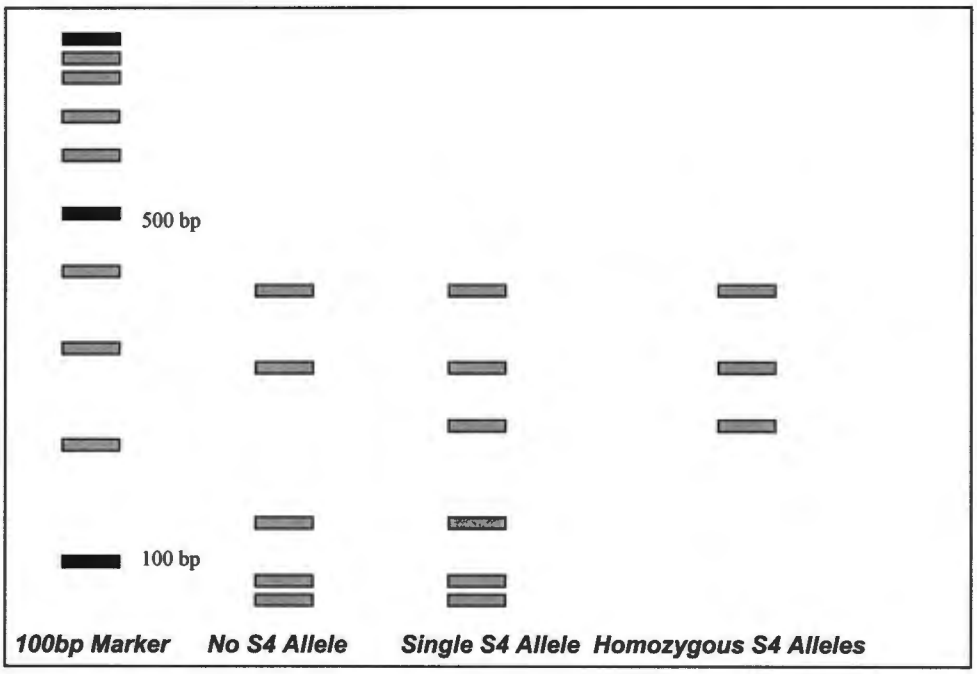


Figure 4.2.d. The banding patterns using *Msp* I restriction enzyme, followed by *Pst* I.

The smaller fragments are more difficult to visualise when running the gel. The resulting banding patterns were assessed and correlated with the apparent phenotype, determined previously. Any discrepancies resulted in the genotyping being repeated. The occurrence/distribution of alleles was tested using the **Hardy Weinberg equation** for multiple alleles in population genetics.

4.3. RESULTS

4.3.1. DNA EXTRACTION AND PURITY

DNA from each patient sample was extracted using the salting out procedure described in section 4.2.1. DNA concentration was determined using the Beer Lambert Law.

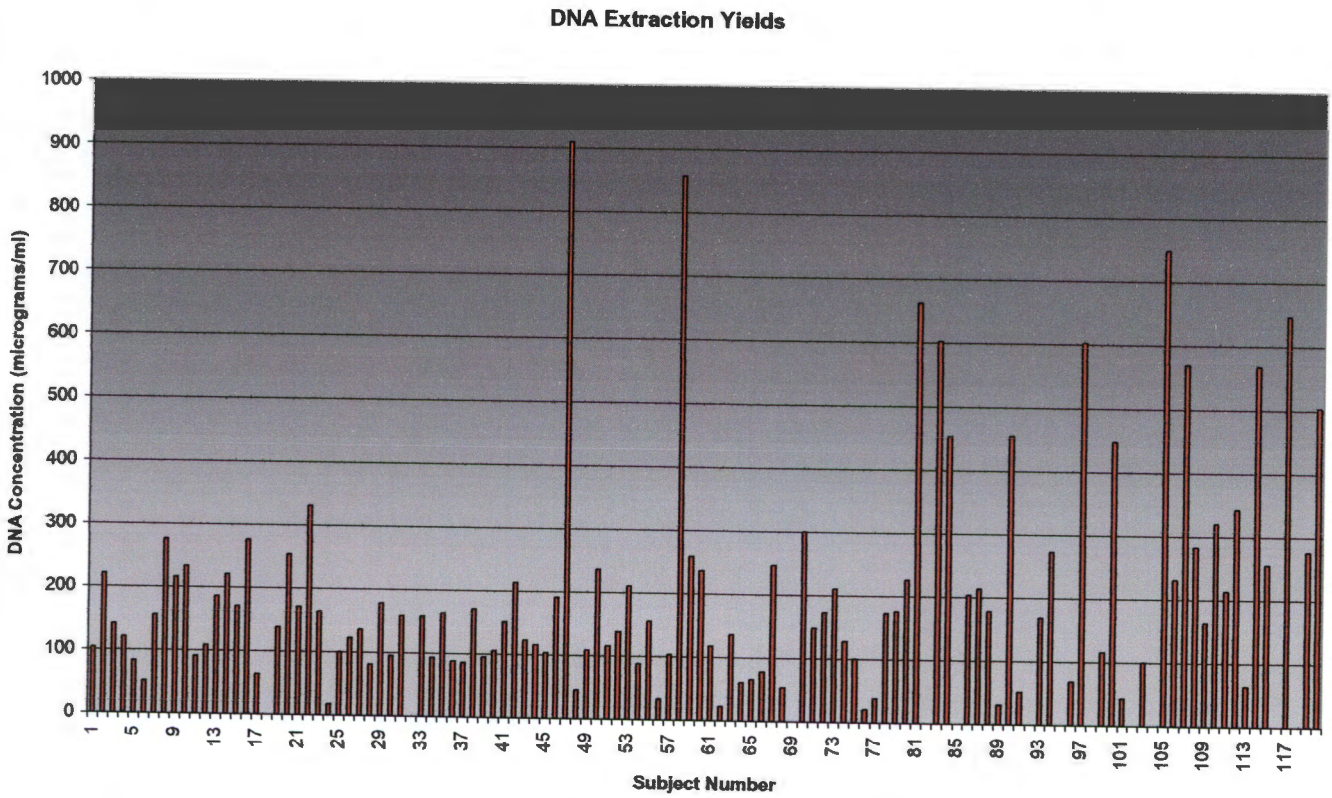
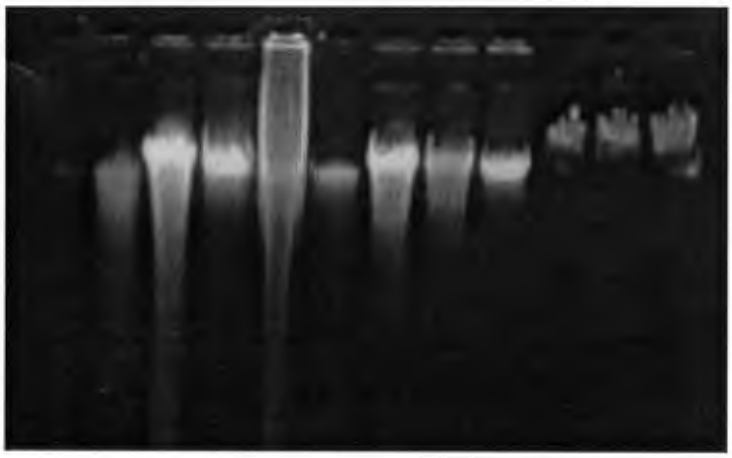


Figure 4.3.1a. The gross DNA yields for each individual to be genotyped calculated using the Beer Lambert law.

The range of yields was broad, extending from 910 μg/ml down to 17.5 μg/ml. This is related to the number of white blood cells present in the sample and could be indicative of the patient's immune response. Certain samples were difficult to extract. A DNA integrity gel was run in order to gauge the integrity of the extracted DNA, prior to PCR. Samples 4, 8, 20, 23, 24, 49, 52 and 107 were tested, having been pelleted during DNA extraction.



Loading order from left: 8, 20, 4, 23, 24, 49, 52, 107, λ-DNA (250 ng), λ-DNA (500 ng) and λ-DNA (1000 ng).

Figure 4.3.1b. Testing the integrity of extracted DNA – A representative gel.

All the above samples contain significant amounts of high molecular weight DNA, of good integrity suitable to undergo amplification (see figure 4.3.1a.). Standards of lambda DNA were also run. Sample 23 showed a considerable amount of smearing, indicative of sheared DNA. This sample was later discarded due to its failure to amplify. The absence of RNA is noted by the absence of a diffuse haze toward the end of the gel.

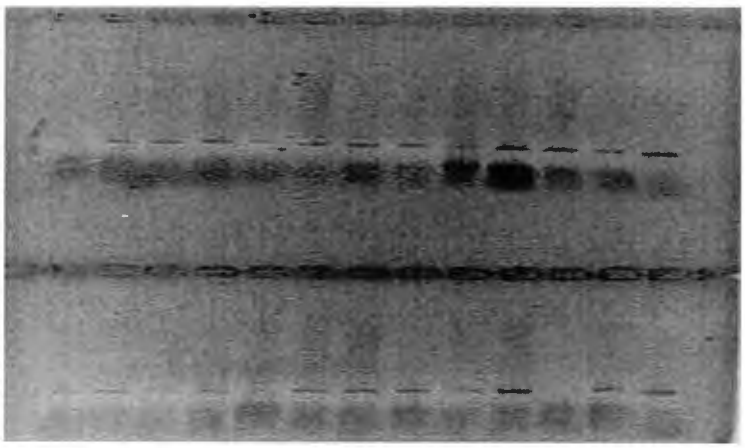
Most samples do show smearing, indicating a certain amount of DNA-shearing, the DNA appears to be intact, even though concentration is low.

4.3.2. DNA AMPLIFICATION

DNA amplification utilised a rapid PCR method using primers specific to conserved sections of the *NAT2* gene sequence.

Samples, which failed to amplify after 4 attempts and were discarded, included 4, 8, 23, 24, 68, 70, 75, 80 and 115.

Below is a representative image of a gel run in order to check the amplification of each DNA sample, following the application of the rapid PCR method. In this batch, samples 24, 33 and 41 failed to amplify. This is shown by the absence of a band in columns 1, 9 (row 1) and 5 (row 2). Shearing is indicated by the haze near the end of the gel.

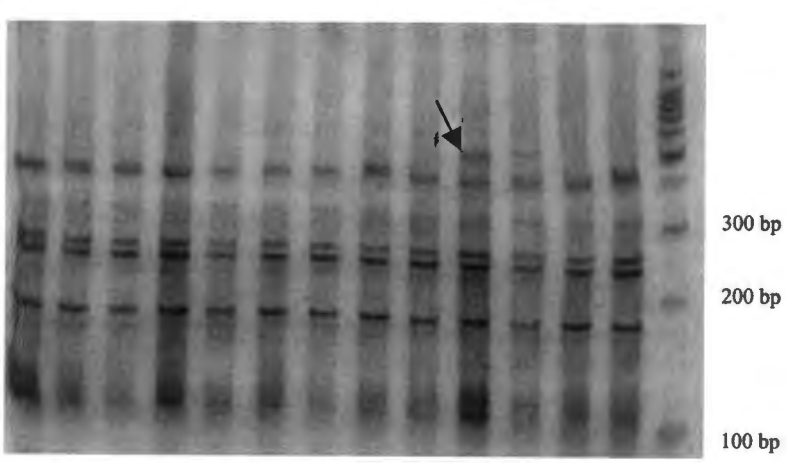


Loading Order: **LEFT to RIGHT**
24, 25, 26, 27, 28, 29, 30, 31, 33, 34, 35, 36, standard marker
37, 38, 39, 40, 41, 42, 43, 44, 45, 45, negative control, positive control, standard marker

Figure 4.3.2a. PCR Amplification

4.3.3 DNA RESTRICTION ENZYME DIGESTION

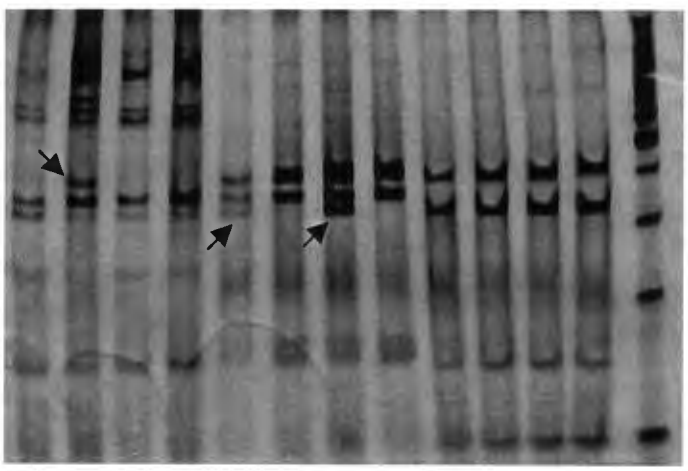
Restriction enzymes *Kpn* I (S1), *Taq* I (S2), *Bam* HI (S3) and *Msp* 1 (S4) were used to identify slow mutant alleles. The technique used to ascertain each individual's *NAT2* genotype worked well. Banding patterns specific to each enzyme and each allele were easily recognised, with the aid of transillumination. One hundred and two patients were successfully genotyped, although there were certain individuals whose genotype did not correlate with their phenotype. It became evident that, as the silver nitrate solution aged, the quality of the silver staining deteriorated. This was resolved by preparing new solutions.



Loading Order: **LEFT to RIGHT**
1, 2, 3, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 100 bp DNA Ladder

Figure 4.3.3a. PAGE of *Taq* I digests 1: Samples 1-15

Lanes 10 and 11 are evidence of S2 heterozygotes, displaying the fragment seen at approximately 500bp. A S2 homozygote would be missing the doublet as well as the lower band of 170bp. Arrows indicate the bands of interest, the bands appearing due to a missing restriction enzyme cutting site.



Loading Order: **LEFT to RIGHT**

(*Bam* HI) 6, 7, 9, 10; (*Kpn* I) 6, 7, 9, 10; (*Msp* I) 6, 7, 9, 10; 100 bp DNA ladder

Figure 4.3.3b. PAGE of mixed digests: Samples 6-10

In lane 2, it is evident that there has been a mutation, indicated by the absence of a restriction site resulting in a larger DNA fragment of approximately 400bp (indicated above using an arrow). This is indicative of the S3 allele, the presence of all the other common bands pointing towards an heterozygote. Lanes 5 and 7 present what is expected for a S1 heterozygote (also indicated using arrows), displaying an extra fragment of approximately 320bp, but there is no evidence of a S4 allele being present in any one of these samples. Certain digests were repeated following incomplete amplification.

4.3.4. ANALYSIS OF RESULTS

Table 4.3.4i. The final allele allocations for 102 subjects, shown with predetermined apparent phenotype

Subject No.	*Kpn*I S1	*Taq*I S2	*Bam*HI S3	*Msp*I S4/M4	Apparent Phenotype	*NAT2** Genotype
1					F	FF
2	•				I	S1F
3*					I	FF
4	PCR Failed					
5	•				I	S1F
6	•				I	S1F
7			•		I	S3F
8	PCR Failed					
9	•				I	S1F
10					F	FF
11	•				I	S1F
12		•		•	S	S2S4
13	•	•			S	S1S2
14	••				S	S1S1
15					F	FF
16	•				I	S1F
17	•				I	S1F
18	Missing					
19	•				I	S1F
20		•	•		S	S2S3
21					F	FF
22	•	•			S	S1S2
23	PCR Failed					
24	PCR Failed					
25			•		I	S3F
26		•		•	S	S2S4
27		•		•	S	S2S4
28					F	FF
29	•	•			S	S1S2
30					F	FF
31					F	FF
32	Excluded – only on rifampicin					
33*	•				F	S1F
34*	••				I	S1S1
35					F	FF
36*		•			F	S2F
37					F	FF
38		•	•		S	S2S3
39					F	FF
40					F	FF
41	•	•			S	S1S2
42		•			I	
43*	•				F	S1F
44	•	•			S	S1S2
45					F	FF

46				•	I	S4F
47*					I	FF
48				•	I	S4F
49	•				I	S1F
50					F	FF
51	•				I	S1F
52		•			I	S2F
53					F	FF
54					F	FF
55		•			I	S2F
56	•			•	S	S1S4
57					F	FF
58		•			I	S2F
59					F	FF
60		•			I	S2F
61					F	FF
62			•		I	S3F
63			•		I	S3F
64	•				I	S1F
65*	•				F	S1F
66	•				I	S1F
67	•				I	S1F
68	PCR Failed					
69	Missing data					
70	PCR Failed					
71			•		I	S3F
72		•			I	S2F
73			•		I	S3F
74*		•			F	S2F
75	PCR Failed					
76	•		•		S	S1S3
77					F	FF
78		•		•	S	S2S4
79	•	•			S	S1S2
80	PCR Failed					
81*	•				S	S1F
83*					S	S3F
84		•			I	S2F
85	Excluded – Vomited after medication					
86					F	FF
87	•				I	S1F
88					F	FF
89				•	I	S4F
90	•				I	S1F
91	•			•	S	S1S4
92	•				I	S1F
93	•				I	S1F
94*					I	FF
95		•	•		S	S2S3
96					F	FF
97		•			I	S2F
98	•				I	S1F
99*	•				F	S1F

100*					I	FF
101	•				I	S1F
102	87 repeated at 2 months					
103					I	FF
104					F	FF
105	•				F	S1F
106						FF
107	•				I	S1F
108	•				I	S1F
109	•				I	S1F
110					F	FF
111*	•				F	S1F
112*	••				S	S1S1
113	•				I	S1F
114	•	•			S	S1S2
115	PCR Failed					
116	Tube broke in centrifuge during extraction					
117		••			S	S2S2
118	Missing data					
119*					I	FF
120					F	FF

* denotes non-correlators

Out of the 114 patients successfully phenotyped in chapter 3, 102 were successfully genotyped. Certain samples failed to extract, owing to the degradation of the white blood cells during storage and were discarded. Others did not amplify, although the DNA appeared to be of good quality and quantity. Each amplified sample was restricted and the banding patterns assessed to determine the presence or absence of each slow allele. No assay was implemented to assay for the wild type allele - this was assumed to be present in the absence of a slow allele.

