DETERMINATION OF ISONIAZID IN HUMAN URINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING DERIVATIZATION WITH SOME ALDEHYDES

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ABSTRACT

Isoniazid or isonicotinic acid hydrazide (INH) is the most commonly used antituberculosis drug for the prevention of clinical tuberculosis. A validated High Performance Liquid Chromatographic (HPLC) method was developed for the analysis of isoniazid in human urine by pre-derivatization with salicylaldehyde to form a hydrazone which was then detected. Chromatographic analyses were carried out on an Eclipse® XDB-C18 column employing a mixture of 45:55 (v/v) methanol and 10 mM acetate buffer with 2 mM EDTA (pH 4.0) in the mobile phase and using UV detection at 289 nm. This condition provided separation of the hydrazone from endogenous matrices in the urine and also the excess reagent, with only filtration and dilution of the sample as a pretreatment. The method developed in this work was validated with respect to various parameters. Linear calibration curves in the concentration range of 2-100 μg/mL in spiked urine had a coefficient of determination (r^2) of 0.9994. The limit of detection was equal to the calculated intercept of the regression line i.e. the blank value, plus three times the standard error of y-residuals of isoniazid which was 1.30 μg/mL. This method also provided good recovery and stability. The method is stable with minimal within-day and between-day variation. The method was applied to the analysis of isoniazid in human urine samples, which were found to have isoniazid in the range 4.99-57.6 μg/mL.

KEY WORDS: ISONIAZID/HYDRAZONE/HUMAN URINE/HPLC

64 pages

การวิเคราะห์ปริมานไอโซไนซิดในปัสสาวะมนุษย์ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงโดยการทำอนุพันธ์กับอัลดีไฮด์บางชนิด

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บทคัดย่อ

ไอโซไนซิดเป็นยาที่นิยมใช้ในการป้องกันรักษาโรควัณโรค ซึ่งเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงถูกพัฒนาขึ้นสำหรับการวิเคราะห์ยาไอโซไนซิดในตัวอย่างปัสสาวะมนุษย์โดยใช้ปฏิกิริยาการเตรียมอนุพันธ์กับซาลิซิลอัลดีไฮด์และทำการตรวจวัดในรูปของไฮดราโซน การวิเคราะห์ทางโครมาโทกราฟีเกิดขึ้นบนคอลัมน์ Eclipse® XDB-C18 ร่วมกับสารละลายผสมของเมทานอล และ 10 มิลลิโมลาร์อะซิเตทบัฟเฟอร์ที่มี 2 มิลลิโมลาร์เอธทีลีนไดเอมีนเตทตระอะซิเตต ในอัตราส่วนร้อยละ 45:55 โดยปริมาตรเป็นวัฏภาคเคลื่อนที่โดยมีการตรวจวัดการดูดกลืนแสงที่ความยาวคลื่น 289 นาโนเมตร โดยสภาวะดังกล่าวสามารถแยกไฮดราโซนออกจากสิ่งรบกวนอื่นที่อยู่ในปัสสาวะมนุษย์ โดยมีการเตรียมตัวอย่างเพียงการกรองปัสสาวะและทำให้เจือจางเท่านั้น กราฟมาตรฐานสำหรับการวิเคราะห์ที่ช่วงความเข้มข้น 2-100 ไมโครกรัมต่อมิลลิลิตรมีค่าสัมประสิทธิ์ความเป็นเส้นตรงเท่ากับ 0.9994 ค่าขีดจำกัดต่ำสุดของไอโซไนซิดที่สามารถตรวจวัดได้ คือ 1.30 ไมโครกรัมต่อมิลลิลิตร และมีความแม่นยำดีทั้งการตรวจวัดในวันเดียวกันและระหว่างวัน ระบบที่พัฒนาขึ้นยังให้ผลการทดลองที่ยอมรับได้ในปัจจัยที่เกี่ยวกับ ค่าการวิเคราะห์คืนกลับ และความเสถียร วิธีดังกล่าวยังได้นำไปประยุกต์ใช้ในการวิเคราะห์หาปริมาณไอโซไนซิดในตัวอย่างปัสสาวะ ผลการทดลองชี้ให้เห็นว่าวิธีวิเคราะห์ที่นำเสนอนี้สามารถนำไปใช้ได้กับตัวอย่างจริงซึ่งพบปริมาณไอโซไนซิดในช่วงความเข้มข้นระหว่าง 4.99-57.6 ไมโครกรัมต่อมิลลิลิตร

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LIST OF ABBREVIATIONS

µL	Microliter
µl/mL	Microgram per milliliter
µM	Micromolarity
µm	Micrometer
2-NBA	2-Nitrobenzaldehyde
2-NIH	2-Nitrobenzaldehyde isonicotinoyl hydrazone
4-NBA	4-Nitrobenzaldehyde
4-NIH	4-Nitrobenzaldehyde isonicotinoyl hydrazone
AA	Ascorbic acid
AgNPs	Silver colloidal nanoparticles
BIH	Benzaldehyde isonicotinoyl hydrazone
CA	Cinnamaldehyde
CE	Capillary electrophoresis
CIH	Cinnamaldehyde isonicotinoyl hydrazone
CTAB	Cetyltrimethyl ammonium bromide
DA	Dopamine
DAD	Diode array detector
DPP	Differential pulse polarlography
ECF	Ethyl chloroformate
EDTA	Ethylenediaminetetraacetic acid
EGDMA	Ethylene glycol dimethacrylate
FI-CL	Flow injection chemiluminescence
FID	Flame ionization detection
g	Gram
GA	Genetic algorithm
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HMDE	Hanging mercury drop electrode

LIST OF ABBREVIATIONS (cont.)

HPLC	High performance liquid chromatography
HZ	Hydrazine
i.d.	Inner diameter
INH	Isoniazid or isonicotinoyl hydrazide
ISP	Ion-selective piezoelectric
LC	Liquid chromatography
LOQ	Limit of quantitation
MAA	Metharylic acid
mAU	Milliabsorbance unit
MEKC	Micellar electrokinetic capillary chromatography
MIP	Molecular imprinted polymer
mL	Milliliter
ml/min	Milliliter per minute
mM	Millimolarity
MS	Mass spectrometry detector
MWCPE	Multi-walled carbon nonotubes paste electrode
NBS	N-bromosuccinimide
NCS	N-chlorosuccinimide
nM	Nanomolarity
NQS	1,2-naphthoquinone-4-sulphonate
^{o}C	Temperature in degree of Celsius
PDT	3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine
PHZ	Phenylhydrazine
PIH	Peridoxal isonicotinoyl hydrazone
PLS	Partial least squares
RSD	Relative standard deviation
SA	Salicylaldehyde
SD	Standard deviation
SDCC	Sodium dichloroisocyanurate

LIST OF ABBREVIATIONS (cont.)