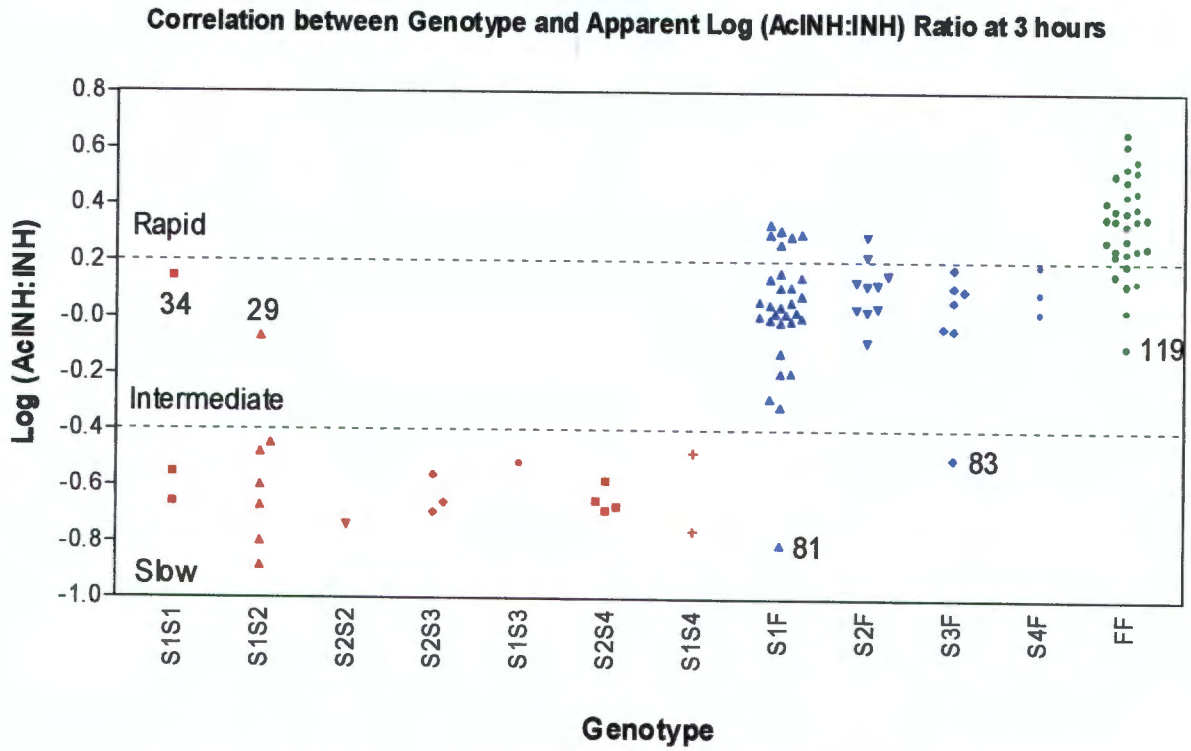


Figure 4.3.4a. Genotype - Phenotype Relationship using the 3-hour level model (n = 102)

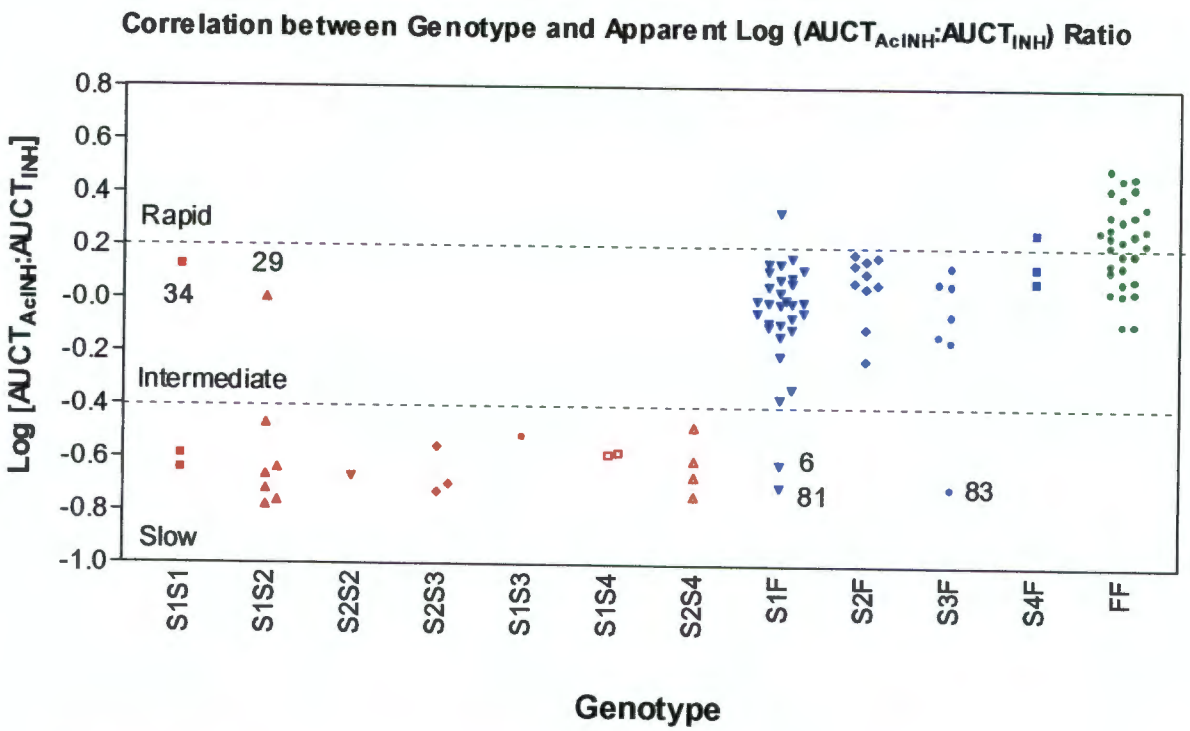


Figure 4.3.4b. Genotype - Phenotype Relationship using the AUCT ratio model (n = 102).

The correlation between phenotype and genotype is very close. The exact percentage of correlation was calculated to be 88.2% for both models, using extreme (difference from upper or lower limit > 0.2 units) outliers for the calculation.

Table 4.3.4ii. The incidence of all alleles according to accepted nomenclature[87]

	Trivial Name	Genotype	No. of Subjects
Slow	S1/S1	NAT2*5/NAT2*5	3
	S1/S2	NAT2*5/NAT2*6A	7
	S1/S3	NAT2*5/NAT2*7B	1
	S1/S4	NAT2*5/NAT2*14	2
	S2/S2	NAT2*6A/NAT2*6A	1
	S2/S3	NAT2*6A/NAT2*7B	3
	S2/S4	NAT2*6A/NAT2*14	4
Intermediate	S1F	NAT2*4/NAT2*5	30
	S2F	NAT2*4/NAT2*6A	10
	S3F	NAT2*4/NAT2*7B	7
	S4F	NAT2*4/NAT2*14	3
Rapid	FF	NAT2*4/NAT2*4	31

From table 4.3.4ii, the incidence of the rapid acetylator allele is apparently similar to that of the slow acetylator allele. This indicates a marked Asiatic influence in this population, in contrast to Caucasian populations. The S4 (M4) allele is very rare in this population, as is the S3 allele - with no homozygotes being recorded, but they have been detected at low frequencies, concurring with Bell and Taylor *et al.* that S4 may be of African origin.[80] The predominant slow acetylator allele is S1, common in Caucasian populations, followed by S2, also relatively common.

The specific frequencies of the slow acetylator alleles are ***S1 (50.0%); S2 (28.26%); S3 (11.96%)*** and ***S4 (9.78%).***

As seen below in figures 4.3.4c and 4.3.4d, the correlation of the marker values used to determine phenotype, as determined by phenotype and by genotype, is good. The increased size of the error bars for the genotype-governed allocations indicates the presence of non-correlators, but these are small and offer no significant overlap. The trimodality is still significant at $p < 0.0001$ (Mann Whitney test).

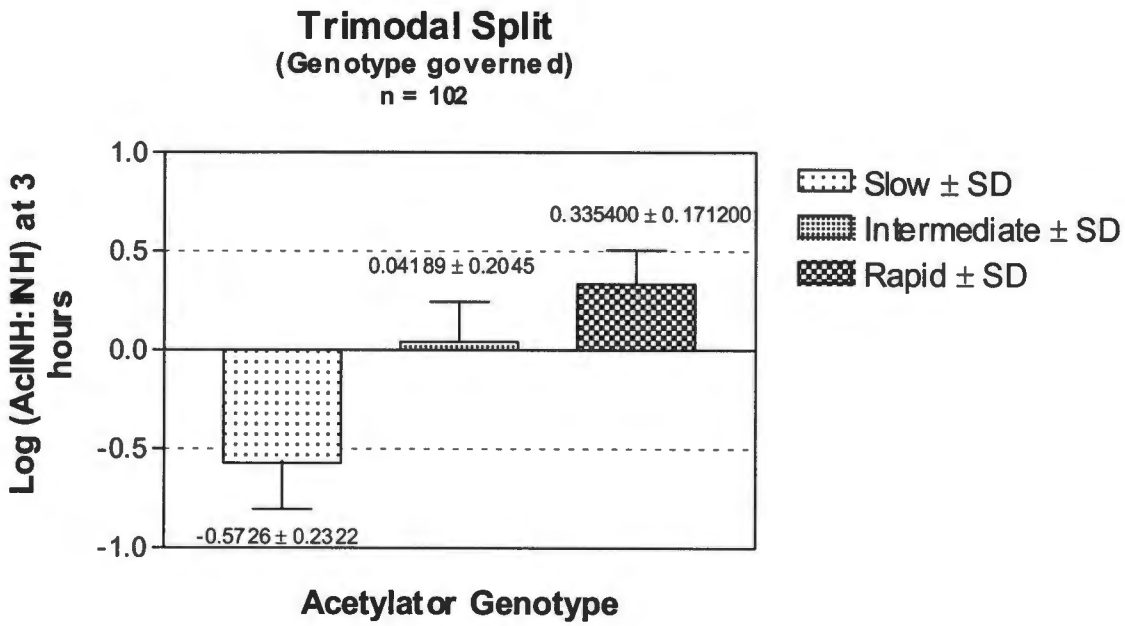


Figure 4.3.4c. The comparison between the trimodal splits governed by genotype and the mean ratios for each subgroup - Genotype

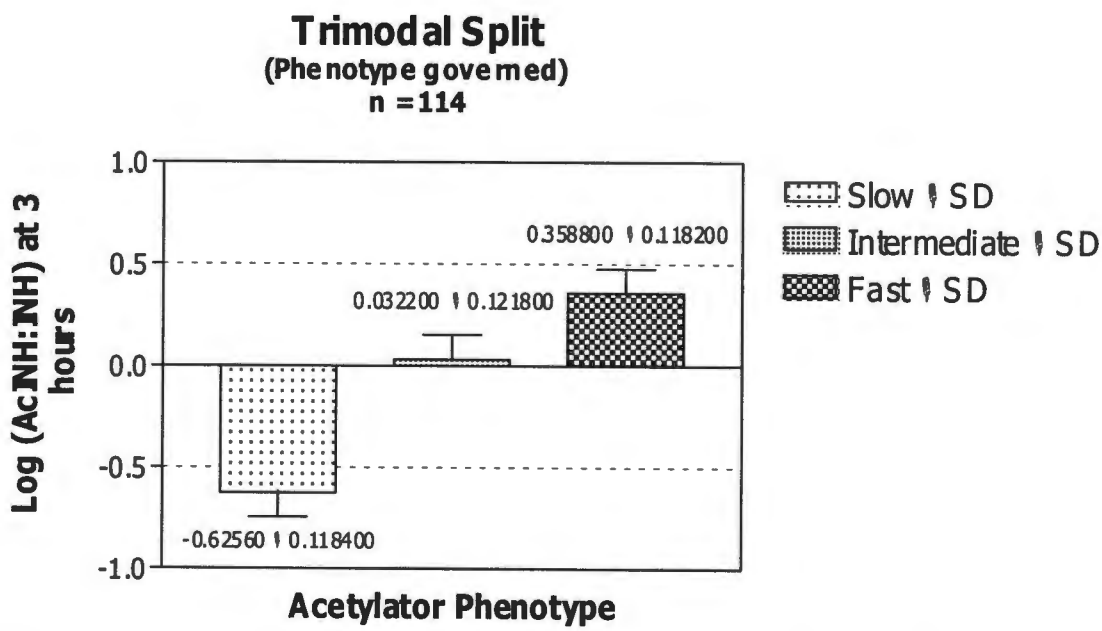


Figure 4.3.4d. The comparison between the trimodal splits governed by phenotype and the mean ratios for each subgroup – Phenotype.

The Hardy Weinberg equation, suitable for multiple alleles, was applied to this population and the incidence of each set of alleles measured to ensure that the population constituted a well-defined gene pool.

The gene frequency of the slow allele (s) was calculated to be 0.4368 for the complete population at Brewelskloof. This frequency value was then used to calculate the expected frequencies and distributions of various genotypes, using the equation:

$$S^2 : 2s\,(1\text{-}s) : (1\text{-}s)^2$$

Calculations were later adjusted to consider a discrete population of coloured individuals only, although this is not a true reflection of the tuberculosis population at Brewelskloof hospital.

Table 4.3.4iii. The results of the application of the Hardy Weinberg equation and its principles

A. Complete Sampled Population at Brewelskloof Hospital (n=102)

Acetylator status		*Allele contributions*		*Genotype frequencies*	
Phenotype	**Genotype**	**slow (s)**	**fast (1-s)**	**Observed**	**Calculated**
Slow	S_xS_x	0.205		0.205	0.203
Intermediate	FS_x	0.245	0.245	0.490	0.495
Fast	FF		0.304	0.304	0.302
Total		**0.450**	**0.549**	**1.00**	**1.00**

B. Eliminating the black and white participants (i.e. non-coloured individuals) (n=91)

Acetylator status		*Allele contributions*		*Genotype frequencies*	
Phenotype	**Genotype**	**slow (s)**	**fast (1-s)**	**Observed**	**Calculated**
Slow	S_xS_x	0.187		0.187	0.188
Intermediate	FS_x	0.247	0.247	0.494	0.492
Fast	FF		0.319	0.319	0.320
Total		**0.434**	**0.566**	**1.00**	**1.00**

4.4. DISCUSSION

The overall outcome of the genotyping procedure supported the phenotyping methods very well, emphasising the apparent trimodality of isoniazid acetylation, in this population, confirming the report of Parkin and Vandenplas *et al.*[37] An *88.2%* correlation was evident between the assigned phenotype and the genotype of subjects (See figures 4.3.4a and b). This supports *(a)* the precision of the Log (AcINH:INH) value at three hours when used as a marker for acetylator status determination as well as *(b)* the usefulness of the assay, allowing a single sample to be analysed rapidly, without derivatisation, in order to estimate the individual's acetylator status.

The DNA extraction was successful, extracting the DNA from all but two samples. The concentrations of DNA were varied, possibly relating to the severity of illness in the patient, extracting the DNA from the leukocytes in the blood. The overall integrity was good, with very little protein and RNA remaining in the sample. Having used the optical density as an indication of DNA purity, high OD_{260} values seemed to have little effect on DNA amplification. Those samples, which should have been heavily contaminated with protein, (data not shown) proceeded to amplify well and digestions were successful. Samples, which failed to amplify after numerous attempts, were discarded and the successful samples were followed though. Reasons for amplification failure might have included low DNA extraction yields (i.e. samples 4 and 8) or a nucleotide substitution within the gene sequence complementary to the primer, inhibiting the primers from annealing optimally.

The individuals, whose genotype did not appear to correlate with their phenotype, could have been misclassified due to the rapid and intermediate phenotype separation not being discrete. This is supported by the fact that not a single individual skipped a group - i.e. a slow misclassified as a rapid acetylator. Also, no digestion was performed by *Dde1*, which has been known to indicate the presence of the mutated allele S1c, which would allow an intermediate acetylator to be classified as a slow acetylator genotypically.[68] However, this allele is reported to be rare in the coloured population by Parkin and Vandenplas *et al.*[37]

Previously, it has been shown that non-correlation is a marker for new allotypic variants,[80, 88] although no sequencing was performed on the individuals in question due to time constraints. Allusions to new acetylator alleles, although rare, have been made by Lin and Han *et al.*[30] This may account for a small proportion of discordance,

especially in this "new" population. Certain subjects did display anomalous bands upon restriction enzyme digestion. This may be an indication of further mutations within the population. Further work may be required to address that issue.

Comparing the phenotypic trimodal split of individuals to the genotypic split, the Log (AcINH:INH) ratios are highly comparable (See figures 4.3.4c and d). The mean ratio for slow acetylators phenotypically is –0.6256 versus a genotypically determined ratio of –0.5726; intermediate acetylators display a phenotypic mean of 0.04189 compared to 0.03020 (genotypic); and rapid acetylators 0.3588 to 0.3354.

Once again, the trimodality of the human acetyltransferase polymorphism has been demonstrated, taking into consideration the co-dominance of the fast and the slow alleles, constituting a separate acetylation group. Previous authors have acknowledged the existence of heterozygotes, but often classified those individuals as rapid heterozygotes. This report has confirmed the presence of a separate sub-group of acetylators, those being the heterozygotes or intermediate acetylators.

The predominance of intermediate acetylators may seem elevated in this population, but the prevalence of all alleles correlated closely with what was predicted by the application of the Hardy Weinberg equation, suitable for population genetics and multiple alleles. The exact correlations for the coloured population at Brewelskloof hospital for slow acetylators were 18.7% (18.8%), 49.4% (49.2%) for intermediate acetylators and 31.9% (32.0%) for rapid acetylators. The calculated percentages are represented in brackets. This determines the sampling population to be a well-defined gene pool.

One of the first papers published by Dufour and Knight *et al.*[12] quoted gene frequencies in Caucasians, African-Americans and Japanese populations. Although the distribution was trimodal, the frequency of the slow allele in Caucasians and African-Americans was almost three times that of the rapid allele, whereas in this population the frequency of both alleles is highly comparable (43.4% vs. 56.6% in the coloured sampling population). This is an indication that this population draws from many influences.

It has been stated previously that this population differs from the African-American population in its composition, due to the Eastern or Malay influence, boosting the incidence of fast alleles, which are known to predominate in these populations.[67] Lin and Han *et al.* confirm that the S3 allele is Asiatic[83] adding credence to the Malay

influence in this population. The incidence of this allele in this population is 12% of slow alleles. The S1 allele is highly prevalent (50% of slow alleles) and this indicates the Caucasian influence on this population.[30]

4.5. FINAL CONCLUSIONS

Genotyping the phenotyped patients served to support the findings in Chapters 2 and 3. The developed assay was used to determine the drug levels, which were the used to calculate various pharmacokinetic parameters, which were subsequently used to determine the acetylator phenotype of the sampled patients.

A correlation of 88.2% was noted between phenotype and genotype, and any discrepancy could be due to external, non-patient factors, such as impaired liver function or undetected alleles, such as S1c. The possibility of new allelic variants must not be ignored. This population is understudied, and may be the source of new slow acetylator mutations, as yet undiscovered, and these being responsible for the discordance observed. Further research is required. The gene pool was determined to be well-defined (i.e. random mating) and heterogeneous.

CHAPTER FIVE

THE INFLUENCE OF ACETYLATOR STATUS

5.1. INTRODUCTION

Much debate has existed over the years as to whether acetylator status dictates the risk of isoniazid hepatotoxicity. Monoacetylhydrazine induces liver damage via the generation of a reactive metabolite.[89, 90] It has been mentioned that the increased incidence of drug-induced hepatitis in certain population may be due to the increased incidence of viral hepatitis.[91] Elevated serum aminotransferase levels associated with isoniazid administration, occur especially when administered with rifampicin, coupled with increased age and previous history of abnormal liver function.[92, 93, 94]

During a retrospective study performed by Mitchell and Thorgeirsson *et al.* (1975), a large number of subjects with drug-induced hepatitis were rapid acetylators. This lead to the hypothesis that rapid acetylators were more prone to hepatic injury, and that this injury was caused by prolonged exposure to an acylating agent, formed during microsomal activation of monoacetylhydrazine.[95]

In contrast, a study conducted to assess the association between acetylator status and drug-induced hepatitis by Dickinson and Bailey *et al.* found that rapid acetylators had a very low risk of developing hepatitis (4%).[96] Slow acetylators under 35 years of age and rapid acetylators above 35 years of age had a moderate risk (13%) and slow acetylators over 35 years had the highest (37%).[96] This study assessed various contributing factors, such as age, mildly abnormal baseline liver enzymes, race, sex and concomitant therapy. The incidence of hepatotoxicity in this study are high when comparing them to those found in other studies.

Mitchell's findings were disputed by many. A paper was published, which showed the lack of hepatotoxicity and acetylator phenotype correlation in 8 controlled clinical trials, involving 3 000 South Indian patients, measuring serum aspartate aminotransferase (AST) in 850 trial subjects and comparing this to their acetylator phenotype.[36]

Following the discovery that the enzyme responsible for detoxifying monoacetylhydrazine was the same one known to acetylate isoniazid,[49] the theory of Mitchell and Thorgeirsson *et al.*[95] was brought into dispute. If a single enzyme were responsible for both of these processes, then the exposure of rapid compared to slow acetylators would be similar, and this was proven by assessing the urinary concentrations of isoniazid metabolites.[9, 97, 98, 99]

Another theory followed. If a large dose (10 mg/kg) of isoniazid is administered to an individual, the enzyme responsible for metabolism (*N*-acetyltransferase 2) becomes saturated.[49] Thus a large fraction of the administered dose will be available to the monooxygenase pathway, shown to produce hepatic necrosis via the formation of a reactive metabolite of acetylhydrazine.[90] Administering drugs such as phenobarbital has induced the monooxygenase pathway.[97] Concomitantly administering drugs, which induce the pathway with isoniazid, would cause an accumulation of acetylhydrazine, now made available to an induced monooxygenase pathway, ready to form a reactive acetylhydrazine metabolite and cause increased hepatic necrosis.[97] The authors of this article (Lauterburg and Smith *et al.*) state that only individuals who possess remarkably slow acetylation capacity are at risk, thus accounting for the lower than expected incidence of hepatic injury in slow acetylators, when concomitantly administered with rifampicin.[97]

Subsequent to this paper, Jenner and Ellard published findings that the addition of rifampicin did not influence the incidence of drug-induced hepatotoxicity, citing among others, that the incidence of jaundice (1%) was similar to previously documented cases prior to the introduction of rifampicin.[100] Peretti and Karlaganis *et al.* added another dimension to this debate – showing that the administration of isoniazid does tend to inhibit the acetylation of monoacetylhydrazine, possibly allowing a greater proportion to become available to the monooxygenase pathway.[101] There has been no consistent evidence that the combination of rifampicin and isoniazid is particularly hepatotoxic in man.[102]

A recent study in Japan correlated elevated serum aminotransferase levels with slow NAT2* genotype. The criteria for determining elevated levels were that they had to be 1.5 X upper limit of normal and 2 X level prior to administration. Aminotransferase levels were taken at 0, 2, 4, 8 and 12 weeks. Each study population subgroup (i.e. acetylator phenotype) was homogeneous in terms of race, sex and age, coupled with the fact that the genotype spread was predicted by the Hardy-Weinberg Law, there was a 100% incidence of raised aminotransferase levels in slow acetylators.[94] There

have been numerous previous reports of elevated serum aminotransferase activity, but it has also been reported that these levels returned to normal after continuing therapy.[93, 103] In the instance of hepatotoxicity in individuals receiving isoniazid chemotherapy, vigilance is necessary due to the likelihood that levels will return to normal and remain normal after therapy continues.[103] It has been suggested that the outcome of hepatotoxicity could be fatal if the patient has been receiving isoniazid therapy for more than two months without vigilance on the part of the clinician.[104]

A distinction should be made between raised levels and levels which are too high to tolerate. Treatment guidelines state that therapy should only be discontinued if the AST level is three times normal, provided the patient remains asymptomatic. Clinically overt hepatitis necessitates the complete discontinuation of treatment.[105]

Table 5.1i. The incidence of hepatotoxicity during a series of studies run by the British Medical Council.[103]

*Country***	*Drug combination***	*Patients assessed*	*Hepatitis*	
			No.*	%
Britain	SHP	444	5 (5)	1
***n* = 2**	SHE	116	1 (0)	1
India	SHP	519	9 (9)	2
***n* = 5**	SH***	78	0	0
	HT	75	2 (2)	3
East Africa	SHT	1665	10 (10)	1
***n* = 5**	HP	125	2 (0)	2
Hong Kong	HP	100	1 (1)	1
	HT	150	8 (8)	5
Singapore	SHT	225	8 (6)	4
	SH	131	2 (1)	2
International	SHT	3563	15 (2)	0.4
***n* = 2**	SH	2394	5 (1)	0.2

* Values in brackets indicate cases of jaundice

** Drugs administered daily - Abbreviations: S = streptomycin, H = isoniazid, P = para-aminosalicylic acid, E = ethambutol, T = thioacetazone

*** Regimen given biweekly

**** All regimens lasted over 12 months, except the international studies (8 and 12 weeks)

In terms of treatment outcome and for the purposes of this study, acetylator status is of no prognostic significance when treatment is given daily and only of doubtful significance when weak twice-weekly regimens are employed.[48]

The variation occurring within the *NAT* gene loci results increased risk of certain types of cancers, depending upon the acetylation phenotype.[68] The development of cancer depends upon a balance between two opposing enzymes – the activating and detoxifying enzymes for the compound and resulting metabolites. In the case of *N*-acetyltransferase, if the enzyme is defective, other enzymes will predominate and from reactive species, which may lead to DNA binding and the formation of tumours.[68] The tissue selectivity depends upon the destination of the reactive end product and the enzymes present, possibly increasing the DNA binding potential of the metabolite.[68] This issue is still broad, with many feasible hypotheses.

This chapter aims to gauge the effect of acetylator status on other patient parameters, such as the incidence of liver toxicity, markers used to gauge treatment progress (i.e. weight gain and clearance of sputum) and the gender distribution of acetylator status.

5.2. MATERIALS AND METHODS

Liver function tests were performed once on all patients enrolled in the study on the day of pharmacokinetic sampling approximately two months after admission to Brewelskloof hospital for TB treatment. Blood for these tests was drawn between the 6 and 8 hours pharmacokinetic samplings via an indwelling canula and analysed by the chemical pathology laboratories at Groote Schuur hospital. Levels of liver enzymes indicate the extent, if any, of incurred liver damage, or the immediate state of the patient's liver. Significant cut-off points are used to gauge the severity of liver function impairment. This study aims to note the absolute elevation of liver enzyme levels, whether or not they are deemed clinically significant.

Tests performed for liver function included alkaline phosphatase (AlkPhos), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin. The levels were regarded as above normal limits if:

- Alkaline phosphatase above 70 units,
- AST and ALT above 25 units,
- Total bilirubin above 17 units.

From the phenotypic results regarding acetylator status, correlations were performed as to whether acetylator status had any effect upon the apparent liver function after two months of anti-tuberculosis drug therapy.

Patient characteristics were obtained from Brewelskloof hospital with the patient's consent. The data included age, weight gain following two months of anti-tuberculosis therapy, sputum clearance after two months and patient gender. Sputum clearance was determined by culturing a sputum sample at two months post therapy initiation.

5.3. RESULTS

As part of the pharmacokinetic study, into which this work fell, all patients were screened for raised liver enzyme levels, possibly in part caused by damage due to drug exposure. There is no direct correlation between isoniazid and raised liver enzymes. Normal fluctuations of liver enzymes are relatively common in individuals. Rifampicin may cause an increase in bilirubin, while all other concomitant medications may affect enzymes levels. The disease state of the patient may also be a mitigating factor. The incidence of raised liver enzymes was correlated with acetylator status.

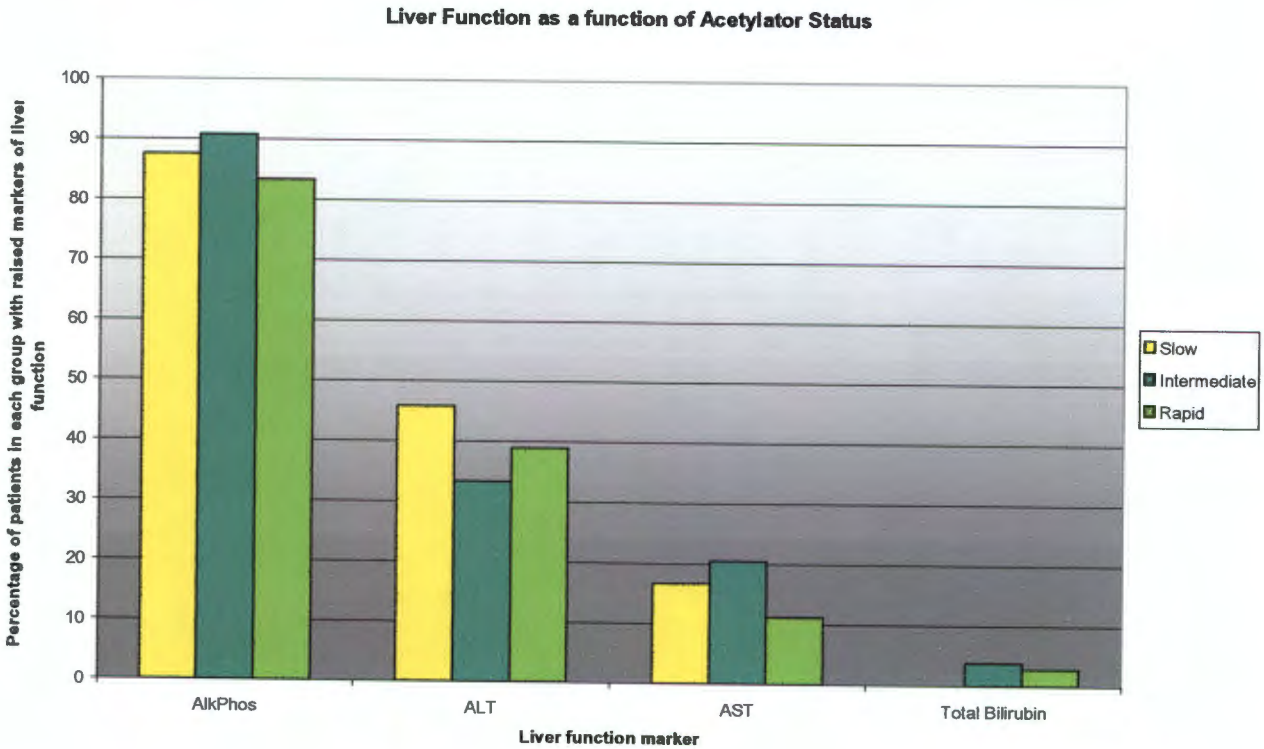


Figure 5.3a. The distribution of raised liver enzymes

As is evident from figure 5.3a, the incidence of raised liver enzyme levels was not related in any way to the assigned acetylator phenotype of the patient.

The alkaline phosphatase levels were generally raised, although could only be regarded as mildly elevated. This in no way indicates isoniazid related liver damage, as alkaline phosphatase levels may be slightly raised due to alcohol intake or in young growing people. The AST and ALT levels are insignificant, neither displaying a two-fold or three-fold increase, necessitating treatment suspension.[105]

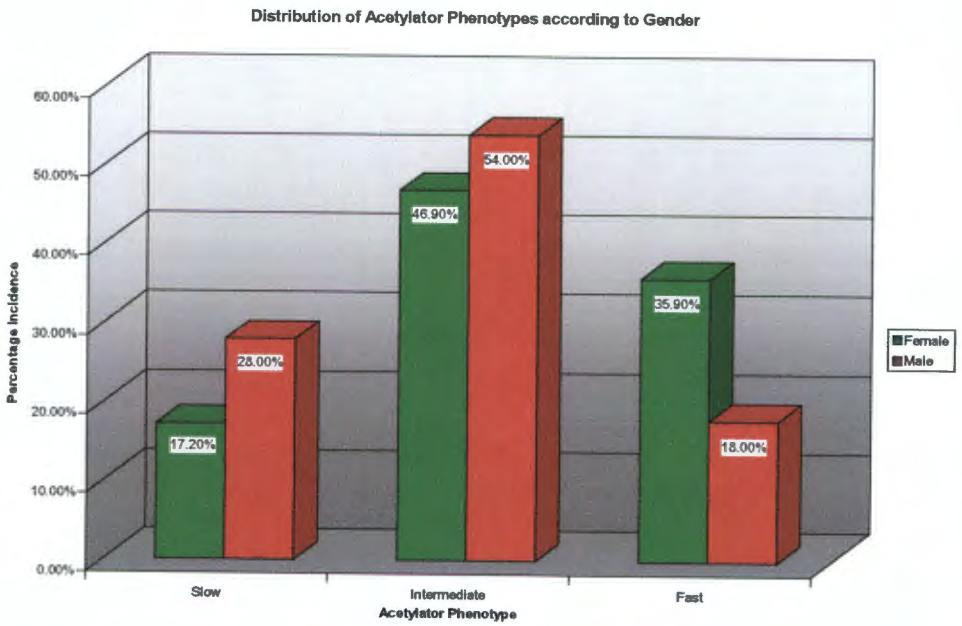


Figure 5.3b. The distribution of acetylator status by gender.

The split of acetylator phenotype according to gender indicated a majority of female patients with rapid phenotypes, and a majority of slow acetylators being male. The ratio of male to female intermediate acetylators was equivalent.

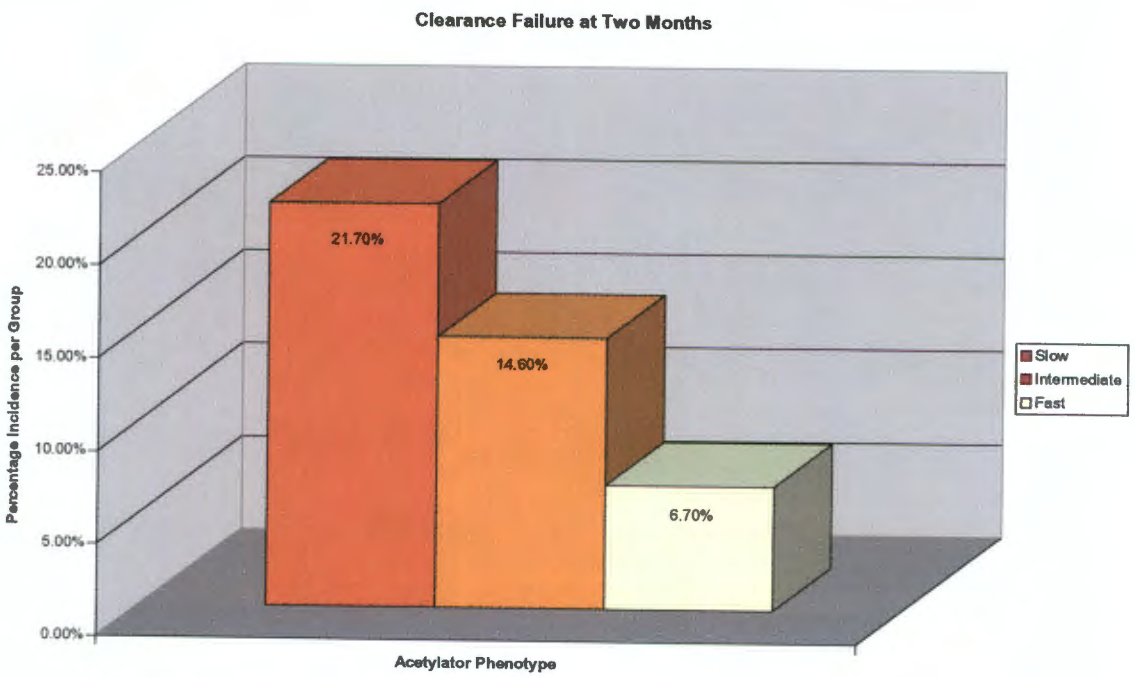


Figure 5.3c. The effect of acetylator status on sputum clearance.

Sputum clearance was implemented as one of the markers indicating possible TB treatment failure in the study. Sputum was collected from each patient at 2 months.

This was then assessed directly before being cultured. Rapid acetylators are known to fare worse than slow acetylators under certain conditions, such as once-weekly therapy.[17, 55] The indication from figure 5.3c is that slow acetylators failed to clear the sputum more often than rapid acetylators. But this conclusion is not statistically significant, as the sample size is too small.

Weight gain, another marker used to gauge response to treatment in the study, was not statistically related to acetylator status, using non-parametric statistics. In general, patients gained weight during the course of the two months they were in hospital, possibly because they were receiving regular meals with proper nutrition.

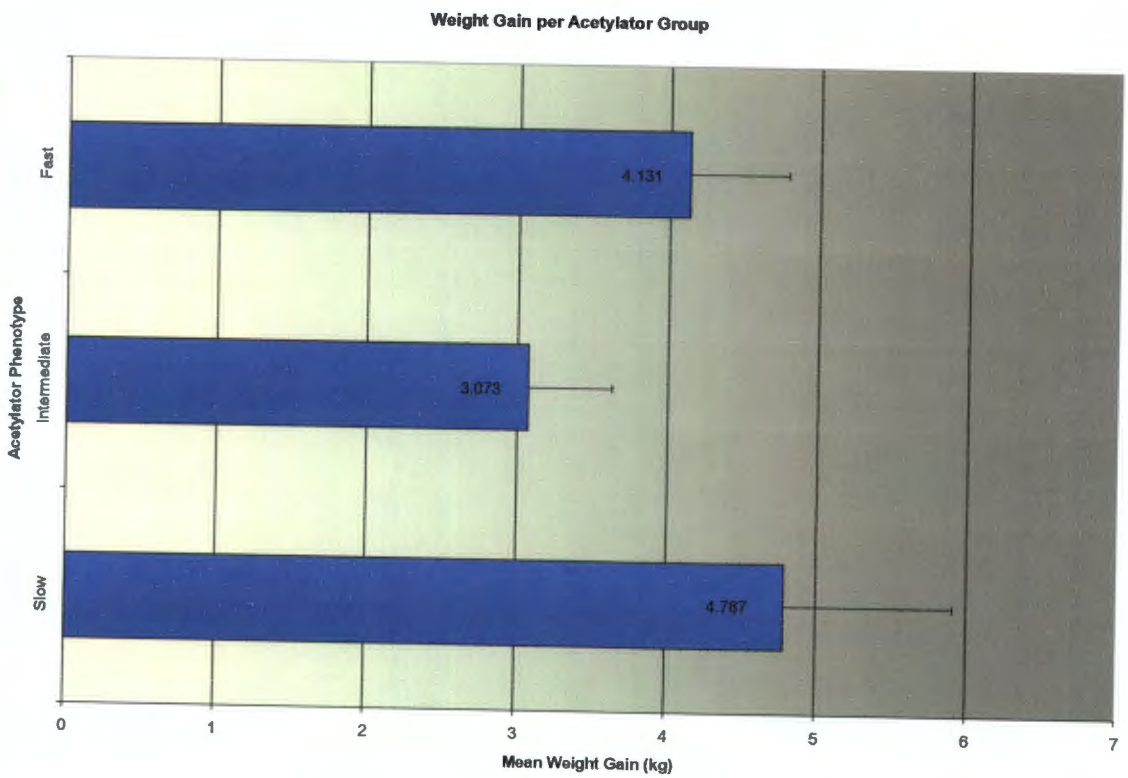


Figure 5.3d. The distribution of acetylator status in terms of weight.

When age was correlated with acetylator phenotype, there was no significant difference between the three acetylator groups. The mean ages for each group were 37.0, 37.0 and 37.57 years for slow, intermediate and rapid acetylators respectively.

Table 5.3i. Analysis of non-correlators for phenotyping confounders

ID	Age	Weight	AlkPhos	AST	ALT	Total Bilirubin	Pheno	Geno	G - P
3	24	37.9	45	23	15	5	I	F	↓
33	33	48.7	68	21	25	7	F	I	↑
36	35	45.4	55	14	11	6	F	I	↑
43	59	47	69	16	14	3	F	I	↑
47	30	47	63	19	23	5	I	F	↓
65	-	-	43	14	10	3	F	I	↑
74	18	43.9	92*	14	9	4	F	I	↑
81	41	51.8	59	16	7	3	S	I	↓
83	55	52	42	16	11	4	S	I	↓
94	35	34	194*	40*	48*	3	I	F	↓
99	44	60.3	47	10	10	11	F	I	↑
100	-	-	62	19	14	5	I	F	↓
111	50	41	81*	34*	48*	2	F	I	↑
119	49	62	38	22	19	8	I	F	↓

* Above normal limits

- Missing data

Although alkaline phosphatase levels were elevated in a significant number of patients, this elevation is not clinically significant. Guidelines state that only if levels are observed to be three times normal, is the asymptomatic patient at risk of hepatotoxicity.[105]

Concomitant medication may influence the phenotypic assignation of a subject. Drugs such as prednisolone and ethanol have been observed to increase acetylation rates in slow acetylators.[16] Only one patient was receiving prednisolone at the time of the sampling, patient 51, and she was classified as an intermediate acetylator, showing no discordance with the determined genotype. Alcohol abuse was not studied in this population, thus judging the effect of concomitant ethanol administration is not possible.

No correlation was found with any patient characteristics and genotype/phenotype discordance.

5.4. DISCUSSION

There are a significant number of individuals with mildly raised liver enzymes, but it is not a function of acetylator status, as demonstrated by the spread of cases between the three groups.

Having sampled a large group of heterogeneous individuals, acetylator status does not play a role in dictating the incidence of raised liver enzymes. However, the incidence of alcohol abuse is high in this population, and perhaps this is masking the results. Another effect of the high incidence of alcohol abuse in this population would be the effect that ethanol has on acetylator phenotyping tests. Alcohol has been found to increase the rate of sulphadimidine acetylation, causing slow acetylators to be misclassified as rapid.[16] Having not determined the incidence of alcohol abuse in this population, it is not possible to quantitate the influence of ethanol.

Using the data available, figure 5.3b. shows the distribution of acetylator status in each gender. From the graph, there is a higher prevalence of female rapid acetylators than male rapid acetylators and in contrast to this, a higher prevalence of slow male acetylators, compared to female slow acetylators. This trait is not sex-linked, it is found on chromosome 8.[75] However, it has been shown previously[106] that females exhibited increased incidence of raised liver enzyme levels. Whether this could be due to the increased incidence of rapid female acetylators, as shown in this study, remains undetermined.

Figure 5.3c. indicates that slower acetylators were seen to fail more often when clearing the sputum of viable *mycobacteria*. This would go against logic, as it is known that only the parent drug, isoniazid, is active against the bacterium. Slow acetylators would be subjected to a significantly prolonged exposure to this drug, thus benefiting more in terms of drug action. Compliance would also be a confounding factor, even within a hospital population.

Weight gain (or change in weight) was not related in any way to the individual's acetylator status using a non-parametric Spearman correlation (figure 5.3d).

For the pharmacokinetic study, a set of markers was decided upon to gauge response to treatment after two months of therapy. These included weight gain, sputum clearance (direct and culture), chest x-ray improvement and symptom alleviation. Using these as guides after this initial treatment, there is no correlation

between treatment response and acetylator phenotype. After a follow-up study, this may be possible, the only way to accurately assess outcome being after the completion of treatment.

These markers do not serve as absolute indicators, because there are too many confounding factors. With weight gain, it would be difficult to assess this without considering the background of the patient. Receiving three full meals a day might not be possible at home, and this may outweigh the effect of the drug on clearing the symptoms.

The role of concomitant medication may play a role in the discordance of phenotype with genotype. Prednisolone has been observed to increase the rate of acetylation in slow acetylators, leading to apparent discordance.[16]

Upon analysing the non-correlators (Table 5.3i.), and looking for confounders of phenotypic/genotypic correlation, none of the factors analysed stood out as contributing factors to non-correlation. The ages and weights were evenly spread in terms of the entire dataset, and only one subject had overtly raised liver enzymes, indicating impaired liver function, which would cause rapid acetylators to be classified as slower acetylators.

Many reasons could be cited for the discordance of these individuals. One could be that they do have slightly impaired liver function, impairing the ability of the liver to metabolise isoniazid, thus phenotypically expressing as a slower acetylator. But raised liver enzymes should indicate this. As mentioned previously, the allele S1c has not been assayed for owing to the rarity of it in this population [personal comm with C. Werely], but this could be contributing to a reduced phenotype compared to the genotype. Possibilities exist that would include new genetic alleles, but this should be investigated further.