SDS	Sodium dodecylsulphate
SIH	Salicylaldehyde isonicotinoyl hydrazone
TB	Tuberculosis
TCCA	Trichloroisocyanuric acid
UV-VIS	Ultraviolet visible
v/v	Volume by volume

THE RELEVANCE OF THE RESEARCH WORK TO THAILAND

Tuberculosis (TB) continues to be the leading cause of death by infectious disease. Annually this disease kills more than 1.8 million people world-wide, and in Thailand there was estimated at 1.4%, with approximately 100,000 new TB cases developing each. To treat TB, isoniazid was first used in 1952 and continues to be one of the most effective first-line TB drugs. Isoniazid is a product that activated in the mycobacterial cell. The down-stream effect of this inhibition is that the protective mycolic acid lipid coating of *M.tuberculosis* can no longer be produced, resulting in bacterial death. Because isoniazid must be taken for a long time there is a severe and sometimes fatal risk of developing liver toxicity. Therefore, to control the quantity of isoniazid accumulated and to protect from its toxicity, quantitation of isoniazid is required.

This work presents an alternative simple method for monitoring of isoniazid in human urine.

CHAPTER I
INTRODUCTION

1.1 The important of isonaizid

Isoniazid or isonicotinic acid hydrazide (INH) (Figure 1.1) was first chemically synthesized in 1912 by Meyer and Mally at Charles University in Prague [1]; however, its use in the treatment of tuberculosis (TB) was not recognized until 1952. INH is an important front-line anti-TB drug that forms the basis of modern TB chemotherapy. It is almost an ideal drug for the treatment of TB because it is inexpensive, highly specific bactericidal to *Mycobacterium tuberculosis*, easily administered, and relatively nontoxic.

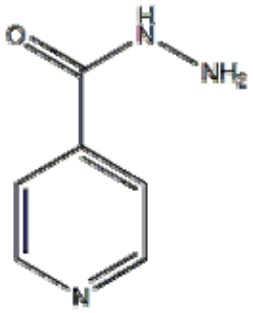


Figure 1.1 Structure of isoniazid

1.1.1 Antibacterial activity

The minimal tuberculostatic concentration is 0.025 to 0.05 µg/mL [2]. The bacteria undergo one or two divisions before multiplication is arrested. The drug is remarkably selective for mycobacteria, and concentrations in excess of 500 µg/mL are required to inhibit the growth of other microorganisms. Isoniazid is highly effective for the treatment of experimentally induced tuberculosis in animals and is strikingly superior to streptomycin. Unlike streptomycin, isoniazid penetrates cells with ease and is just as effective against bacilli growing within cells as it is against those growing in culture media.

In the mouse model of TB infection, bacterial colony counts in infected organs decrease quickly in the first few days of INH treatment. However, the early bactericidal activity of INH becomes slower after the first 2 to 3 weeks in mice. The number of colony-forming units stabilizes at a low level after 2 to 3 months of treatment with INH. Longer treatment with INH monotherapy may lead to the emergence of INH–resistant organisms and the number of colony-forming unit in the infected organs may increase again. The addition of a second antituberculous drug, such as paraaminosalicylic acid or streptomycin, prevents the emergence of INH-resistant organisms.

1.1.2 Bacteria resistance

When tubercle bacilli are grown in vitro in increasing concentrations of isoniazid, mutants are readily selected that are resistant to the drug [2]. The most common mechanism of isoniazid resistance is mutations in catalase-peroxidase that decrease the drug's activity, preventing conversion of the prodrug isoniazid to its active metabolite. However, some low-level resistant strains with catalase activity can develop a higher level of resistance through multiple mutations and may still retain catalase activity. The degree of INH resistance varies from low (0.2 µg/mL) to as high as 100 µg/mL. Because INH is the most commonly used antituberculosis drug, resistance to INH is also the most frequent among drug-resistance clinical isolates. INH resistance can be as high as 20% to 30% in some cities and the percentage of INH-resistant strains varies in different geographic regions.

As with the other agents, treatment with INH alone leads to the emergence in vivo of resistant strains. The shift from primarily sensitive to mainly insensitive microorganism occasionally occurs within a few weeks after therapy is started; however, the time of appearance of this phenomenon varies considerably from case to another. Approximately one in 10^6 tubercle bacilli will be genetically resistant to INH since tuberculous cavities may contain as many as 10^7 to 10^9 microorganism, it is not surprising that treatment with INH alone results in the selection of these resistant baterial. Mutations in the following genes (*katG*, *inhA*, *kasA*, *ndh*, *ahpC*) are associated with INH resistance.

1.1.3 Mechanism of action

A primary action of INH is to inhibit the biosynthesis of mycolic acids (Figure 1.2) which are long, branched lipids that are attached to a unique polysaccharide, galactan, to form part of the mycobacterial cell wall [3]. Mycolic acids are unique to mycobacteria, explaining the high degree of selectivity of the antimicrobial activity of INH. Mutations of the *katG* gene that result in an inactive catalase-peroxidase cause high-level INH resistance, since the prodrug cannot be activated by the catalase-peroxidase. Exposure to INH leads to a loss of acid-fastness and a decrease in the quantity of methanol-extractable lipids in the microorganisms.

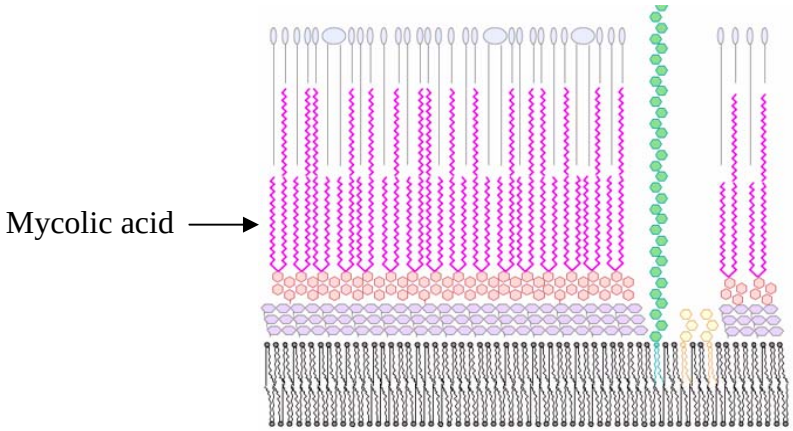


Figure 1.2 Schematic diagram of mycobacterial cell wall.

1.1.4 Drug treatment regimens

Effective drug regimens are available to treat tuberculosis [4]. Drug selection is based on the prevention of development of drug-resistant organisms and drug toxicity. General guidelines include: monitoring the prescribed therapy regimen closely to avoid client noncompliance, to detect side effects or adverse reactions, and to register progress of the treatment program.

Table 1.1. Recommended dosage for initial treatment of tuberculosis

	Total daily dose of INH	**Twice-weekly dose of INH**
Children	5 mg/kg : max 300mg/kg	15 mg/kg : max 900mg/kg
Adults	10-20mg/kg : max 300 mg/kg	15 mg/kg : max 900mg/kg

1.1.5 Absorption, distribution and excretion

INH is readily absorbed when administered either orally or parentrally [2]. INH diffuses readily into all body fluids and intracellular compartments. After oral administration of the usual dose of 5 mg/kg INH or 300 mg per day, a peak serum concentration of 5 µg/mL is achieved in 1 to 2 hours. INH concentrations in the lungs and cerebrospinal fluid are similar to that achieved in the serum. INH is metabolized in the liver primarily by acetylation and dehydrazination. Its penetrates well into caseous material. The concentration of the agent is initially higher in the plasma and muscle than in the infected tissue.

Up to 70% of a dose of INH is excreted in the urine within 24 hours, mostly as metabolites. The main excretory products in human beings are the result of enzymatic acetylation (acetylisoniazid) and enzymatic hydrolysis (isonicotinic acid). Small quantities of an isonicotinic acid conjugate (probably isonicotinyl glycine), one or more isonicotinyl hydrazone, and traces of N-methylisoniazid also are detectable in urine. Slow acetylators excrete 37% of the drug as free INH or its hydrazone conjugates and 63% as actyl-INH and its metabolites. In contrast, rapid acetylators excrete 94% as free INH as acetyl-INH and its metabolites and only 2.8% as free INH and 3.6% as hydrazone conjugate. Liver disease can reduce acetylation rates. The rate of acetylation does not significantly alter the efficacy of the drug with current INH dosing.

Human populations show genetic heterogeneity with regard to the rate of acetylation of isoniazid. The distribution of slow and rapid inactivators of the drug is bimodal owing to differences in the activity of an acetyltransferase (Figure 1.3). The rate of acetylation signiflicantly alters the concentrations of the drug that are achieved

in plasma and its half-life in the circulation. The half-life of the drug may be prolonged in the presence of hepatic insufficiency.

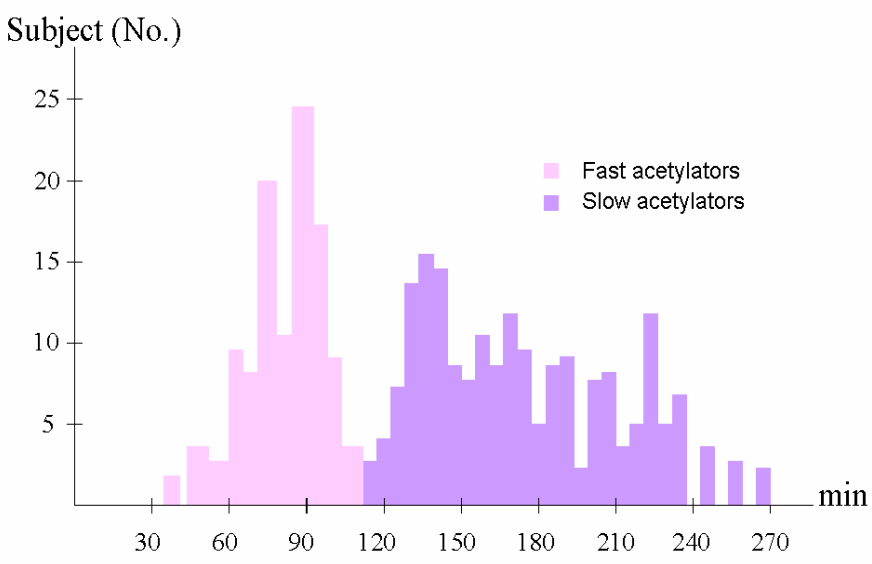


Figure 1.3 Biomodal distributions of serum isoniazid half-life in a large group of patient [2].

The frequency of each acetylation phenotype is dependent upon race but not influenced by gender or age. High acetyltransferase activity (fast acetylators) is inherited as an autosomal dominant trait; “fast acetylators” of INH are either heterozygous or homozygous. The average concentration of active INH in the circulation of fast acetylators is about 30% to 50% of that present in persons who acetylate the drug slowly.

The half-life of isoniazid varies from less than 1 to more than 4 hours (Figure 1.3) [5]. The mean half-life in the fast acetylators is approximately 70 min, whereas 2 to 5 hours is characteristic of slow acetylators. However, a sufficient amount of drug can be administered to fast acetylators to achieve a therapeutic effect equal to that seen in slow acetylators. A dosage reduction is recommended for slow acetylators with hepatic failure.

The clearance of INH is dependent to only a small degree on the status of renal function, but patients who are slow inactivators of the drug may accumulate toxic concentrations if their renal function is impaired.

Thus, analysis of INH has an important role. Doctor can prescribe drug suitable for each patient, depending on whether they are low or high acetylators to minimize drug toxicity and ensuring appropriate dosage regimen, especially in the case of drug resistance.

1.1.6 Side effect of isoniazid

The principal side effect is gastric irritation, which is more common in adults and women [6]. This effect is reduced by commencing with a low dose and gradually increasing to the full dose, by the use of antacids and by taking the drug at bedtime. Hypersensitivity reaction and hepatitis also occur. Rare effects include hypothyroidism, menstrual irregularities, alopecia, convulsion, deafness, diplopia, peripheral neuropathy, mental disturbances (including depression) and, in male patients, impotence and gynecomastia.

CHAPTER II
OBJECTIVES

The purpose of this research is to develop a simple high performance liquid chromatography (HPLC) with diode array detector (DAD) for the determination of isoniazid in the form of hydrazone in human urine.

The objectives of this study include:

(i) To study the various aldehydes and experimental conditions to derivatize isoniazid to give the isonicotinoyl hydrazones.

(ii) To investigate the analytical performance of HPLC method for determination of the hydrazone.

(iii) To apply the developed methods for analysis of isoniazid in human urine.

CHAPTER III
LITERATURE REVIEW

3.1 Analytical method for determination of isoniazid

Several techniques have been developed to determine isoniazid in various types of sample. Each technique has some advantages and disadvantages depending on the application it is used. In this chapter, the different techniques available for measurement of isoniazid, divided into two categories, non-chromatographic and chromatographic methods, are described.