5.5. FINAL CONCLUSIONS

Acetylator status played no role in determining the incidence of raised liver enzyme levels in this population. The incidence of toxicity was not age-related, nor did it correlate with the weight gain observed after the first two months of drug therapy. There was a difference in the distribution of phenotype with respect to gender, female patients in general exhibiting a faster phenotype than males. Sputum clearance showed an insignificant trend towards favouring rapid acetylators at two months.

No explanation in terms of patient related characteristics could be offered for the discordance of phenotype and genotype. This raises suspicion that there may be new allelic variants present in this population, responsible for reduced acetylation. Further genetic analysis (sequencing) is needed for this to be confirmed.

STUDY CONCLUSIONS

DEVELOPMENT

A plasma-based, high performance liquid chromatographic assay has been developed to measure the levels of isoniazid and *N*-acetylisoniazid in patients with tuberculosis. The assay utilises a simple solid phase extraction, simultaneously extracting both isoniazid and *N*-acetylisoniazid, before resolving the peaks using a C8 column on HPLC.

The assay has been validated in terms of its quality and has been determined to be specific, accurate, precise and linear, with detection limits of 300 ng/ml and 150 ng/ml for isoniazid and *N*-acetylisoniazid respectively. The recovery is good and consistent, in excess of 70% for isoniazid and 90% for *N*-acetylisoniazid. The coefficients of variation were maintained below 10% and the coefficients of determination (r^2) above 0.990.

The result is a timesaving and effective assay, suitable for high volume pharmacokinetic analysis.

IMPLEMENTATION

In determining markers for acetylator phenotype, the ratio of AcINH:INH at three hours proved to be the most accurate, as well as the most convenient, displaying a trimodal distribution.

Statistical analysis of the resulting pharmacokinetics revealed significant separation of the slow acetylators from the intermediate and rapid acetylators in all cases, with separation the latter two groups only evident in AUCT and AUCINF. Cmax and half-life were bimodally distributed, failing to draw significance between the intermediate and rapid acetylators. Area under the curve (total and to infinity) was conclusively trimodal, significant difference being shown between all three phenotypes. Of concern is the significant reduction in AUC experienced by rapid acetylators, as well as the relative risk of rapid acetylators to fail to achieve therapeutic levels of isoniazid, in this population even while dosing for weight. Analysis of the elimination rate constant for isoniazid in each individual indicated the codominance of the alleles,

in that the prediction of the intermediate mean rate constant could be made within 7% of the observed mean.

VALIDATION

Genotypic analysis served to support the phenotypic criteria for acetylator status determination, as well as the assay development. The assay was developed to determine plasma levels of isoniazid and *N*-acetylisoniazid, which were used to determine the phenotypic characteristics of acetylator status in patients with tuberculosis.

A correlation of 88.2% was observed between phenotype and genotype. Discordance could be due to patient characteristics or the evolution of new allelic variants. Further investigation into DNA sequencing is required. The distribution of alleles correlated closely to that predicted by the Hardy-Weinberg equation, proving the population to be a heterogeneous sampling of a well-defined gene pool.

ACETYLATOR STATUS INFLUENCE

Various patient factors were investigated in terms of acetylator status. It was found that acetylator status played no role in determining the incidence of liver toxicity. Acetylator status was not age-related, nor did it influence the weight gain of patients within the first two months of treatment. There was a difference in the distribution of phenotype with respect to gender, female patients in general, exhibiting a faster phenotype than male patients. Sputum clearance showed an insignificant trend towards favouring rapid acetylators after two months of therapy.

No explanation could be offered for the discordance of phenotype with genotype, in terms of patient characteristics, confirming the suspicion that the discordance could be related to new allelic variants within this population, requiring DNA sequencing.

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APPENDICES

I. PATIENT CONSENT FORMS AND DATA COLLECTION

II. INDIVIDUAL PATIENT PHARMACOKINETIC CURVES

III. PHARMACOKINETIC DATA FOR ISONIAZID AND *N*-ACETYLISONIAZID

IV. ELIMINATION AND FORMATION RATE CONSTANTS

V. INDIVIDUAL PATIENT DETAILS

APPENDIX I

PATIENT CONSENT FORMS AND DATA COLLECTION

Subject no._____

CONSENT TO PARTICIPATE IN A CLINICAL STUDY
PHARMACOKINETICS OF ANTITUBERCULOSIS DRUGS IN PATIENTS RESPONDING POORLY TO THERAPY.

I agree to take part in the above-mentioned study.
I understand that the study is to improve the understanding of how drug levels, tuberculosis sensitivity to drug levels and other individual patient factors influence the effectiveness of TB treatment.

I understand that the study involves the documentation of personal and treatment details, a physical examination, collection of blood samples (about 25ml of blood), a chest x-ray and sputum collection. Laboratory evaluation of the blood will include testing for the HIV virus, haematological, biochemical and immunological status.

In addition, on one or three occasions: I will fast overnight before serial blood samples totalling about 110ml will be taken over a period of eight hours after the swallowing of my medication the next day. A cannula will be inserted into an arm vein for the collection of these blood samples. Three, 2hour urine collections will also be collected during the 8 hour period(s).

I understand that the study also involves the collection of a sputum samples at 2 months and at 6, 9, 12, 18 and 24 months after the initiation of treatment at Brewelskloof Hospital. I understand that I am obliged to present at the clinic to which I am referred at the required times.

I understand that participation in the study does not hold any treatment benefit.
I have been given the opportunity to ask any questions concerning the study and am satisfied with the explanation or advice given.
I have truthfully answered all the questions necessary to complete the data collection forms.
I agree to comply with the instructions given to me by the investigators or their assistants.
I understand that the information collected will be treated confidentially, and that only the investigators and their assistants will be able to link any of the information collected to my name.
I understand that I am free to withdraw from the study at any time without prejudice to me for doing so.

I am aware that this study has been subjected to review by an ethics committee.

_____	_____	_____
NAME OF SUBJECT	SIGNATURE	DATE
_____	_____	_____
NAME OF INVESTIGATOR	SIGNATURE	DATE

Subject no.:_____

TB PROGRESS RATING AT 2 MONTHS **TOTAL SCORE: /20**

1. SPUTUM **SCORE: /8**

Direct sputum microscopy

Initial / 2 months	+++	++	+	Scanty
+++	0	0	0	0
++	0	0	0	0
+	1	0	0	0
Scanty	2	1	0	0
-	4	4	4	4

Culture

Initial / 2 months	1-7 days	>7-14 days	>14-21 days	>21 days
1-7 days	0	0	0	0
>7-14 days	0	0	0	0
>14-21 days	1	0	0	0
>21 days	2	1	0	0
Negative	4	4	4	4

If only direct microscopy done; multiply score by 2
If only culture cone; multiply score by 2
If initial direct microscopy was negative, use culture score and multiply by 2
If smear and culture done; add the scores
If smear remains positive, but culture is negative at 2 months; allocate a score of 8

2. CHEST X-RAY **SCORE: /6**

chest x-ray on admission is compared with that after 7 to 8 weeks of DOT as an in-patient and assessed by 3 Medical Officers independently.

Unchanged or worse	0
Some improvement	2
Obvious improvement	4
Resolved	6

3. WEIGHT **SCORE: /4**

weight on admission is compared with that after 7 to 8 weeks of DOT as an in-patient.

Decreased, static or increased <1kg	0
Increase < 1kg up to 10%	2
Increase > 10% up to 15%	3
Increase > 15%	4

4. SYMPTOMS: **SCORE (max 2): /2**

symptoms on admission are compared with those after 7 to 8 weeks of DOT as an in-patient.

	No improvement	**Some improvement**	**Markedly improved or resolved**
Cough/ Dyspnoea/ Chest pain/ Night sweats/ Appetite/ Other	0	1	2

Subject no:		**Date:**	
Ward:		**Investigator:**	
Folder number:		**Date of admission:**	

Name:

Age: **Sex:** **male / female**

Race: **A** | **B** | **C** | **W** | **Other**

Home address:

Place of birth:

ELIGIBILITY:

Pulmonary tuberculosis confirmed by positive sputum for M. tuberculosis: direct microscopy or culture

Informed consent

consent to HIV testing

≥ 18 years

On daily inpatient treatment with rifampicin, isoniazid, pyrazinamide, +/- ethambutol, +/- an aminoglycoside for 8 weeks at Brewelskloof Hospital.

not critically ill and no contraindications to multiple blood sampling

no known resistance to isoniazid or rifampicin

Subject no. _____

PK SAMPLING / ADVERSE EVENTS FORM

DATE of PK sampling: / /

Drug administration:

Drug	Dose	Exact admini-stration time	Formulation / brand	Batch no.	Exp. date
Rifampicin					
Isoniazid					
Pyrazinamide					
Ethambutol					
Notes:					

Blood sampling times:

Sample	**Hrs after drug administration**	**Exact time of sampling (hh:mm)**
0	**Pre-drug sample**	
1	**0.5**	
2	**1.0**	
3	**1.5**	
4	**2.0**	
5	**2.5**	
6	**3.0**	
7	**4.0**	
8	**6.0**	
9	**8.0**	

APPENDIX II

INDIVIDUAL PATIENT PHARMACOKINETIC CURVES

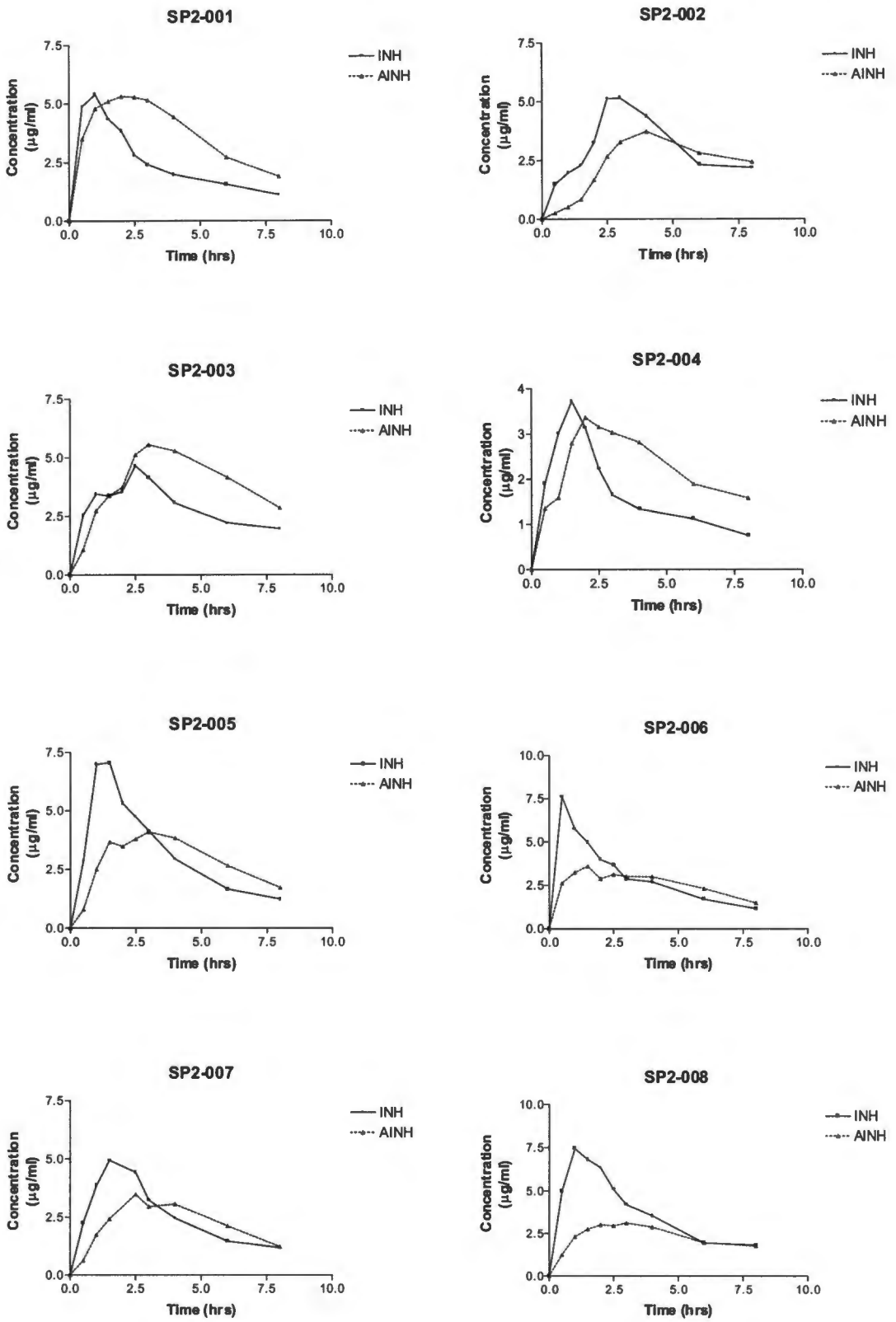

Figure IIa. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 1 - 8

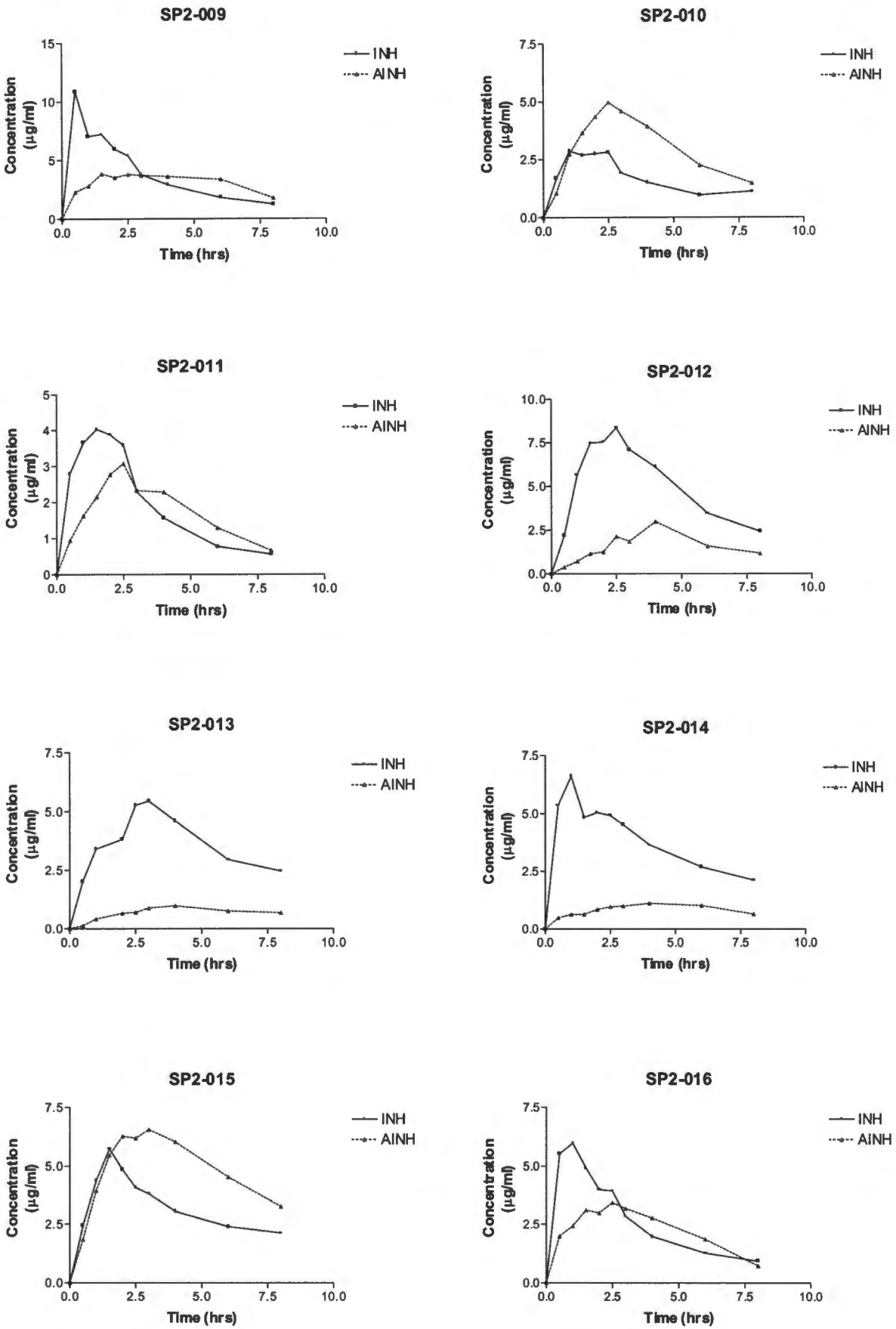

Figure IIb. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 9-16

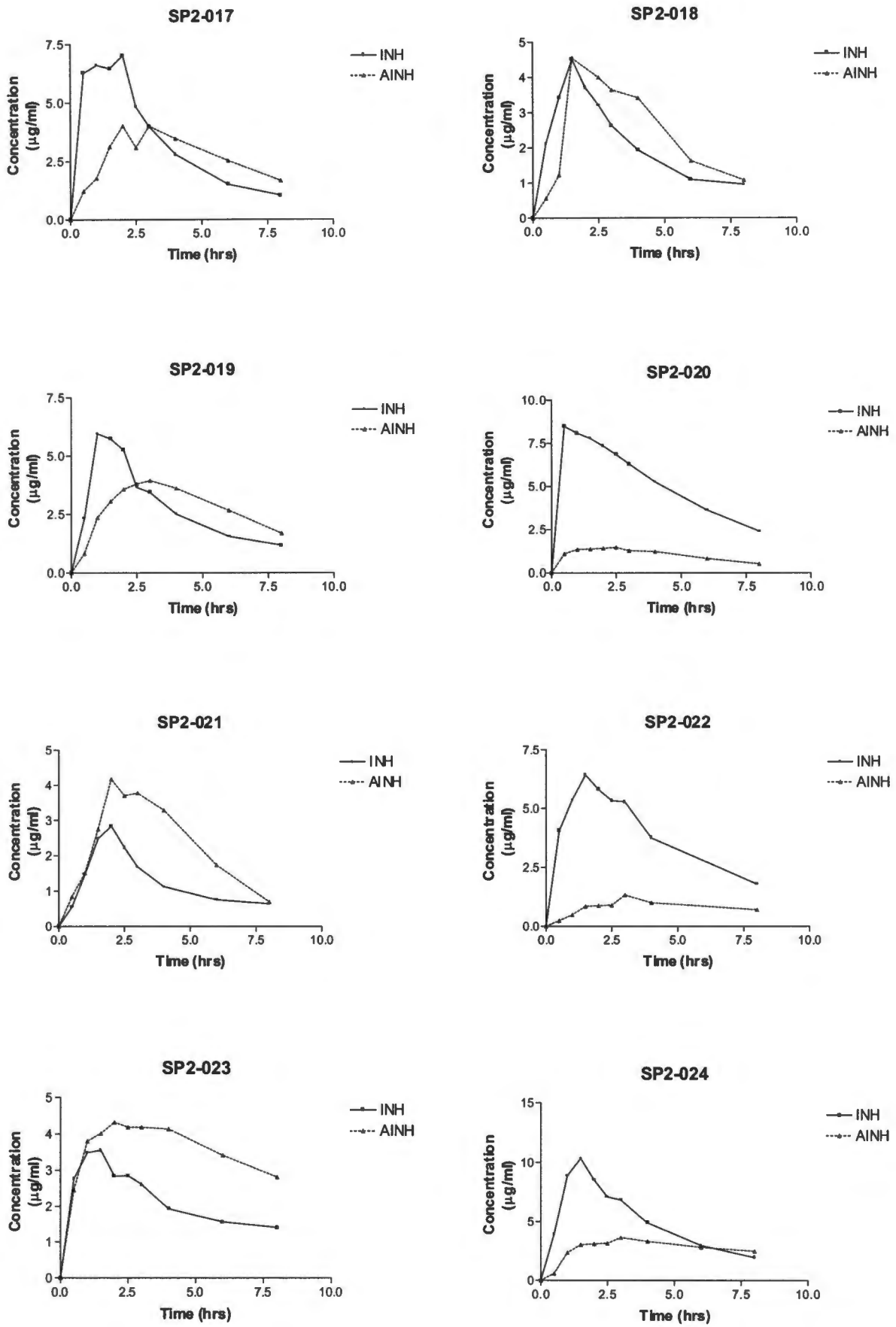

Figure IIc. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 17-24

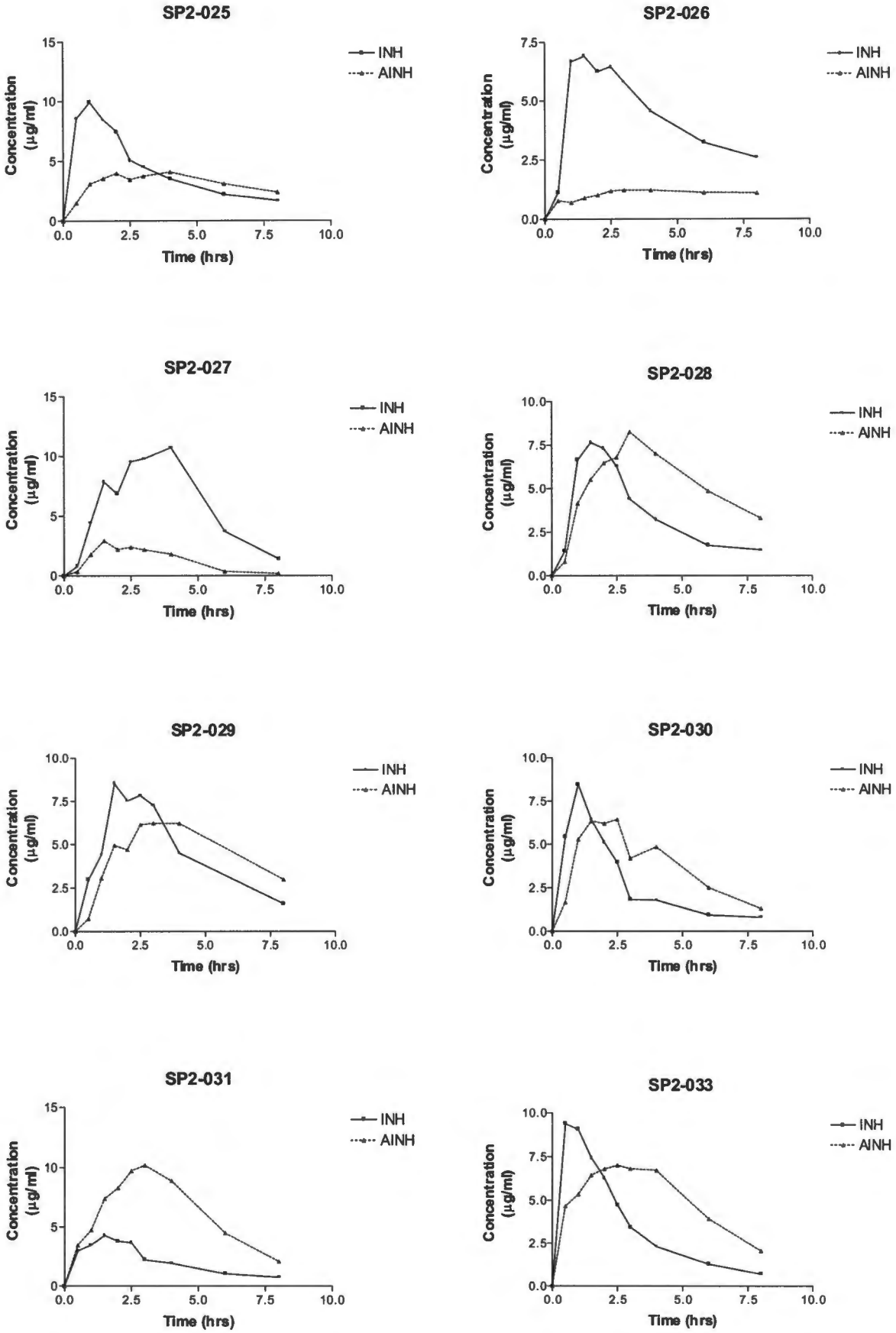

Figure IId. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 25-33

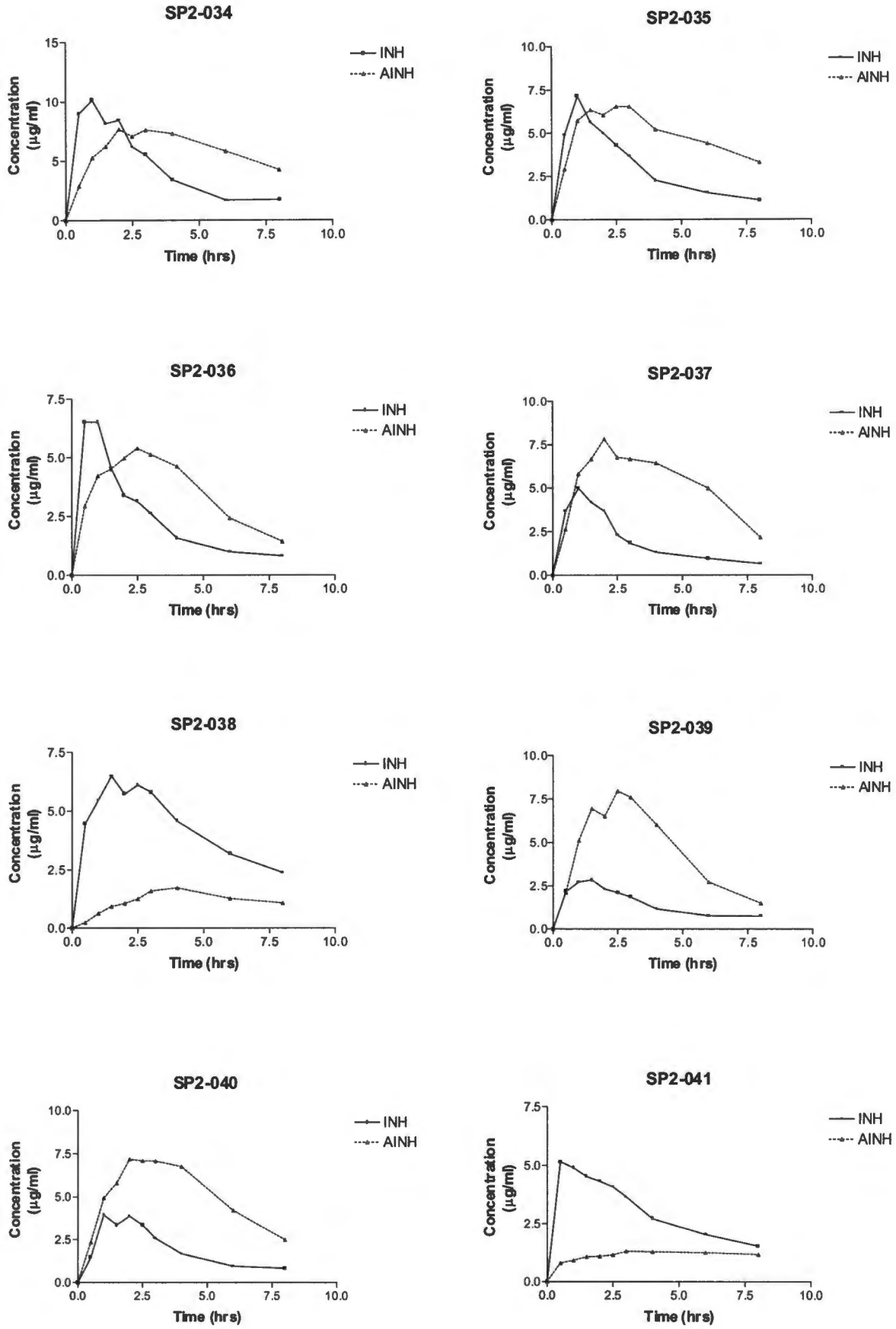

Figure IIe. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 34-41

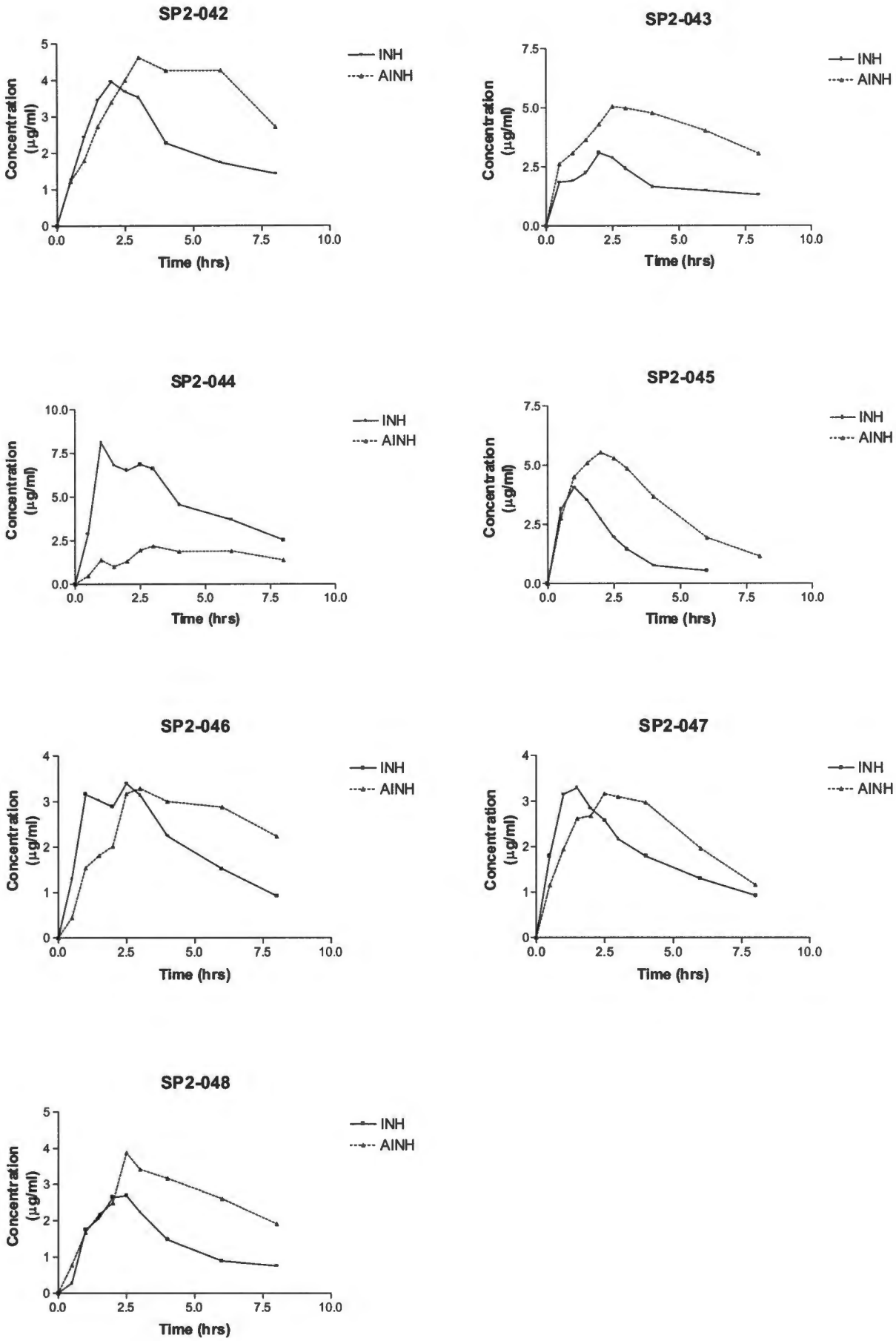

Figure IIf. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 42-49

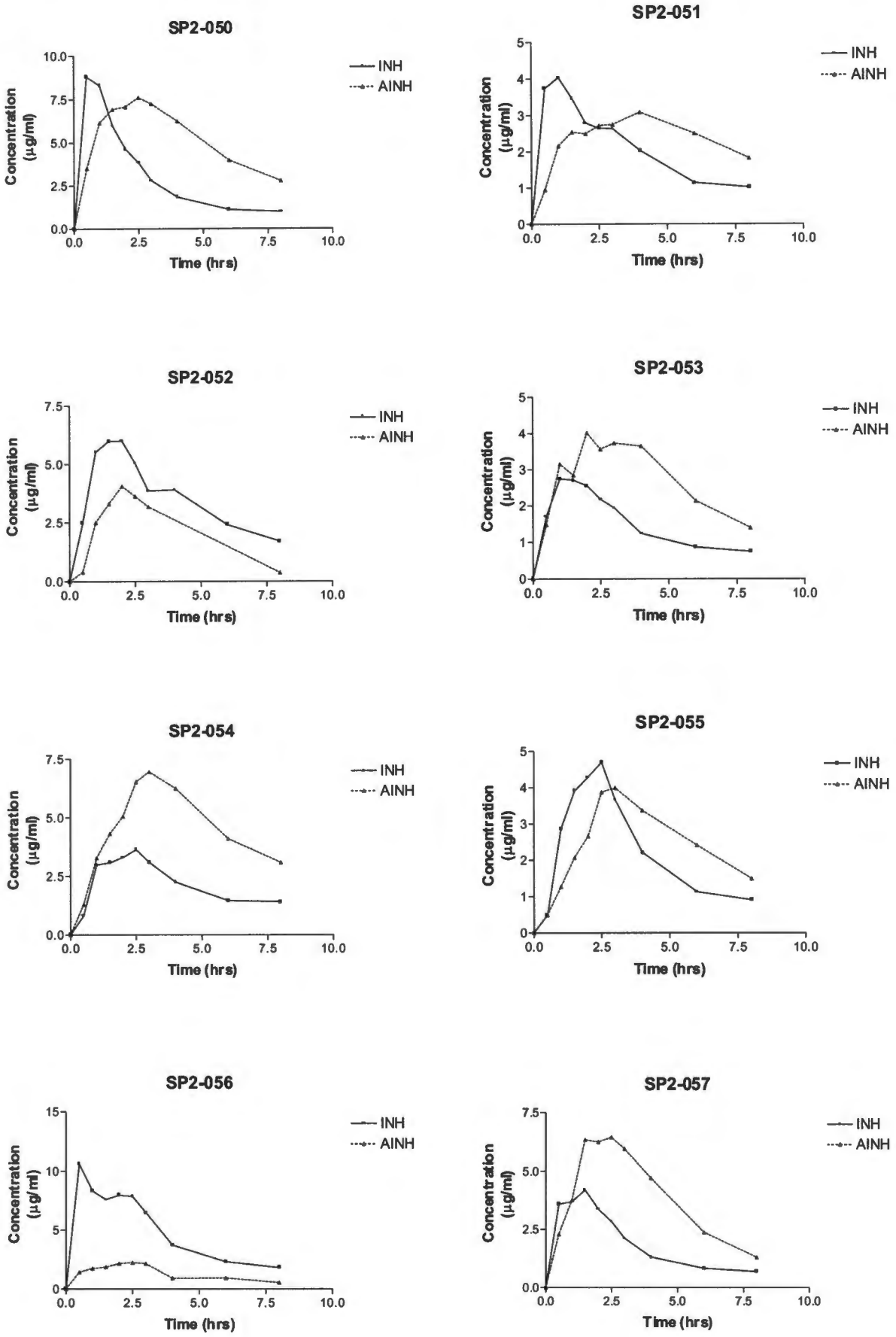

Figure IIg. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 50-57