3.1.1 Non chromatographic method

3.1.1.1 Electrochemical method

In 1999, Yao *et al.* developed ion-selective piezoelectric (ISP) for the direct determination of isoniazid in body fluids [7]. It is based on the sensitive mass response of piezoelectric quartz crystal and selective adsorption/desorption on the modified film. The ISP sensor was fabricated by coating a PVC film containing activant on one electrode of a thickness-shear mode piezoelectric quartz crystal. Three activants, INH-phosphotungstate (I), INH-silicotungstate (II), and INH-$(BiI_4)^-$ (III), were synthesized and applied for the INH assay.

In 2001, Espinosa-Mansilla *et al.* reported a comparative study between the abilities of partial least squares (PSL) and a modification of the novel hybrid linear analysis (HLA/XS method) to resolve a complex mixture of rifampicin, pyrazinamide and isoniazid, widely used in clinical treatment [8]. Statistical parameters, such as the relative error of prediction, were compared with the aim of selecting the best multivariate algorithm to resolve the proposed mixture.

Alonso Lomillo *et al.* developed a simple and rapid method for the polarographic determination of rifampicin, isoniazid and pyrazinamide by differential pulse polarlography (DPP) [9]. A partial least squares (PLS) regression was used for the resolution of the overlapped polarographic signals from mixtures of

the three drugs. A genetic algorithm (GA) was used to select some of the predictor variables (potentials of the polarogram). The PLS model constructed with these selected potentials gave satisfactory results. The procedure was successfully applied to pharmaceutical preparations and biological fluids.

In 2003, Ghoneim *et al.* reported a square-wave adsorptive cathodic stripping voltammetric procedure to assay the drug in bulk, pharmaceutical formulations and biological fluids using a hanging mercury drop electrode (HMDE) [10]. Under the optimized conditions, isoniazid generated irreversible cathodic peak. The procedure was applied to the assay of the drug in tablets (Isocid and T.B. Zide) and spiked human serum and urine.

In 2004, Hamnam *et al.* developed cyclic and square-wave voltammetry at carbon paste electrode in Britton-Robinson buffers [11]. A validated square-wave adsorptive anodic stripping voltammetric procedure was described to assay the isoniazid and rifampicin separately or combined in pharmaceutical preparations without the necessity for sample pretreatment or time-consuming extraction steps prior to the analysis. The proposed procedure was successfully applied to simultaneous assay of both drugs in human serum samples.

In 2006, Quintino *et al.* proposed a new, fast and precise method to analyze isoniazid based on the electrochemical oxidation of the analyte at a glassy carbon electrode in 0.1 M NaOH [12]. The quantification was performed utilizing amperometry associated with batch injection analysis technique. Fast sequential analysis with a wide linear dynamic range, high sensitivity and low limits of detection and quantification were achieved for determination of isoniazid in isoniazid-rifampicin tablets.

In 2007, Shahrokhian *et al.* described the design and preparation of a multi-walled carbon nonotubes paste electrode (MWCPE) [13]. The electrode was shown to be very effective for the detection of isoniazid in the presence of other biological reductant compounds. The electrode exhibited a very good resolution between the voltammetric peak of isoniazid and the peaks of ascorbic acid (AA) and dopamine (DA). A resolution of more than 450 mV between the anodic peak potentials makes the MWCPE suitable for simultaneous detection of isoniazid in the presence of AA or DA in clinical and pharmaceutical preparations.

3.1.1.2 Spectrophotometric method

In 1996, Nagaraja *et al.* used sodium 1,2-naphthoquinone-4-sulphonate (NQS), along with cetyltrimethyl ammonium bromide (CTAB), as a reagent for the determination of INH in alkaline medium and the method is based on the formation of a red-colored product [14]. The method offers the advantages of sensitivity, simplicity and rapidity without the need of extraction or heating.

In 1998, Benetton *et al.* reported two methods developed and validated, a visible spectrophotometric method using the three-point correction technique between 420 and 520 nm for the determination of RIF, and the amplitude of the first-derivative ultraviolet spectrophotometric spectrum at 257 nm for the determination of INH [15] . The methods were rapid, simple and did not require any separation step.

In 2008, *Safavi et al.* were designed an optical sensor to assay the drug in bulk, without the need for sample pretreatment or any time-consuming extraction or evaporation steps prior to the analysis [16]. The sensing membrane consisted of immobilized 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine (PDT) on a triacetylcellulose membrane. The method is based on the reduction of Fe(III) by isoniazid to Fe(II) which forms a complex with PDT. The complex has an absorption maximum at 558 nm.

3.1.1.3 Flow injection analysis (FIA)

FIA is widely utilized because it is an automated and simple technique. Flow injection chemiluminescence (FI-CL) has been successfully applied to detection of many drugs owing to its great sensitivity and wide linear working range with simple instrumentation. CL systems for determination of isoniazid have been reported.

In 1999, Li *et al.* proposed a flow-injection analysis method for isoniazid based on the direct CL reaction of isoniazid and Mn(III) in acidic medium [17]. The unstable Mn(III) is electrogenerated on-line, via a positive Pt electrode, from $MnSO_4$ in H_2SO_4 medium with constant current electrolysis. Furthermore, its concentration can be controlled on-line by regulation of electrolytic current.

In 2000, an indirect fluorimetric determination of isoniazid was reported by Lapa *et al.* [18]. The analytical procedure was based on the oxidation of isoniazid by cerium(IV) and monitoring of the fluorescence intensity of the cerium(III) formed. A time-based sample insertion enabled an effective control of the sampling volume and thus of the dispersion level attained. These features were evaluated in automated dissolution studies.

In 2001, Song *et al.* proposed CL reagents, luminol and ferricyanide, that were both immobilized on Amberlyst A-27 (Rohm and Hass Co.) anion-exchange resin [19]. Using injection of 200 µL sodium phosphate, the reagents on the anion-exchange resin column were eluted and the presence of any isoniazid could be observed. The concentration of isoniazid was quantified via the peak height of the decreased CL intensity. This FI-CL system was applied successfully to the determination of isoniazid in pharmaceutical preparation.

Li *et al.* reported a FI-CL method for determination of isoniazid by oxidation with KIO_4 and KOH as the media of the CL reaction [20]. This system was evaluated for monitoring of dissolution profiles of isoniazid tablets. The undissolved suspended particles in the solution were eliminated via on-line filter. This method provides a new technique for on-line process monitoring of isoniazid and its metabolites in biological body fluids.

Zhang *et al.* reported a new CL sensor for the determination of isoniazid based on its enhancing effect on the CL system between luminol and periodate [21]. In this system luminol and periodate were immobilized electrostatically on anion exchange resin. By passing sample in suitable eluant through the sensor, CL occurred and isoniazid was quantitated by the CL intensity.

In 2003, Safavi *et al.* showed that both N-bromosuccinimide (NBS)-luminol and N-chlorosuccinimide (NCS)-luminol CL systems can be employed for determination of isoniazid [22]. In their latest paper, they reported that sodium dichloroisocyanurate (SDCC)-luminol and trichloroisocyanuric acid (TCCA)-luminol CL systems can be employed for the determination of isoniazid, based on the strong enhancing effect of isoniazid on these CL systems [23]. This was also applied successfully to determination of isoniazid in pharmaceutical preparations.

In 2007, Xiong *et al.* reported a novel FI-CL sensor using isoniazid molecular imprinted polymer (MIP) as recognition element [24]. The MIP material with specific binding sites for isoniazid was fabricated by thermal radical copolymerization using methacrylic acid (MAA) as functional monomer and ethylene glycol dimethacrylate (EGDMA) as cross-linker. MIP was packed into a flow-cell and absorbed isoniazid on-line, which was sensed by its enhancing effect on the CL reaction between potassium periodate and luminol. After CL reaction, absorbed isoniazid was destroyed and removed leaving cavities on the MIPs for adsorption of the next sample of isoniazid. It has been successfully used to determine isoniazid in human urine sample.

In 2009, Haghighi *et al.* investigated the effect of silver colloidal nanoparticles (AgNPs) on the luminol-isoniazid system [25]. It was found that AgNPs could act as a nanocatalyst on the luminol-isoniazid system to generate CL. This method was proposed for the determination of isoniazid without using any oxidant. The effect of reaction conditions on the CL signal intensity was explored in the FI mode of analysis. The proposed FI-CL system was applied for the determination of isoniazid in a pharmaceutical sample.

3.1.2 Chromatographic method

3.1.2.1 Gas chromatography (GC)

In 2008, Khuhawar *et al.* used capillary GC for the determination of INH and hydrazine (HZ) using phenylhydrazine (PHZ) as internal standard, after derivatization with ethyl chloroformate (ECF) and flame ionization detection (FID) [26]. GC separation was carried out on an HP-5 column (30 m x 0.32 mm i.d.). The elution was carried out at an initial column temperature of 150 ºC for 1 min, then heating up to 250 ºC rate of 10 ºC/min, with nitrogen flow rate of 4 ml/min and a split ratio of 10:1. The method was subsequently applied to the determination of INH and HZ in pharmaceutical preparation.

3.1.2.2 Capillary electrophoresis (CE)

In 1999, You *et al.* applied CE for the simultaneous detection of hydrazine, methylhydrazine and isoniazid with a 4-pyridyl hydroquinone self-assembled microdisk platinum electrode [27]. This modified electrode was found to be very stable and reproducible when used continuously as detector for CE are a period of at least 4 weeks, with no apparent loss of response.

3.1.2.3 Micellar electrokinetic capillary chromatography (MEKC)

In 2002, Acedo-Valenzuela *et al.* reported a method for determination of antitubercular drugs by MEKC with UV detection [28]. The optimal separation were carried out at 30 ºC and 20 kV, using a 40 mM borate buffer and 100 mM sodium dodecylsulphate (SDS) adjusted to pH 8.5. The method was applied to the determination of these compounds in different pharmaceuticals.

3.1.2.4 Liquid chromatography (LC)

Liquid chromatography is one of the popular techniques for determination of isoniazid because of its separation power for samples containing complex matrices. Different mode of liquid chromatography combined with many types of detectors, for separating and monitoring of isoniazid have been developed. In this review, liquid chromatography is divided into three main groups of the detector employed: electrochemical detector, mass spectrometry and UV detector.

3.1.2.4.1 Electrochemical detector

In 1998, Delahunty *et al.* developed a system to measure the concentration of INH in plasma 1 hr after a standard 300 mg dose and to detect the low levels typically found in alveolar cells obtained by bronchoalveolar lavage of subjects maintained on a standard regimen of the drug [29]. Following extraction with a chloroform-butanol mixture, the INH was back-extracted into dilute acid which was subsequently analyzed by HPLC using reversed-phase CN column and an acetonitrile-isopropanol mobile phase. In this system, detection of the drug was accomplished with a sealed coulometric detector (+0.6 V) capable of giving a consistent daily response

without adjustment. The results suggest that this rugged HPLC technique can quantitate INH in plasma with good precision and can be used to estimate the very low INH concentrations found in alveolar cells and cell-free lavage recovered from patients undergoing anti-tuberculosis therapy.

3.1.2.4.2 Mass spectrometry detector (MS)

In 2007, Ng *et al.* reported the development and validation of a sample but highly sensitive LC-MS/MS method for the quantification of INH and its metabolite, AcINH, in rat plasma and homogenate of a rat aloveolar marcrophage cell line, NR8383 [30]. The method was applied to the pharmacokinetic study of INH-loaded microparticles in rats, using Aquasil C_{18}, Thermo Hypersil-Keystone. The mobile phase consisted of methanol and 0.1% formic acid in water.

Bhutani *et al.* proposed LC/MS using C-18 column and mobile phase comprising of water:acetonitrile (96:4) for study of INH and three major degradation products [31]. Stress studies with subsequent LC/MS analysis showed that the drug was decomposed to previously known degradation products. The method proved to be simple, accurate, precise, specific and selective.

In 2009, Huang *et al.* reported a method developed to determine INH in human plasma using hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass spectrometry (MS/MS) [32]. Sample preparation is simple, only protein precipitation with acetonitrile followed by centrifugation. Supernatants are directly injected onto a silica column without reconstitution. The method is simple and fast, with advantageous in respect to instability in plasma and loss of drug during evaporation and reconstitution steps.

3.1.2.4.2 UV-visible spectrometric detector (UV)

UV detection is the most commonly used method for detection of isoniazid following separation by liquid chromatography. Many research groups reported the application of this technique for determination of isoniazid in various samples as shown in Table 3.1.

Table 3.1 Review of UV detection of isoniazid following liquid chromatography separation.