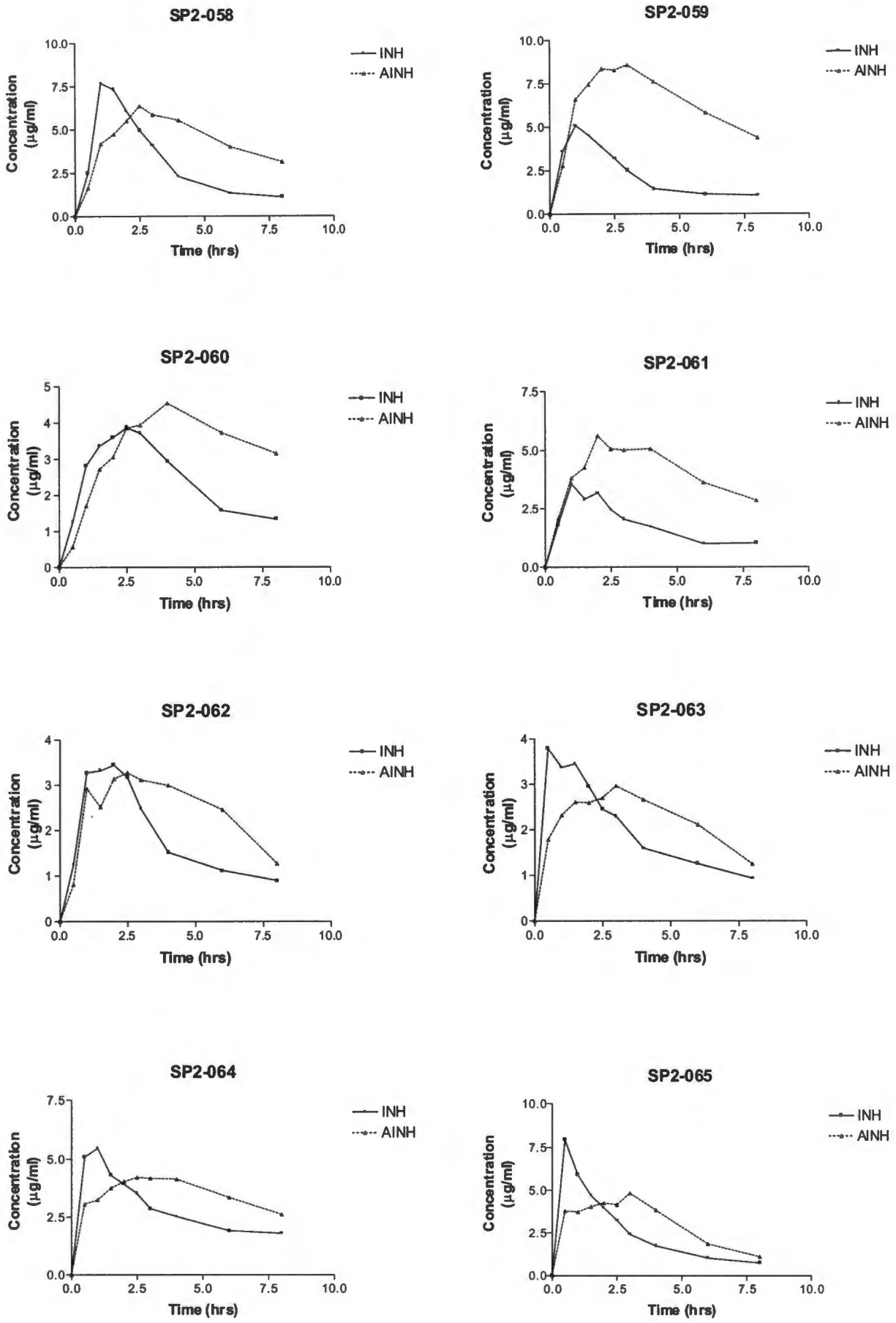

Figure IIh. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 58-65

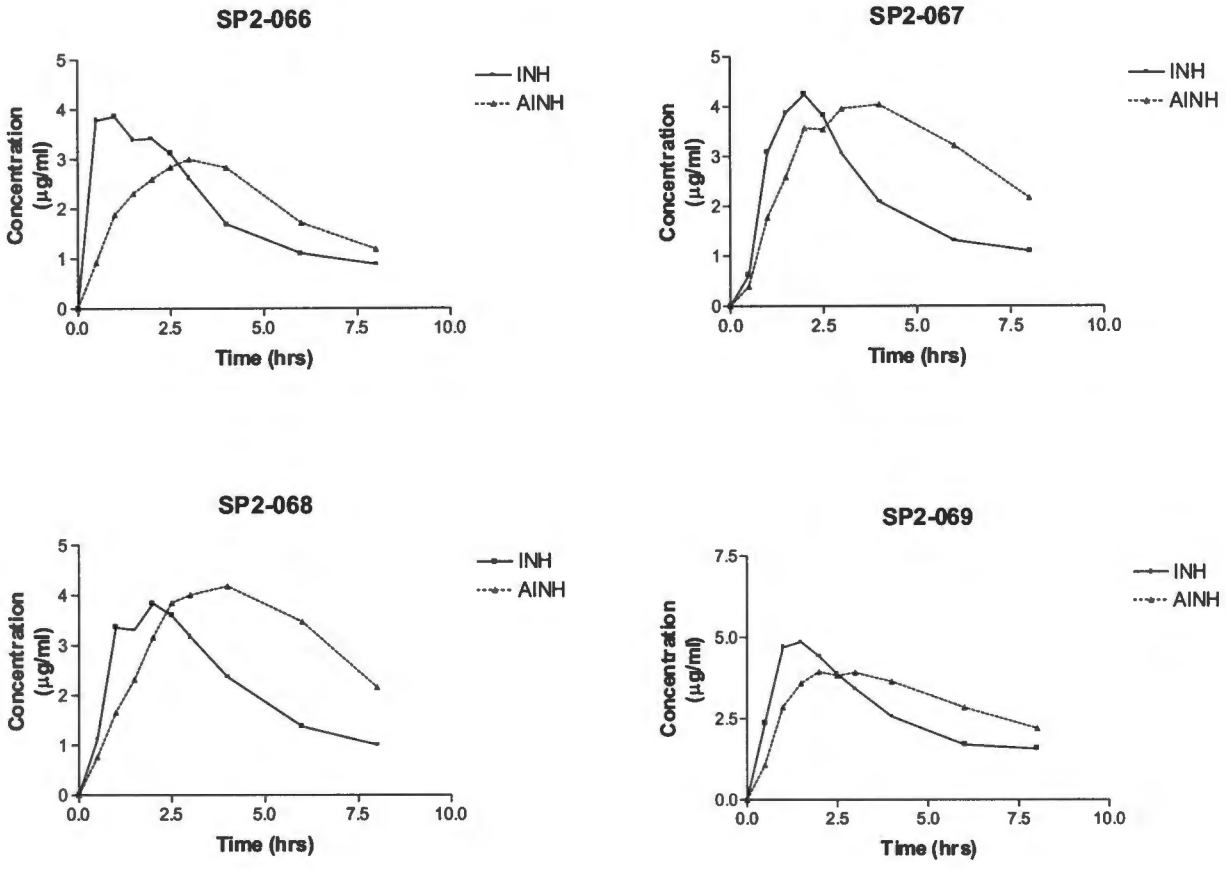

Figure IIi. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 66-69

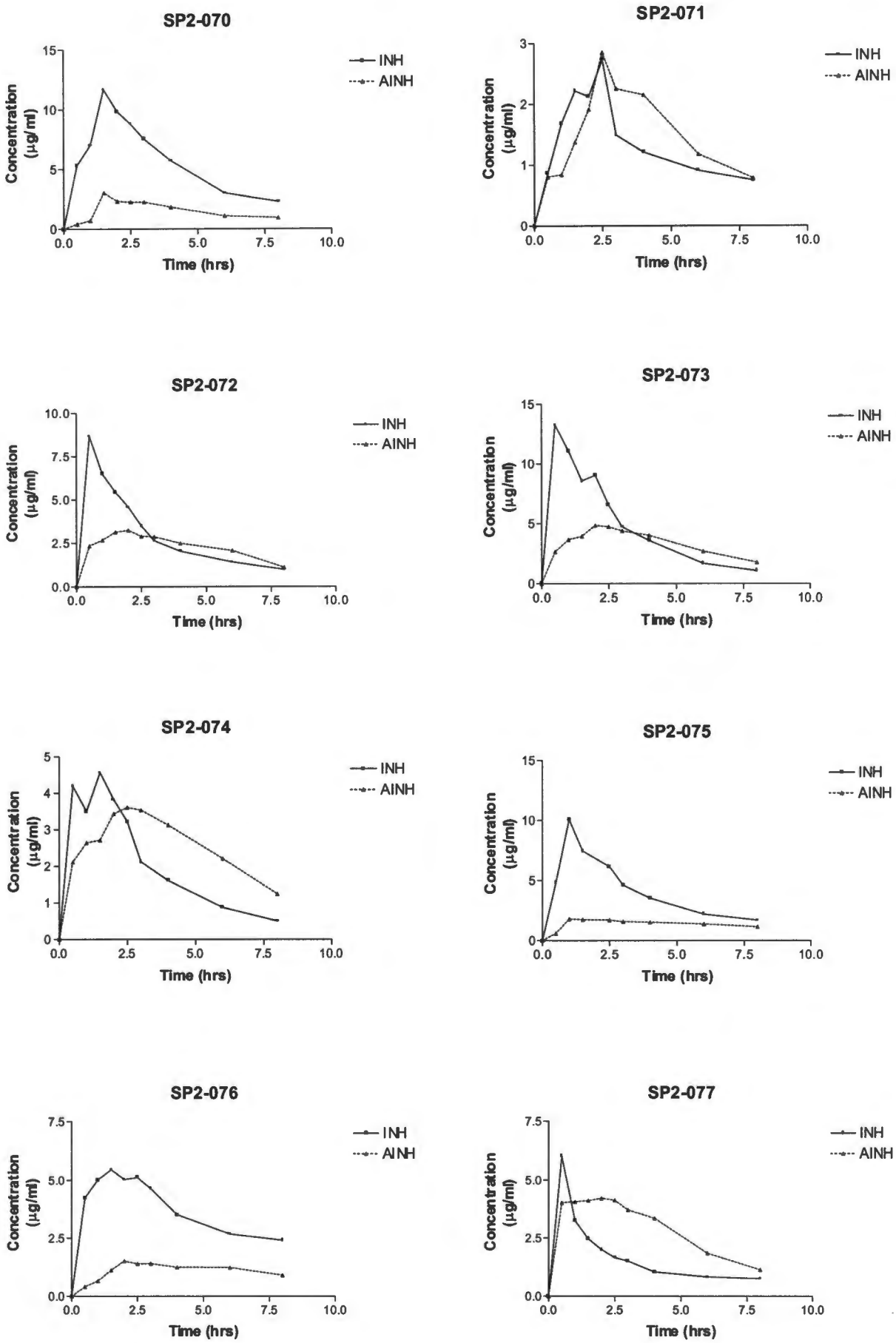

Figure IIj. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 70-77

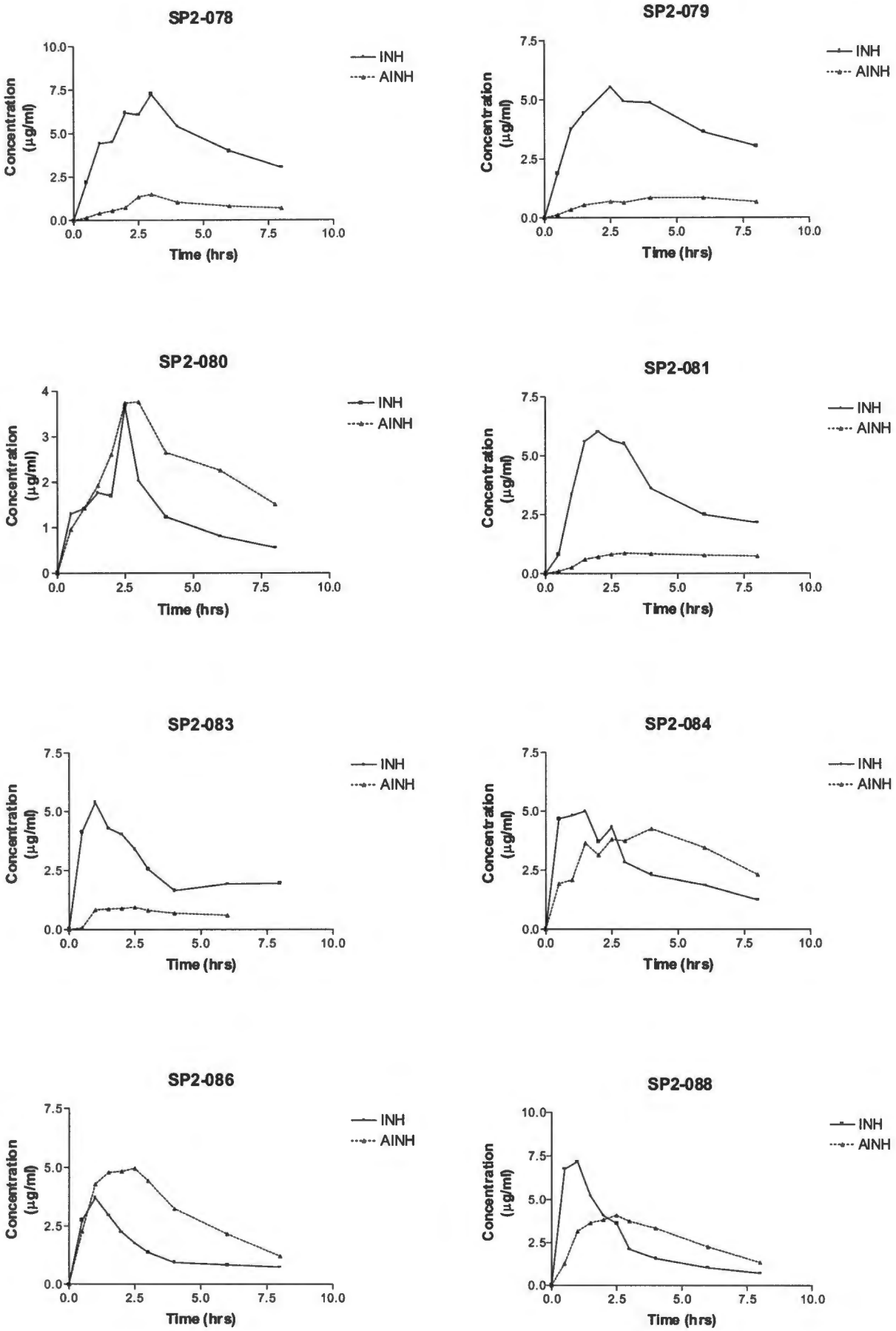

Figure IIk. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 78-88

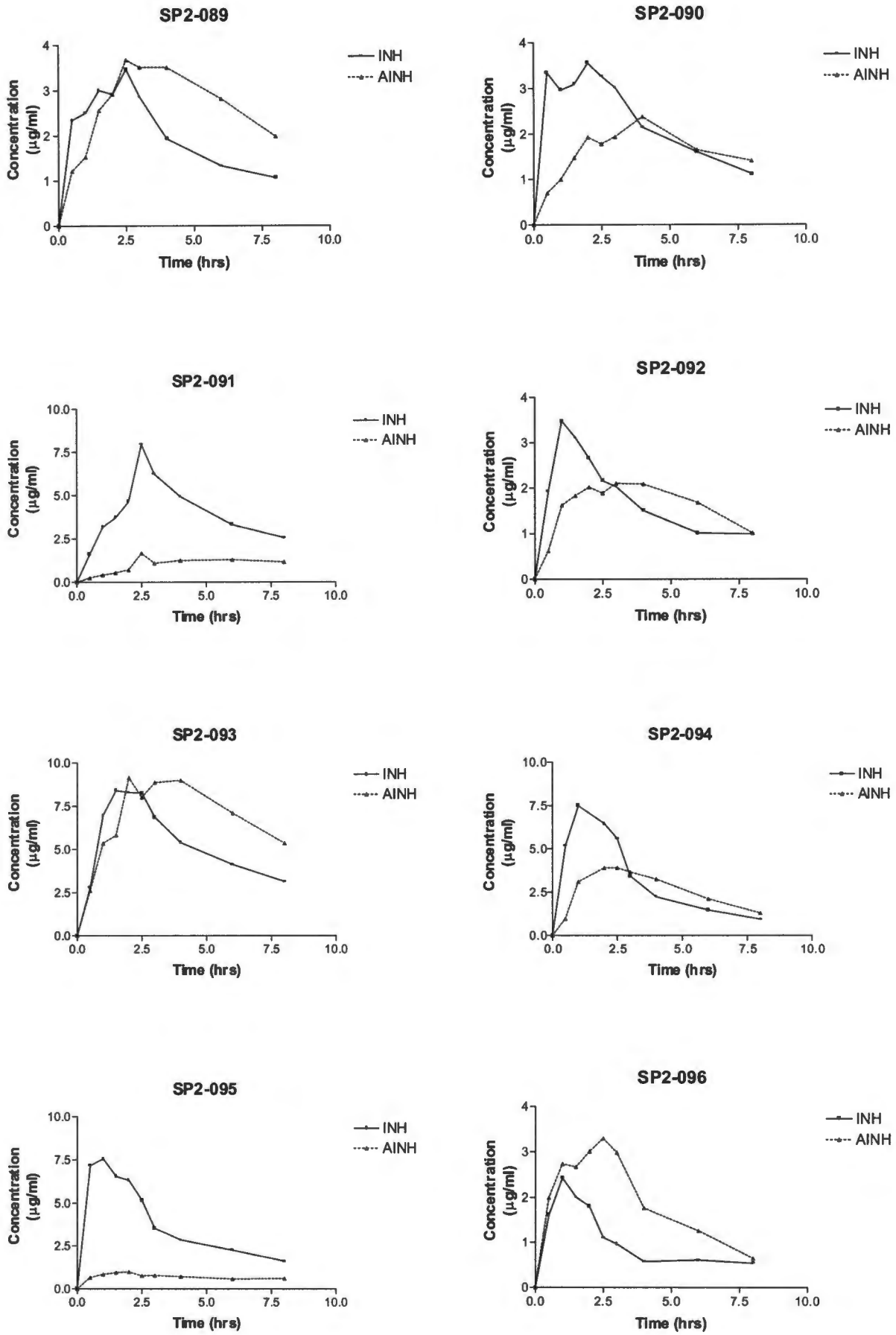

Figure III. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 89-96

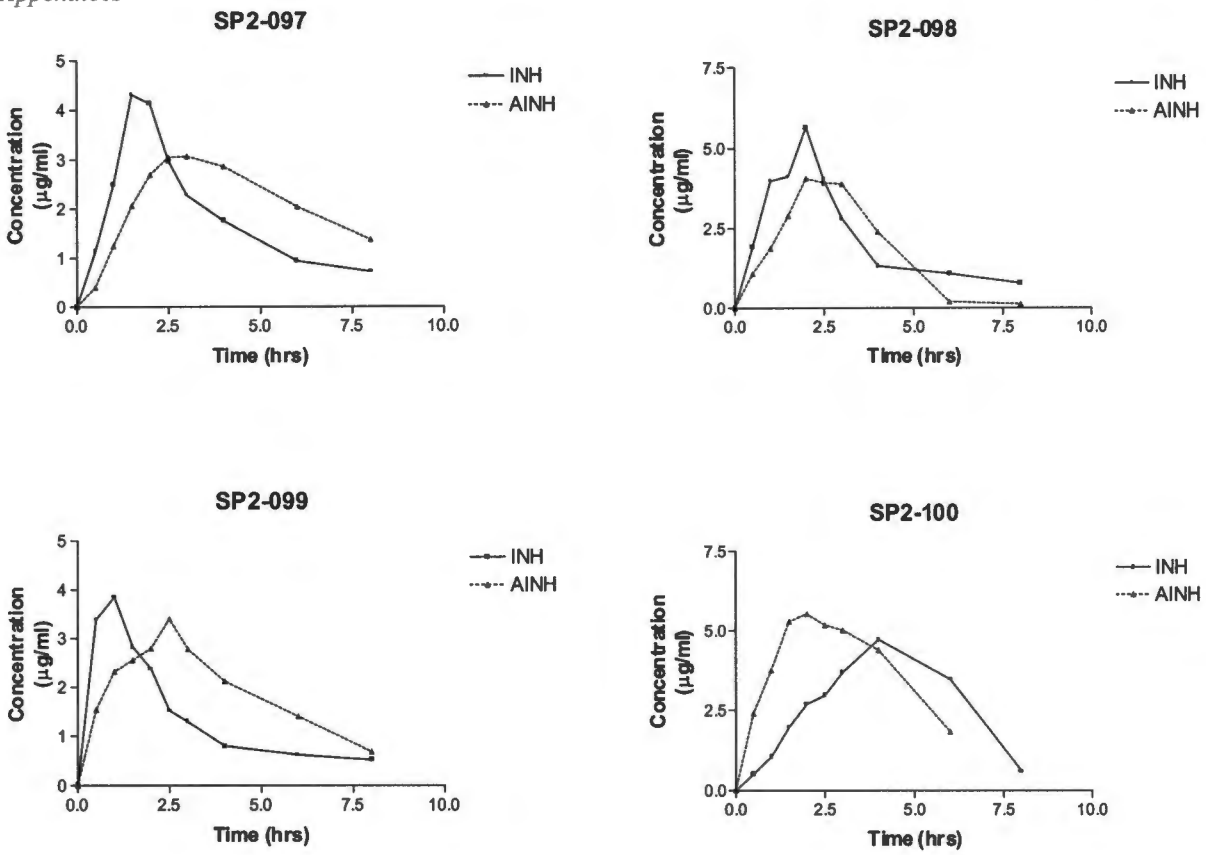

Figure IIm. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 97-100

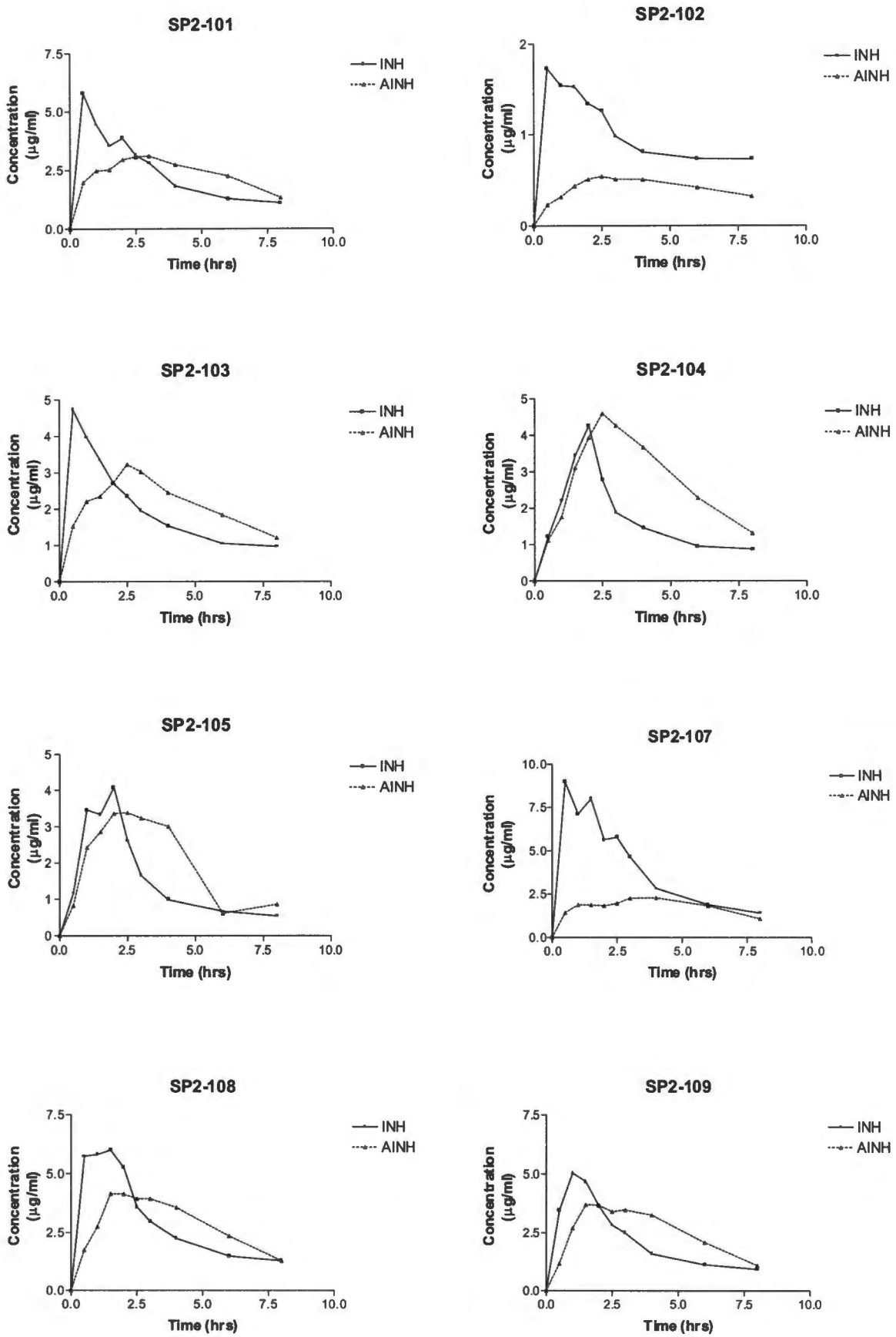

Figure IIn. Pharmacokinetic Profiles for isoniazid and *N*-Acetylisoniazid: Patients 101-109