Column	Mobile phase	Matrix	Sample preparation	Detection wavelength (nm)	LOD	Ref.
Whatman Partesil 5 C8 (250 x 4.6 mm i.d.)	Mixture of 50 mM KH_2PO_4 and acetonitrile-isopropanol; 4:1, v/v	Blood, CSF, Urine	Hydrolysis and Incubation	340	-	[33]
Waters Nova-Pak C18 (125 x 3.9 mm i.d.)	Mixture of water-acetonitrile-tri-ethylamine-acetic acid ;600:400:2:1, v/v	Serum	Precipitated with TCA	340	0.02 mg/L	[34]
Waters Nova-Pak C18 (250 x 4 mm i.d.)	0.01 M methanol-sodium phosphate buffer pH 5.2; 65:35, v/v	Plasma, Urine	Extraction	254	-	[35]
Merck-Lichrocart (250 x 4.6 mm i.d)	Mixture of water/acetonitrile ;50:50, v/v	Biological fluids	Filtration	264	91 µg/L	[36]
YMC-ODS (150 x 4.6 mm i.d.)	Mixture of ethanol-water-chloroform-acetonitrile ;55:40:4:1, v/v	Pharmaceutical preparations, Blood	Precipitation, Filtration, Recrystallization	337	-	[37]
Waters Nova-Pak C18 (150 x 3.9 mm i.d)	Mixture of methanol in phosphate buffer (gradient)	Urine	Dilution	254, 475	5 µg/mL	[38]
Merck Lichrospher 100 RP18 (250 x 4 mm i.d)	Mixture of acetonitrile and 50 mM phosphate buffer pH 3.5 (gradient)	Pharmaceutical preparations	Dilution	254,361,265	3.5 µg/mL	[39]

Table 3.2 Review of electrochemical detection of iodide following liquid chromatography separation (continuted).

Column	Mobile phase	Matrix	Sample preparation	Detection wavelength (nm)	LOD	Ref.
Waters Nova-Pak C18 (150 x 3.9 mm i.d.)	Mixture of 10 mM potassium dihydrogenphosphate (pH 6.24) and acetonitrile (gradient)	Blood	Precipitation	215,248,261 ,475	0.6 mg/L	[40]
Waters X-terra RP18 (150 x 3 mm i.d.)	Mixture of 20 mM 1-hexanesulfonic acid sodium salt solution and ACN (gradient)	Plasma	Deproteination, Evaporation	290	0.24 µg/mL	[41]
Waters Acquity Shield RP18 (50 x 2.1 mm i.d.)	50 mM Phosphate buffer (pH 7.0)	Pharmaceutic al preparations	Dilution	254	-	[42]

CHAPTER IV
MATERIAL AND METHODS

4.1 Instrumentation

Instrument used in the analytical experiments, preparation of solutions and sample treatments are given below in Table 4.1.

Table 4.1 List of instrumentation.

Instrument	Model	Company
Analytical balance	Mettler Model AJ 150 (sens. 0.1 mg)	Zürich (Switzerland)
Micropipette	Eppendorf Model Research	Hamburg (Germany)
pH meter	Thermo Orion Model 420 A+	Beverly Massachusetts (USA)
Ultrasonic bath	NEY Model ULTRAsonik 280H	Yucaipa CA (USA)
Hot plate	Heidolph MR 3001	Schwabach (Germany)

4.1.1 Spectrophotometer

For UV-Visible absorbance studies of the aldehydes and their hydrazones, Perkin Elmer model Lamda 25 UV-Vis spectrophotometer was used.

4.1.2 HPLC instrument

HPLC instrument used in the analytical experiments, are given below in Table 4.2.

Table 4.2 The liquid chromatography system with UV-Visible detector.

Instrument	Model	Company
Pump	Binary gradient pump with on line degasser 1100 series	Agilent (Germany)
Manual Injector	7725i with 20 µL injection loop	Rheodyne (USA)
Detector	Diode-Array Detector 1100 Series	Agilent (Germany)
Data system	HP Chemstation Version A.07.01	Agilent (Germany)

4.2 Reagents and Materials

4.2.1 Reagents

Chemicals and solvents were purchased from various suppliers as listed in Table 4.3. All solvents used in this work were HPLC grade. HPLC grade water (18.2 MΩ/cm) purified by Milli-Q water purification system (Waters, Massachusett, USA) was used for preparation of all solutions.

Table 4.3 List of chemicals and reagents.

Chemicals and reagents	Suppliers
Acetic acid glacial	J.T Beaker (USA)
Ethylenediaminetetraacetic acid disodium salt (EDTA, 99%)	Merck (Germany)
Hydrochloric acid (37 % v/v)	J.T Beaker (USA)
Isoniazid	Fluka (Switzerland)
Methanol (HPLC grade)	Lab-scan (Thailand)
2-Nitrobenzaldehyde	Fluka (Switzerland)
4-Nitrobenzaldehyde	Merck (Germany)
Salicylaldehyde	Fluka (Switzerland)
Sodium acetate (99% assay)	Merck (Germany)
Sodium hydroxide, pellet (99% assay)	Merck (Germany)

Table 4.4 List of hydrazones.

Chemicals and reagents	Suppliers
Cinnamaldehyde Isonicotinoyl Hydrazone (CIH)	In house synthesis
2-Nitrobenzaldehyde Isonicotinoyl Hydrazone (2-NIH)	In house synthesis
4-Nitrobenzaldehyde isonicotinoyl Hydrazone (4-NIH)	In house synthesis
Salicylaldehyde Isonicotinoyl Hydrazone (SIH)	Synthesis [43]

4.2.2 Materials

Materials used in this work are given below.

4.2.2.1 HPLC Column

Eclipse® XDB-C18 column (150 x 4.6 mm i.d., 5µm) with an Eclipse® XDB-C18 guard column (12.5 x 4.6 mm i.d., 5 µm) supplied by Agilent Tecnologies (Waldbornn, Germany) was used.

4.2.2.2 Membrane Filter

Cellulose acetate filter membrane (0.45 µm pore size, 47 mm i.d.) supplied by Agilent Tecnologies (Weldbornn, Germany) were used to remove particular materials from acetate buffer used for mobile phase preparation (Section 4.3.1).

Nylon filter membrane (0.45 µm pore size, 47 mm i.d.) supplied by Thermo-Hypersil (Bellefonte, Pennsylvania, USA) were used to remove particulate material from urine samples (Section 4.10).

4.3 Preparation of Reagents

4.3.1 Acetate buffer solution, 10 mM, pH 4.0, with 2 mM EDTA

Acetate buffer solution (10 mM) pH 4.0 containing 2 mM EDTA was prepared by combining 68.5 mL of 10 mM sodium acetate solution, 431.5 mL of 10 mM acetic acid and 20.0 mL of 0.1 M EDTA. This mixture was then made to final volume of 1,000.00 mL with Milli-Q water. The solution was filtered through 0.45 µm membrane filter to remove discarded materials and then degassed in an ultrasonic bath for 10 min before use.

4.3.2.1 Acetic acid solution, 200 mM

Glacial acetic acid, 6.00 g, was weighed and transferred to 500.00 mL volumetric flask and then made up to volume with Milli-Q water.