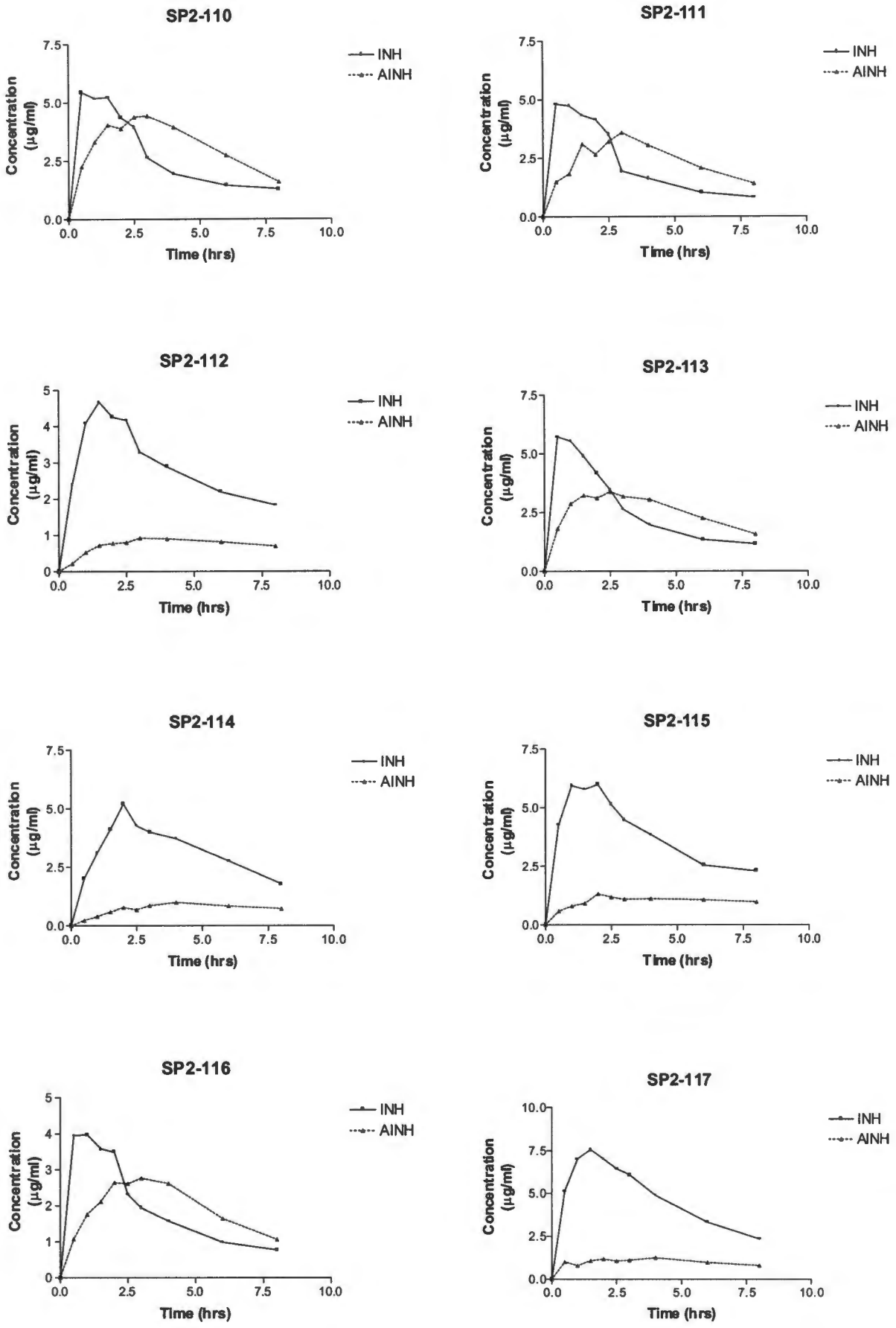

Figure IIo. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 110-117

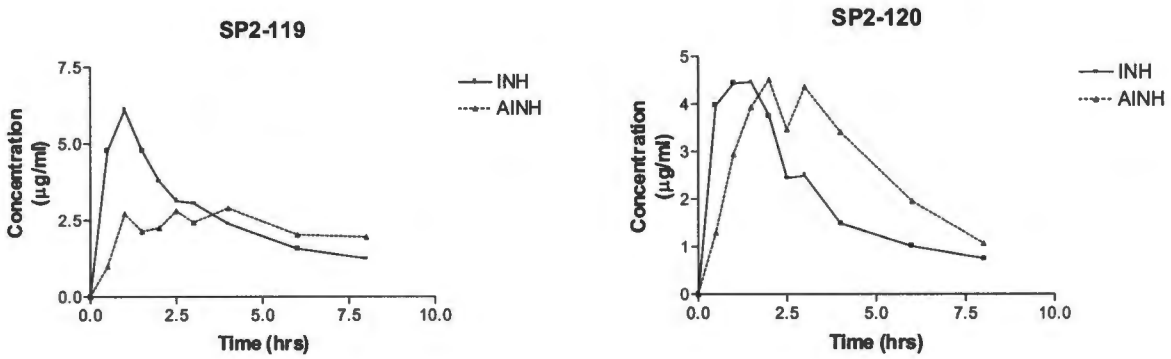

Figure IIp. Pharmacokinetic profiles for Isoniazid and *N*-Acetylisoniazid: Patients 119-120

APPENDIX III

PHARMACOKINETIC DATA FOR ISONIAZID AND *N*-ACETYLISONIAZID

NCA Data - [C:\WINNONLN\INHFIN.WDO]
Final Parameters

Subject	Rsq	Corr(x:y)	Tmax	Cmax	No. points Lambda z	AUClast	Lambda z	t1/2 Lambda z	AUCINF(observed)
1	0.9936	-0.9968	1.0000	5.3887	4.0000	19.6735	0.1507	4.5985	27.0353
2	0.9164	-0.9573	3.0000	5.1442	5.0000	24.3641	0.1795	3.8622	36.5761
3	0.9329	-0.9658	2.5000	4.6536	5.0000	22.8908	0.1599	4.3347	35.1806
4	0.9758	-0.9878	1.5000	3.7231	4.0000	13.2393	0.1474	4.7041	18.3669
5	0.9814	-0.9906	1.5000	7.0659	8.0000	25.5272	0.2662	2.6042	30.1817
6	0.9970	-0.9985	0.5000	7.6100	3.0000	23.7627	0.2113	3.2808	29.2623
7	0.9596	-0.9796	1.5000	4.9407	6.0000	20.3875	0.2343	2.9589	25.5071
8	0.9542	-0.9768	1.0000	7.4252	8.0000	29.5482	0.2209	3.1372	37.8221
9	0.9935	-0.9968	0.5000	10.9152	4.0000	30.2957	0.2200	3.1502	36.0250
10	0.8459	-0.9197	1.0000	2.8605	8.0000	13.1934	0.1684	4.1171	19.9009
11	0.9654	-0.9825	1.5000	4.0330	7.0000	15.1711	0.3325	2.0845	16.8660
12	0.9918	-0.9959	2.5000	8.3571	5.0000	39.5046	0.2271	3.0522	50.2269
13	0.9658	-0.9827	3.0000	5.4547	5.0000	28.4691	0.1553	4.4644	44.3452
14	0.9947	-0.9974	1.0000	6.6103	3.0000	29.7932	0.1367	5.0688	45.2848
15	0.9515	-0.9754	1.5000	5.7446	5.0000	25.0846	0.1205	5.7514	42.7675
16	0.9913	-0.9957	1.0000	5.9724	3.0000	20.7458	0.1900	3.6488	25.6216
17	0.9845	-0.9922	2.0000	7.0282	5.0000	26.8769	0.2814	2.4634	30.5872
18	0.9553	-0.9774	1.5000	4.5148	7.0000	16.5327	0.2483	2.7919	20.3971
19	0.9837	-0.9918	1.0000	5.9599	5.0000	22.1087	0.2168	3.1966	27.4998
20	0.9991	-0.9996	0.5000	8.4863	5.0000	41.5722	0.1898	3.6519	54.2711
21	0.9218	-0.9601	2.0000	2.8392	7.0000	9.9108	0.2404	2.8827	12.5891
22	0.9899	-0.9949	1.5000	6.4308	6.0000	30.4446	0.1987	3.4891	39.4995
23	0.9386	-0.9688	1.5000	3.5580	8.0000	17.1625	0.1419	4.8836	27.1102
24	0.9979	-0.9989	1.5000	10.3290	3.0000	39.8010	0.2337	2.9657	48.0434
25	0.9862	-0.9931	1.0000	9.9238	5.0000	34.5300	0.2052	3.3783	42.7565
26	0.9816	-0.9908	1.5000	6.9087	7.0000	33.9769	0.1593	4.3522	50.2804
27	0.9992	-0.9996	4.0000	10.7368	3.0000	47.0639	0.5050	1.3725	49.8836
28	0.9496	-0.9745	1.5000	7.6503	7.0000	27.7375	0.2788	2.4857	33.0317
29	0.9884	-0.9942	1.5000	8.5263	4.0000	35.5836	0.2911	2.3810	41.0984
30	0.9139	-0.9560	1.0000	8.4830	4.0000	21.4307	0.1847	3.7538	25.7972
31	0.9803	-0.9901	1.5000	4.2883	4.0000	16.5669	0.2278	3.0423	19.8887
33	0.9999	-0.9999	0.5000	9.3776	3.0000	27.7873	0.2990	2.3184	30.1307
34	0.9304	-0.9646	1.0000	10.1407	9.0000	35.3352	0.2718	2.5504	41.7245
35	0.9970	-0.9985	1.0000	7.1503	3.0000	23.8394	0.1763	3.9313	30.1876

NCA Data - [C:\WINNONLN\INHFIN.WDO]
Final Parameters

Subject	Rsq	Corr(x:y)	Tmax	Cmax	No. points Lambda z	AUClast	Lambda z	t1/2 Lambda z	AUCINF(observed)
36	0.9432	-0.9712	0.5000	6.5205	9.0000	19.1835	0.2990	2.3185	21.9429
37	0.9970	-0.9985	1.0000	4.9756	3.0000	15.3267	0.1707	4.0596	19.1995
38	0.9934	-0.9967	1.5000	6.4888	5.0000	34.0990	0.1754	3.9510	47.7432
39	0.9243	-0.9614	1.5000	2.8561	8.0000	11.4812	0.2186	3.1709	14.8863
40	0.9413	-0.9702	1.0000	3.9474	8.0000	15.1826	0.2532	2.7373	18.4687
41	0.9999	-0.9999	0.5000	5.1593	3.0000	23.9375	0.1433	4.8375	34.6527
42	0.9907	-0.9953	2.0000	3.9665	3.0000	18.3272	0.1156	5.9978	30.6759
43	0.9966	-0.9983	2.0000	3.0935	3.0000	14.5638	0.0599	11.5642	36.2782
44	0.9698	-0.9848	1.0000	8.1111	5.0000	37.3344	0.1796	3.8583	51.4705
45	0.9473	-0.9733	1.0000	4.0809	7.0000	10.4650	0.4388	1.5797	11.6953
46	0.9950	-0.9975	2.5000	3.3985	5.0000	16.5570	0.2378	2.9150	20.4151
47	0.9999	-1.0000	1.5000	3.2941	3.0000	14.6662	0.1638	4.2318	20.3393
48	0.9489	-0.9741	2.5000	2.6867	6.0000	11.1364	0.2334	2.9694	14.3648
49	0.9992	-0.9996	1.0000	3.2379	4.0000	15.3472	0.1501	4.6184	22.8851
50	0.9299	-0.9643	0.5000	7.5890	9.0000	20.5733	0.3192	2.1718	23.2354
51	0.9647	-0.9822	1.0000	3.4850	9.0000	14.4028	0.1974	3.5111	18.8443
52	0.9911	-0.9955	2.0000	5.1749	3.0000	23.9563	0.2066	3.3549	31.0741
53	0.9502	-0.9748	1.0000	2.3713	8.0000	10.1437	0.2119	3.2706	13.1941
54	0.9073	-0.9525	2.5000	3.1354	6.0000	14.6237	0.1708	4.0582	21.7738
55	0.9476	-0.9735	2.5000	4.0549	6.0000	14.9997	0.2949	2.3505	17.6716
56	0.9520	-0.9757	0.5000	9.1423	9.0000	32.8220	0.2486	2.7882	39.1079
57	0.9380	-0.9685	1.5000	3.6322	8.0000	12.6975	0.2828	2.4512	14.7828
58	0.9552	-0.9773	1.0000	6.6341	8.0000	21.1754	0.3101	2.2352	24.2123
59	0.8910	-0.9439	1.0000	4.3935	8.0000	15.1372	0.2457	2.8208	18.8591
60	0.9590	-0.9793	2.5000	3.3330	5.0000	16.5048	0.2136	3.2450	21.8630
61	0.9234	-0.9609	1.0000	3.0778	8.0000	12.2215	0.1950	3.5540	16.7735
62	0.9918	-0.9959	2.0000	3.4389	3.0000	14.4874	0.1315	5.2691	21.3051
63	0.9970	-0.9985	0.5000	3.7896	3.0000	15.5655	0.1336	5.1901	22.5721
64	0.9192	-0.9587	1.0000	5.4784	9.0000	22.7479	0.1573	4.4070	34.1670
65	0.9817	-0.9908	0.5000	7.9556	4.0000	20.1115	0.2364	2.9318	23.2399
66	0.9654	-0.9826	1.0000	3.8597	8.0000	16.3546	0.2308	3.0034	20.1761
67	0.9483	-0.9738	2.0000	4.2376	7.0000	16.9521	0.2271	3.0519	21.7821
68	0.9894	-0.9947	2.0000	3.8346	6.0000	17.2932	0.2368	2.9266	21.5190
69	0.9518	-0.9756	1.5000	4.8678	8.0000	21.5014	0.1828	3.7916	30.1081

NCA Data - [C:\WINNONLN\INHFIN.WDO]
Final Parameters

Subject	Rsq	Corr(x:y)	Tmax	Cmax	No. points Lambda z	AUClast	Lambda z	t1/2 Lambda z	AUCINF(observed)
70	0.9862	-0.9931	1.5000	11.6764	7.0000	43.9203	0.2613	2.6522	52.6099
71	0.9874	-0.9937	2.5000	2.7334	3.0000	10.3166	0.1205	5.7510	16.5222
72	1.0000	-1.0000	0.5000	8.6863	3.0000	23.1946	0.1846	3.7557	28.4776
73	0.9857	-0.9928	0.5000	13.2757	9.0000	37.6203	0.3494	1.9839	40.6713
74	0.9996	-0.9998	1.5000	4.5638	4.0000	15.9094	0.2917	2.3765	17.6237
75	0.9779	-0.9889	1.0000	10.0673	4.0000	32.5289	0.2005	3.4573	41.0311
76	0.9533	-0.9764	1.5000	5.4603	7.0000	28.9554	0.1379	5.0275	46.4417
77	0.9506	-0.9750	0.5000	6.0306	3.0000	12.8059	0.0795	8.7165	22.2898
78	0.9993	-0.9996	3.0000	7.2467	3.0000	36.2670	0.1449	4.7846	57.1791
79	0.9772	-0.9885	2.5000	5.5447	5.0000	31.6261	0.1082	6.4059	59.6757
80	0.9989	-0.9994	2.5000	3.6612	3.0000	10.4685	0.1996	3.4722	13.2498
81	0.9455	-0.9724	2.0000	6.0311	6.0000	27.3855	0.1885	3.6770	38.7677
83	0.6943	-0.8333	1.0000	5.3773	9.0000	20.8052	0.1438	4.8212	34.3294
84	0.9825	-0.9912	1.5000	5.0024	4.0000	21.7941	0.1564	4.4323	29.7747
86	0.9997	-0.9998	1.0000	3.7182	3.0000	11.5691	0.0593	11.6837	24.0269
88	0.9984	-0.9992	1.0000	7.1775	3.0000	20.1625	0.1980	3.5011	23.7846
89	0.9798	-0.9898	2.5000	3.4652	3.0000	15.8718	0.1503	4.6133	22.9129
90	0.9964	-0.9982	2.0000	3.5509	3.0000	17.9027	0.1641	4.2250	24.6925
91	0.9852	-0.9926	2.5000	7.9475	4.0000	31.8069	0.1778	3.8989	46.2431
92	0.9263	-0.9624	1.0000	3.4770	8.0000	13.4721	0.1919	3.6113	18.6353
93	1.0000	-1.0000	1.5000	8.4048	3.0000	41.9968	0.1341	5.1702	65.4853
94	0.9999	-1.0000	1.0000	7.4941	3.0000	25.6758	0.2146	3.2307	30.0974
95	0.9915	-0.9957	1.0000	7.5649	4.0000	29.4576	0.1495	4.6352	40.3283
96	0.7711	-0.8781	1.0000	2.4234	8.0000	7.8180	0.2196	3.1568	10.2661
97	0.9675	-0.9836	1.5000	4.3044	5.0000	14.4485	0.2578	2.6884	17.2418
98	0.9804	-0.9901	2.0000	5.6607	3.0000	16.9046	0.1330	5.2108	22.7686
99	0.9886	-0.9943	1.0000	3.8499	3.0000	10.9049	0.1082	6.4054	15.7024
100	0.8593	-0.9270	4.0000	4.7237	3.0000	21.9932	0.5137	1.3494	23.1716
101	0.9466	-0.9729	0.5000	5.7935	9.0000	18.9296	0.2232	3.1057	23.9008
102	0.8567	-0.9256	0.5000	1.7315	9.0000	7.8573	0.1276	5.4331	13.5675
103	0.9274	-0.9630	0.5000	4.7249	9.0000	15.4000	0.2193	3.1605	19.8198
104	0.9134	-0.9557	2.0000	4.2736	4.0000	13.3580	0.1541	4.4991	19.0852
105	0.9722	-0.9860	2.0000	4.0868	3.0000	11.9198	0.1499	4.6243	15.5202
107	0.9928	-0.9964	0.5000	8.9817	3.0000	30.6876	0.1758	3.9421	38.6591

NCA Data - [C:\WINNONLN\INHFIN.WDO]
Final Parameters

Subject	Rsq	Corr(x:y)	Tmax	Cmax	No. points Lambda z	AUClast	Lambda z	t1/2 Lambda z	AUCINF(observed)
108	0.9433	-0.9713	1.5000	6.0031	5.0000	23.0954	0.1878	3.6914	29.9352
109	0.9725	-0.9862	1.0000	5.0477	3.0000	17.2729	0.1352	5.1260	24.1285
110	0.9165	-0.9574	0.5000	5.4441	9.0000	21.1668	0.2223	3.1179	27.0032
111	0.9777	-0.9888	0.5000	4.8143	4.0000	17.6099	0.1764	3.9288	22.3326
112	0.9899	-0.9949	1.5000	4.6746	4.0000	22.7808	0.1194	5.8063	38.0977
113	0.9468	-0.9730	0.5000	5.7208	9.0000	20.6664	0.2381	2.9115	25.5265
114	0.9884	-0.9942	2.0000	5.2015	3.0000	25.2557	0.1835	3.7768	34.9939
115	0.9609	-0.9802	2.0000	5.9937	8.0000	30.1077	0.1551	4.4680	44.9160
116	0.9797	-0.9898	1.0000	3.9800	5.0000	15.2375	0.1997	3.4701	19.1130
117	0.9991	-0.9995	1.5000	7.5702	3.0000	37.5132	0.1834	3.7792	50.3177
119	0.9813	-0.9906	1.0000	6.1039	6.0000	21.5573	0.1847	3.7526	28.3580
120	0.9931	-0.9965	1.5000	4.4583	3.0000	16.3930	0.1713	4.0461	20.7761

NCA Data - [C:\WINNONLN\AINHFIN.WDO]
Final Parameters

Subject	Rsq	Corr(x:y)	Tmax	Cmax	No. points Lambda z	AUClast	Lambda z	t1/2 Lambda z	AUCINF(observed)
1	0.9944	-0.9972	2.0000	5.3053	4.0000	29.8147	0.2056	3.3718	39.0556
2	0.9649	-0.9823	4.0000	3.7300	3.0000	19.0897	0.1067	6.4982	41.9129
3	0.9836	-0.9918	3.0000	5.5449	3.0000	31.2785	0.1543	4.4910	49.7606
4	0.9787	-0.9893	2.0000	3.3603	6.0000	17.9882	0.1320	5.2513	29.9796
5	0.9978	-0.9989	3.0000	4.0778	3.0000	22.9544	0.1994	3.4760	31.6043
6	0.9774	-0.9886	1.5000	3.5997	3.0000	20.5945	0.1750	3.9605	29.0842
7	0.9883	-0.9941	2.5000	3.5029	3.0000	17.9711	0.2287	3.0313	23.3687
8	0.9417	-0.9704	3.0000	3.1050	4.0000	18.4936	0.1208	5.7397	33.2258
9	0.7808	-0.8837	1.5000	3.8285	5.0000	24.7796	0.1248	5.5549	38.9673
10	0.9935	-0.9968	2.5000	4.9739	5.0000	23.6907	0.2266	3.0589	30.2508
11	0.9977	-0.9988	2.5000	3.0789	3.0000	13.7186	0.3097	2.2379	15.8576
12	0.9576	-0.9786	4.0000	2.9847	3.0000	13.0450	0.2323	2.9836	18.1177
13	0.9419	-0.9705	4.0000	0.9662	3.0000	5.4674	0.0897	7.7236	12.9861
14	0.8681	-0.9317	4.0000	1.1012	3.0000	6.8043	0.1363	5.0837	11.4857
15	0.9981	-0.9990	3.0000	6.5541	3.0000	38.1290	0.1549	4.4745	59.0919
16	0.9366	-0.9678	2.5000	3.4148	5.0000	17.9141	0.2698	2.5695	20.6124
17	0.9930	-0.9965	2.0000	4.0186	4.0000	21.5578	0.1741	3.9811	31.1452
18	0.9731	-0.9865	1.5000	4.5465	5.0000	19.4982	0.2510	2.7610	23.7950
19	0.9866	-0.9933	3.0000	3.9463	3.0000	22.2113	0.1908	3.6336	31.0484
20	0.9993	-0.9996	2.5000	1.4584	3.0000	8.3077	0.2138	3.2421	10.7557
21	0.9900	-0.9950	2.0000	4.1730	3.0000	18.4251	0.3875	1.7888	20.2268
22	0.9235	-0.9610	3.0000	1.3370	3.0000	6.6161	0.1146	6.0487	12.8292
23	1.0000	-1.0000	2.0000	4.3175	3.0000	28.3785	0.0964	7.1891	57.5690
24	0.9936	-0.9968	3.0000	3.6368	4.0000	21.8658	0.0776	8.9347	53.6607
25	0.9997	-0.9999	4.0000	4.0826	3.0000	25.3158	0.1343	5.1604	43.0764
26	0.9134	-0.9557	3.0000	1.2279	4.0000	8.3951	0.0240	28.8312	54.2900
27	0.9639	-0.9818	1.5000	2.9286	5.0000	10.0684	0.5155	1.3446	10.4102
28	0.9998	-0.9999	3.0000	8.2659	3.0000	41.6055	0.1882	3.6832	59.1628
29	0.9647	-0.9822	3.0000	6.2287	3.0000	35.9806	0.1575	4.4008	54.9367
30	1.0000	-1.0000	2.5000	6.4369	3.0000	29.7165	0.3271	2.1193	33.7231
31	0.9990	-0.9995	3.0000	10.1893	3.0000	48.9494	0.3611	1.9193	54.7678
33	0.9979	-0.9989	2.5000	7.0074	3.0000	40.2096	0.2954	2.3467	47.1954
34	0.9913	-0.9957	2.0000	7.6714	3.0000	47.0974	0.1362	5.0887	78.2180
35	0.9799	-0.9899	3.0000	6.5404	5.0000	38.6038	0.1255	5.5234	64.8747