4.3.2.2 Acetic acid solution, 10 mM

This solution was prepared by transferring 25.00 mL of stock 200 mM acetic acid and making to final volume of 500.00 mL with Milli-Q water. This solution was used as stock solution to prepare 10 mM acetate buffer (Section 4.3.1).

4.3.2.3 EDTA solution, 0.1 M

Solid disodium EDTA (3.72 g) was dissolved in Milli-Q water and then made up to volume in a 100.00 mL volumetric flask. This solution was used as a stock solution for preparation of aqueous mobile phase (Section 4.3.1).

4.3.2.4 Sodium acetate solution, 10 mM

Solid sodium acetate (0.6804 g) was dissolved in Milli-Q water and made up to 500.00 mL. This solution was used as stock solution for preparation of acetate buffer (Section 4.3.1).

4.3.2.5 Sodium hydroxide solution, 6 M

Sodium hydroxide pellet (ca. 6 g) was dissolved in Milli-Q water and made up to 25.00 mL. This solution was used for adjustment of pH of reagent solution.

4.4 Preparation of Standard Reagents, and Standard Solutions

4.4.1 Stock standard isoniazid (INH), 1000 µg/mL

INH 2.5 g (known accurate weight) was dissolved and made up to 25.00 mL in a volumetric flask with Milli-Q water.

4.4.2 Stock cinnamaldehyde (CA) standard solution, 1 M in methanol

Accurate weight (3.43 g) of cinnamaldehyde was dissolved in 25.00 mL of methanol.

4.4.3 Stock salicylaldehyde (SA), 2-nitrobenzaldehyde (2-NBA) and 4-nitrobenzaldehyde (4-NBA), 1 M in methanol

All stock standard were prepared in the same manner as described in Section 4.4.2 except that 3.053 g of SA, 3.778 g of 2-NBA and 3.778 g of 4-NIH were used, respectively.

4.4.4 Stock cinnamaldehyde isonicotinoyl hydrazone (CIH) standard solution, 1000 µg/mL in methanol

The recrystallized CIH solid (2.5 g) was dissolved and made up to 25.00 mL with methanol.

4.4.5 Stock salicylaldehyde isonicotinoyl hydrazone (SIH), 2-nitrobenzaldehyde isonicotinoyl hydrazone (2-NIH) and 4-nitrobenzaldehyde isonicotinoyl hydrazone (4-NIH) standard solution, 1000 µg/mL in mathanol

All stock standards were prepared in the same manner as described in Section 4.4.4.

4.4.6 Working isoniazid (INH) standard solution

A 2.5 mL of stock standard INH (1000 µg/mL) (Section 4.4.1) was transferred to 25.00 mL volumetric flask and then made up to final volume with human urine for preparation of 100 µg/mL INH standard solution. This solution was used for the optimization studies in the derivatization of INH with aldehyde to form the hydrazone (Section 4.7).

4.4.7 Working cinnamaldehyde (CA) standard solution

A 1.25 mL of stock standard CA (1 M) (Section 4.4.2) was transferred to 25.00 mL volumetric flask and made up with methanol for preparation of 50 mM CA standard solution.

4.4.8 Working salicylaldehyde (SA), 2-nitrobenzaldehyde (2-NBA) and 4-nitrobenzaldehyde (4-NBA) standard solution

All working standard solution was prepared as described as in Section in 4.4.7.

4.4.9 Reference cinnamaldehyde isonicotinoyl hydrazone (CIH) standard solution

An aliquot of 1 M stock CIH (Section 4.4.4) was diluted with mobile phase, 45:55 (v/v) methanol-acetate buffer (10 mM , containing 2 mM EDTA, pH 4.0), to give 10 µg/mL reference solution for the chromatographic studies of the determination of isoniazid as the form of hydrazone. This reference solution was used in the study of the hydrazone (Section 4.5.1).

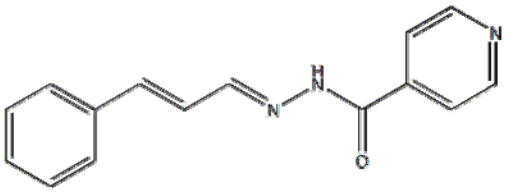
4.4.10 Reference salicylaldehyde isonicotinoyl hydrazone (SIH), 2-nitrobenzaldehyde isonicotinoyl hydrazone (2-NIH) and 4-nitrobenzaldehyde isonicotinoyl hydrazone (4-NIH) standard solution

All working standard solution was prepared as described in Section 4.4.9.

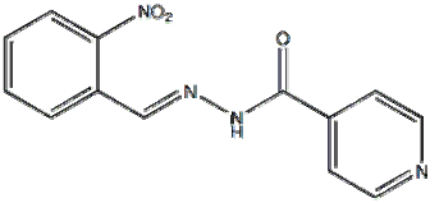
4.5 Synthesis of Isonicotinoyl Hydrazone as Reference Compounds

4.5.1 Isonicotinoyl hydrazones

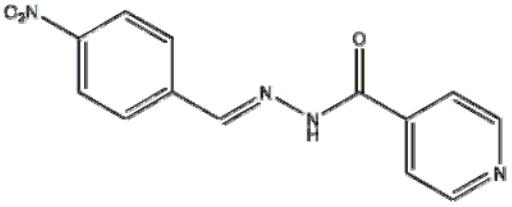
Three hydrazones were prepared in a good yield by mixing together equimolecular quantities of nearly saturated methanol solutions of isonicotinic acid hydrazide and hydrazone. The mixture was heated on a hot plate at 45-50 ºC for 20 minutes and allowed to stand for 24 hours at room temperature. The yellow crystalline product was filtered from the solution, washed with cold methanol and air dried. The product was recrystallized at least 2 times using hot methanol. The structures of hydrazone are shown in Figure 4.1. The different starting materials are listed in Table 4.5.



Cinnamaldehyde isonicotinoyl hydrazone (CIH)



2-Nitrobenzaldehyde isonicotinoyl hydrazone (2-NIH)



4-Nitrobenzaldehyde isonicotinoyl hydrazone (4-NIH)

Figure 4.1 Structures of isonicotinoyl hydrazone

Table 4.5 Starting materials for hydrazone synthesis

Starting materials	Product
2-Nitrobenzaldehyde(2-NBA) / isoniazid	2-Nitrobenzaldehyde isonicotinoyl hydrazone (2-NIH)
4-Nitrobenzaldehyde (4-NBA) / isoniazid	4-Nitrobenzaldehyde isonicotinoyl hydrazone (4-NIH)
Cinnamaldehyde (CA) / isoniazid	Cinnamaldehyde isonicotinoyl hydrazone (CIH)

4.6 Sample Preparation

Blank urine samples from normal subjects were filtered through 0.45 µm nylon membrane filter before use.