NCA Data - [C:\WINNONLN\AINHFIN.WDO]
Final Parameters

Subject	Rsq	Corr(x:y)	Tmax	Cmax	No. points Lambda z	AUClast	Lambda z	t1/2 Lambda z	AUCINF(observed)
36	0.9966	-0.9983	2.5000	5.3918	3.0000	28.0481	0.2928	2.3669	32.9301
37	0.8777	-0.9369	2.0000	7.8223	6.0000	41.6397	0.1901	3.6461	53.0285
38	0.9684	-0.9841	4.0000	1.7275	3.0000	9.4552	0.1175	5.8994	18.6449
39	0.9946	-0.9973	2.5000	7.9659	4.0000	35.9865	0.3363	2.0611	40.4170
40	0.9991	-0.9995	2.0000	7.1819	3.0000	40.0115	0.2497	2.7765	49.9739
41	Missing	Missing	3.0000	1.3079	0.0000	4.1392	Missing	Missing	Missing
42	0.7792	-0.8827	3.0000	4.6130	4.0000	27.6379	0.0963	7.1958	55.7871
43	0.9818	-0.9909	2.5000	5.0492	3.0000	31.2953	0.1116	6.2126	58.6260
44	0.8269	-0.9093	3.0000	2.1906	4.0000	12.6969	0.0805	8.6071	29.7716
45	0.9981	-0.9991	2.0000	5.5384	4.0000	25.7828	0.2906	2.3852	29.7588
46	0.9076	-0.9527	3.0000	3.2880	4.0000	19.4466	0.0712	9.7332	50.8018
47	0.9956	-0.9978	2.5000	3.1656	3.0000	17.6446	0.2356	2.9415	22.5661
48	0.9835	-0.9917	2.5000	3.8805	5.0000	19.9161	0.1200	5.7762	35.8487
49	0.7939	-0.8910	2.0000	2.5961	6.0000	15.1247	0.1048	6.6154	26.7460
50	0.9966	-0.9983	2.5000	6.5458	4.0000	35.4352	0.1967	3.5235	47.5918
51	0.9862	-0.9931	4.0000	2.6582	3.0000	16.3176	0.1320	5.2517	28.1968
52	0.9991	-0.9995	2.0000	3.4879	3.0000	14.2826	0.4186	1.6558	15.0520
53	0.9953	-0.9977	2.0000	3.4584	3.0000	18.4938	0.2391	2.8984	23.5411
54	0.9908	-0.9954	3.0000	5.9892	4.0000	31.0952	0.1696	4.0880	46.7448
55	0.9917	-0.9958	3.0000	3.4410	4.0000	16.8356	0.1944	3.5654	23.4619
56	0.8709	-0.9332	2.5000	1.9144	6.0000	8.6475	0.2441	2.8401	10.5120
57	0.9987	-0.9994	2.5000	5.5464	3.0000	25.9153	0.3232	2.1449	29.3622
58	0.9908	-0.9954	2.5000	5.4758	5.0000	30.1458	0.1301	5.3265	50.8479
59	0.9998	-0.9999	3.0000	7.3838	3.0000	43.5470	0.1385	5.0031	70.7397
60	0.9972	-0.9986	4.0000	3.9028	3.0000	22.6364	0.0907	7.6426	52.5753
61	0.9906	-0.9953	2.0000	4.8425	3.0000	27.4671	0.1431	4.8435	44.6728
62	0.8819	-0.9391	2.5000	3.2693	5.0000	19.3176	0.1607	4.3136	27.2307
63	0.9533	-0.9764	3.0000	2.9665	4.0000	17.6814	0.1687	4.1091	25.1090
64	0.9983	-0.9991	2.5000	4.2104	3.0000	27.8200	0.1139	6.0829	50.8553
65	0.9939	-0.9970	3.0000	4.8256	4.0000	24.1993	0.3043	2.2775	27.8266
66	0.9925	-0.9963	3.0000	2.9930	3.0000	16.3556	0.2176	3.1851	21.7910
67	0.9765	-0.9882	4.0000	4.0372	3.0000	23.5383	0.1558	4.4503	37.4402
68	0.9400	-0.9695	4.0000	4.1761	3.0000	24.2344	0.1645	4.2126	37.3766
69	0.9999	-0.9999	2.0000	3.9388	3.0000	23.9225	0.1258	5.5115	41.4222

NCA Data - [C:\WINNONLN\AINHFIN.WDO]
Final Parameters

Subject	Rsq	Corr(x:y)	Tmax	Cmax	No. points Lambda z	AUClast	Lambda z	t1/2 Lambda z	AUCINF(observed)
70	0.9610	-0.9803	1.5000	3.0287	7.0000	11.8533	0.1799	3.8525	17.0211
71	0.9824	-0.9911	2.5000	2.8488	5.0000	11.9505	0.2312	2.9979	15.3405
72	0.9310	-0.9649	2.0000	3.2405	6.0000	18.2674	0.1629	4.2538	25.0658
73	0.9996	-0.9998	2.0000	4.8396	3.0000	26.3464	0.2045	3.3902	34.9915
74	0.9739	-0.9869	2.5000	3.6075	4.0000	20.2505	0.2082	3.3287	26.2226
75	0.9794	-0.9896	1.0000	1.7971	7.0000	11.1731	0.0617	11.2290	29.8379
76	0.8946	-0.9458	2.0000	1.5083	6.0000	8.7867	0.0753	9.2053	20.6623
77	0.9963	-0.9982	2.0000	4.2072	3.0000	22.8872	0.2715	2.5528	27.0532
78	0.9865	-0.9932	3.0000	1.4845	3.0000	6.5520	0.1004	6.9064	13.4087
79	0.7833	-0.8851	4.0000	0.8465	3.0000	5.2597	0.0594	11.6741	16.5020
80	0.9637	-0.9817	3.0000	3.7632	5.0000	18.1413	0.1643	4.2183	27.3361
81	0.9897	-0.9949	3.0000	0.8602	4.0000	5.3930	0.0314	22.0865	28.8341
83	0.9449	-0.9721	2.5000	0.9407	3.0000	4.0420	0.0933	7.4258	10.4948
84	0.9678	-0.9838	4.0000	4.2546	3.0000	25.6940	0.1526	4.5418	40.8345
86	0.9959	-0.9979	2.5000	4.9645	5.0000	24.3160	0.2522	2.7480	29.1203
88	0.9934	-0.9967	2.5000	4.0895	3.0000	21.6544	0.2275	3.0469	27.5561
89	0.9822	-0.9911	2.5000	3.6715	3.0000	21.4347	0.1431	4.8448	35.2520
90	0.9483	-0.9738	4.0000	2.3775	3.0000	13.1346	0.1322	5.2450	23.7384
91	0.4713	-0.6865	2.5000	1.6664	3.0000	8.1682	0.0177	39.2409	73.5850
92	0.9489	-0.9741	3.0000	2.1026	3.0000	13.0678	0.1804	3.8428	18.6840
93	0.9972	-0.9986	2.0000	9.1369	3.0000	55.1417	0.1296	5.3488	96.4266
94	0.9986	-0.9993	2.5000	3.9057	3.0000	20.8572	0.2308	3.0034	26.4650
95	0.8274	-0.9096	2.0000	0.9869	5.0000	5.5108	0.0542	12.7839	16.6049
96	0.9786	-0.9893	2.5000	3.2947	5.0000	14.8998	0.2899	2.3907	17.1239
97	0.9981	-0.9991	3.0000	3.0693	3.0000	16.7648	0.1835	3.7773	24.2495
98	0.9350	-0.9669	2.0000	4.0603	5.0000	13.9579	0.7169	0.9669	14.1312
99	0.9892	-0.9946	2.5000	3.3970	5.0000	15.0352	0.2783	2.4909	17.4819
100	0.9553	-0.9774	2.0000	5.5218	3.0000	23.2397	0.3507	1.9767	28.4528
101	0.9449	-0.9720	3.0000	3.1051	4.0000	18.7670	0.1626	4.2625	26.9861
102	0.9897	-0.9948	2.5000	0.5430	3.0000	3.3283	0.1137	6.0956	6.1623
103	0.9954	-0.9977	2.5000	3.2262	5.0000	16.8412	0.1762	3.9329	23.7181
104	0.9982	-0.9991	2.5000	4.5941	3.0000	21.8543	0.2556	2.7115	27.0186
105	0.7838	-0.8853	2.5000	3.3869	6.0000	15.4484	0.2988	2.3199	18.3292
107	0.9510	-0.9752	4.0000	2.2800	3.0000	14.2757	0.1898	3.6529	19.9005

NCA Data - [C:\WINNONLN\AINHFIN.WDO]
Final Parameters

Subject	Rsq	Corr(x:y)	Tmax	Cmax	No. points Lambda z	AUClast	Lambda z	t1/2 Lambda z	AUCINF(observed)
108	0.9510	-0.9752	4.0000	2.2800	3.0000	14.2757	0.1898	3.6529	19.9005
109	0.9894	-0.9947	1.5000	3.7013	3.0000	20.0806	0.2754	2.5166	24.0190
110	0.9885	-0.9942	3.0000	4.4224	3.0000	25.2228	0.2255	3.0732	32.3160
111	1.0000	-1.0000	3.0000	3.5842	3.0000	18.9742	0.1916	3.6182	26.3770
112	0.9877	-0.9939	3.0000	0.9181	3.0000	5.8745	0.0637	10.8864	16.8024
113	0.9971	-0.9985	2.5000	3.3781	3.0000	20.2242	0.1659	4.1781	29.6823
114	0.9985	-0.9993	4.0000	0.9825	3.0000	5.8170	0.0767	9.0338	15.2377
115	0.9537	-0.9766	2.0000	1.3156	3.0000	7.9710	0.0341	20.3008	36.3650
116	0.9997	-0.9998	3.0000	2.7664	3.0000	15.4547	0.2254	3.0751	20.1689
117	0.9942	-0.9971	4.0000	1.2520	3.0000	8.0476	0.1095	6.3294	15.4251
119	0.8204	-0.9058	4.0000	2.8984	3.0000	17.6252	0.0988	7.0150	37.3818
120	0.9995	-0.9997	2.0000	4.5111	3.0000	21.4205	0.2899	2.3914	25.1078

APPENDIX IV

ELIMINATION AND FORMATION RATE CONSTANTS

Table A. The calculated rate constants for each individual in the study

Subject No.	Elimination Rate Constant (h^{-1})	Formation Rate Constant (h^{-1})	Phenotype
1	**0.1993**	**-0.3802**	**Rapid**
2	**0.1738**	**-1.1371**	**Intermediate**
3	**0.1459**	**-1.1475**	**Intermediate**
4	**0.2058**	**-0.7243**	**Rapid**
5	**0.2543**	**-1.526**	**Intermediate**
6	**0.2130**	**-0.3162**	**Intermediate**
7	**0.2317**	**-1.342**	**Intermediate**
8	**0.2183**	**-0.7809**	**Intermediate**
9	**0.2598**	**-0.5205**	**Intermediate**
10	**0.1706**	**-1.245**	**Rapid**
11	**0.3356**	**-0.8204**	**Intermediate**
12	**0.2238**	**-1.113**	**Slow**
13	**0.1570**	**-1.535**	**Slow**
14	**0.1431**	**-0.2632**	**Slow**
15	**0.1337**	**-1.085**	**Rapid**
16	**0.2632**	**-0.4472**	**Intermediate**
17	**0.2814**	**-0.9405**	**Intermediate**
18	**0.2483**	**-2.087**	
19	**0.2487**	**-1.317**	**Intermediate**
20	**0.1767**	**-0.2260**	**Slow**
21	**0.2186**	**-1.182**	**Rapid**
22	**0.2001**	**-1.248**	**Slow**
23	**0.1290**	**-0.4930**	**Rapid**
24	**0.2499**	**-1.621**	**Intermediate**
25	**0.2464**	**-0.8516**	**Intermediate**
26	**0.1612**	**-0.1302**	**Slow**
27	**0.5050**	**-2.148**	**Slow**
28	**0.2761**	**-1.928**	**Rapid**
29	**0.2766**	**-1.907**	**Intermediate**
30	**0.3226**	**-1.336**	**Rapid**
31	**0.2763**	**-0.7540**	**Rapid**
32		**Excluded**	

33	0.3705	-0.3274	Rapid
34	0.2776	-0.7690	Intermediate
35	0.2592	-0.7753	Rapid
36	0.2919	-0.4296	Rapid
37	0.2757	-0.9283	Rapid
38	0.1755	-1.354	Slow
39	0.2077	-1.187	Rapid
40	0.2546	-0.9048	Rapid
41	0.1739	-0.2959	Slow
42	0.1777	-0.8032	Intermediate
43	0.1345	-0.3266	Rapid
44	0.1766	-0.7473	Slow
45	0.4388	-0.6121	Rapid
46	0.2380	-1.419	Intermediate
47	0.1786	-0.8219	Intermediate
48	0.2140	-1.035	Intermediate
49	0.1551	-1.664	Intermediate
50	0.2852	-0.6811	Rapid
51	0.1960	-0.9851	Intermediate
52	0.1876	-2.112	Intermediate
53	0.2154	-0.6509	Rapid
54	0.1570	-1.202	Rapid
55	0.2760	-1.423	Intermediate
56	0.2689	-0.2608	Slow
57	0.2730	-1.018	Rapid
58	0.3094	-1.061	Intermediate
59	0.2353	-0.9873	Rapid
60	0.2163	-1.566	Intermediate
61	0.1675	-0.7170	Rapid
62	0.2202	-1.131	Intermediate
63	0.1866	-0.3769	Intermediate
64	0.1392	-0.2033	Intermediate
65	0.3020	-0.06426	Rapid
66	0.2329	-0.9143	Intermediate
67	0.2260	-1.882	Intermediate
68	0.2389	-1.114	Intermediate
69	0.1828	-1.210	
70	0.2573	-1.972	Slow
71	0.1369	-0.5392	Intermediate
72	0.2714	-0.2927	Intermediate
73	0.3473	-0.3955	Intermediate

74	0.3335	-0.2478	Rapid
75	0.2255	-1.079	Slow
76	0.1390	-1.022	Slow
77	0.2031	-0.02336	Rapid
78	0.1449	-1.262	Slow
79	0.1052	-1.459	Slow
80	0.2460	-0.6967	Rapid
81	0.1875	-1.839	Slow
82		Excluded	
83	0.1276	-2.631	Slow
84	0.1891	-0.6403	Intermediate
85		Excluded	
86	0.2027	-0.7500	Rapid
87		Excluded	
88	0.3033	-1.055	Rapid
89	0.1911	-0.7490	Intermediate
90	0.1947	-0.7518	Intermediate
91	0.1778	-0.7767	Slow
92	0.1825	-1.090	Intermediate
93	0.1671	-0.8039	Intermediate
94	0.3156	-	Intermediate
95	0.2177	-0.3629	Slow
96	0.1968	-0.2908	Rapid
97	0.2832	-1.641	Intermediate
98	0.2751	-0.9807	Intermediate
99	0.2583	-0.5087	Rapid
100	0.5137	--0.7898	Intermediate
101	0.2104	-0.2447	Intermediate
102	0.1210	-0.6418	Intermediate
103	0.1917	-0.4282	Intermediate
104	0.1953	-1.024	Rapid
105	0.2652	-1.247	Rapid
106		Excluded	
107	0.2518	-0.2734	Intermediate
108	0.2201	-0.8673	Intermediate
109	0.2472	-1.148	Intermediate
110	0.2038	-0.5835	Rapid
111	0.2723	-0.7291	Rapid
112	0.1442	-1.169	Slow
113	0.2366	-0.5812	Intermediate
114	0.1554	-0.9611	Slow

115	0.1509	-0.4583	Slow
116	0.2419	-0.6747	Intermediate
117	0.1860	-0.0630	Slow
118		Excluded	
119	0.1987	-0.7708	Intermediate
120	0.2545	-1.111	Rapid

APPENDIX V

INDIVIDUAL PATIENT DETAILS

Table B. Individual patient details

Subject Number	Age	Weight @ 2 months	Gender	Race	HIV Status
001	50	45.1	F	C	-
002	22	43.2	F	C	-
003	24	37.9	F	C	-
004	33	46.9	F	B	+
005	50	60	M	C	-
006	26	68	M	C	-
007	31	46	M	C	+
008	21	33.4	F	C	-
009	36	46.4	F	C	-
010	20	48.5	F	C	-
011	21	46	F	C	-
012	31	38.5	F	C	-
013	19	50	M	C	-
014	31	66.3	M	C	+
015	71	35.1	F	C	-
016	39	52.5	F	C	+
017	56	39.4	F	C	-
018	47	48	F	C	-
019	36	38.2	F	C	+
020	35	-	M	W	+
021	-	-	F	C	o
022	31	55.4	F	C	-
023	63	55.1	M	C	-
024	22	39.5	F	B	+
025	51	42	F	C	+
026	40	50	M	C	-
027	41	41.4	F	C	-
028	26	50	F	C	-
029	22	28.5	F	B	-
030	27	37.5	F	C	-
031	42	30.6	F	C	-
033	33	48.7	F	C	-
034	71	40.4	F	C	-
035	-	-	M	C	-
036	35	45.4	F	C	-
037	28	46.1	F	C	-
038	38	51.3	F	C	-
039	36	-	F	C	-
040	26	50.6	F	C	-
041	57	60	M	C	-
042	30	44	M	B	+
043	59	47	M	C	+
044	-	-	F	C	-
045	37	37.2	F	C	-
046	37	43	M	C	-
047	30	47	M	C	-
048	35	53	M	C	-

049	22	54	M	B	+
050	55	31.1	F	C	-
051	40	54	M	C	-
052	47	46	M	C	-
053	34	50	M	C	-
054	23	47.5	F	B	+
055	31	46.6	F	C	-
056	39	30	F	C	-
057	29	40.6	F	C	-
058	22	37.4	F	C	-
059	45	39	F	C	-
060	35	36	M	C	-
061	48	45	M	C	-
062	31	46	M	C	-
063	43	52	M	C	-
064	40	85.5	F	C	-
065	26	-	F	C	-
066	45	49	M	C	-
067	39	40	M	C	-
068	22	36	M	C	-
069	40	47	M	C	-
070	33	42	M	C	-
071	61	41.3	F	C	-
072	42	50.8	F	C	-
073	21	35.7	F	C	-
074	18	43.9	F	C	-
075	33	40.9	F	C	-
076	40	53	M	C	-
077	28	52	M	C	-
078	39	56	M	C	-
079	35	47	M	C	-
080	43	59.5	F	C	-
081	41	51.8	F	C	-
083	55	52	M	C	-
084	46	46	M	C	-
086	28	40.1	F	C	-
088	36	36	M	C	-
089	48	44	M	C	-
090	26	49	M	C	+
091	28	37.5	M	C	-
092	27	68.5	M	B	-
093	37	35.7	F	C	-
094	35	34	F	C	-
095	42	48.5	F	C	-
096	32	60.9	F	C	-
097	20	45.8	F	B	-
098	-	-	F	C	-
099	44	60.3	F	C	-
100	-	-	M		-
101	42	46.5	M	C	-
102	18	49	F	C	-
103	30	60	M	C	-
104	30	52.8	F	C	-
105	23	53.7	F	C	-
107	58	36.5	F	C	-
108	58	43.4	F	C	-
109	26	46.8	F	C	-
110	44	41.5	F	C	-
111	50	41	M	C	-

112	53	68	M	C	-
113	58	50	M	C	-
114	22	50	M	C	-
115	-	-	M	C	-
116	19	50	M	C	-
117	21	46	M	B	-
119	50	-	M	C	-
120	40	37	M	C	-