4.7 Derivatization of Hydrazone in Urine

The following aldehydes to form hydrazone aldehydes were studied as derivatization reagent for isoniazid: cinnanmaldehyde (CA), salicylaldehyde (SA), 2-nitrobenzaldehyde (2-NBA) and 4-nitrobenzaldehyde (4-NBA). Experiments to select the aldehyde and the optimal conditions in urine are described below.

Urine containing aldehyde and INH at mole ratio of 20:1 was prepared as follow. A 0.2 mL aliquot of stock standard solution of aldehyde (75.7 mM in methanol) was added to 1.0 mL of filtered urine containing INH at concentration 2-100 µg/mL, and then 140 µL of Milli-Q water. These solutions were prepared for study of the effect of concentration of acid, temperature and time of reaction.

4.7.1 UV-Visible spectrum

The UV-Visible spectrum of the aldehydes and synthesized isonicotinoyl hydrazones were measured to find the suitable monitoring wavelength for detection in HPLC system. The absorption spectrum of each compound was measured in 45:55 (*v/v*) methanol-10 mM acetate buffer (pH 4.0, containing 2 mM EDTA).

4.7.2 Concentration of acid

The effects of various concentration of acid in derivatization steps to derivatize INH to hydrazone at room temperature were studied.

4.7.3 Temperature

The effect of temperature on the formation of hydrazone was studied. The temperatures were room temperature and heat at 50 ˚C.

4.8 Chromatographic Condition

HPLC with isocratic elution was employed for the separation of hydrazone in human urine samples on a silica reverse-phase column [44]. The column was first equilibrated for 30 min with mobile phase before analysis. The following chromatographic condition was employed for this study.

Table 4.6 Chromatographic condition for the study of hydrazones

Parameter	**Condition**
Mobile phase	45:55 (v/v) methanol-acetate buffer (10 mM , containing 2 mM EDTA, pH 4.0)
Column	Endcapped C18 reversed-phase column (Eclipse® XDB –C18) with an Eclipse® XDB-C18 guard column (150 x 4.6 mm i.d., 5 µm)
Flow rate	1.0 mL/min
Injection volume	20 µL
Detection	UV absorbance at 289 nm

4.9 Analytical Performance

To validate the method for determination of isoniazid in form of the hydrazone, the following parameters were measured.

4.9.1 Linearity

Calibration curves of isoniazid were investigated, with standard isoniazid spiked in urine and derivatized with excess aldehyde in concentration range 2.0 to 100.0 μg/mL.

4.9.2 Precision

The repeatability of the method was determined by analysis at five different concentrations. Within-day precision was determined by analysis of two samples at each concentration level within 1 day, each sample injected three times. To investigate between-day precision, the solutions were analyzed over a period of three consecutive days. The precision was expressed as the relative standard deviation (%RSD) of the peak area.

4.9.3 Limit of detection (LOD)

The limit of detection is the lowest concentration of an analyte that can be detected. In this work, the detection limit was calculated by the method as proposed by Miller and Miller [45]. The detection limit is equal to the calculated intercept of the regression line i.e. the blank value, plus three times the standard error of y-residuals, i.e. standard deviation of blank value, converted to concentration using the regression equation.

4.10 Optimal procedure for sample preparation

The following steps describe the final standard procedure for sample preparation.

1. Pipette 1.0 mL of filtered urine (sample or spiked urine) to brown vial
2. Add 60 µL of 1 M HCl
3. Add 200 µL of 75.7 mM SA
4. Add 140 µL with Milli-Q water
5. Heat at 50 ºC for 10 min on a water bath
6. Cool to room temperature
7. Load 60 µL into Rheodyne 20 µL injection loop
8. Inject into HPLC column

4.11 Recovery study

A 0.1 mL aliquot of filtered patient urines or standard isoniazid solution was added to give a concentration of 6 µg/mL of added INH. The spiked samples were derivatized and analyzed with HPLC at the optimum condition as given in Table 5.2. The recovery of isoniazid was calculated using the amounts of isoniazid measured in spiked sample and sample. The % recovery is calculated from the following equation:

$$\% \text{ recovery} = \left[\frac{C_{spiked} - C_{sam}}{C_{add}} \right] \times 100 \qquad \ldots(4.1)$$

Where, C_{spiked} is the measured concentration of isoniazid in spiked sample, C_{sam} is the measured concentration of isoniazid of sample, C_{add} is the calculated concentration of isoniazid added to sample.

4.12 Hydrolysis

For the study of the metabolite of INH , acetyl isoniazid, a hydrolysis step was carried out to convert the metabolite to INH [33] The hydrolysis step was carried out as follow.

1. Pipette 1.0 mL of filtered urine (sample or spiked urine) in brown vial
2. Add 100µL of 6 M HCl
3. Heat at 80 ºC for 60 min
4. Add 60 µL of 1 M HCl
5. Add 200 µL of 75.7 mM SA
6. Add 40 µL with Milli-Q water
7. Heat at 50 ºC for 10 min on a water bath
8. Cool to room temperature
9. Load 60 µL into Rheodyne 20 µL injection loop
10. Inject into HPLC column

4.13 Stability

Three different concentrations (low, medium, high) of the isoniazid spiked in human urine samples were stored at -20 ºC to study the effect of storage time on the stability. The spiked samples were prepared as in Section 4.10. The stability profile of isoniazid is given as the plot of the chromatographic peak area with storage time.

CHAPTER V
RESULTS AND DISCUSSION

This chapter describes results of the development of HPLC procedure with UV-Visible absorbance detection for separation and determination of isoniazid, in the form of hydrazone, in human urine. The studies include selection of detection wavelength for the various hydrazones, and the mole ratio between aldehyde and isoniazid. Various validation parameters were evaluated to ensure the suitability of the method.

5.1 Preliminary results

In the preliminary study of the determination of INH in form of hydrazone, the chromatographic condition of Sameenoi, Y. [44] was used. The direct determination of INH in urine has the problem of matrices which elute at about 1-5 min (Figure 5.1 (a)). This is the same retention time range as for INH (Figure 5.1 (b)). A convenient way for determination of INH is to derivatize with aldehyde to give the hydrazone as the detection compound.

Figure 5.1 (c), shows the chromatogram of pure SIH. The retention time of SIH is far from the urine matrices. This work was to select a suitable aldehyde, mole ratio between aldehyde and isoniazid and time of reaction to provide complete conversion to hydrazone.

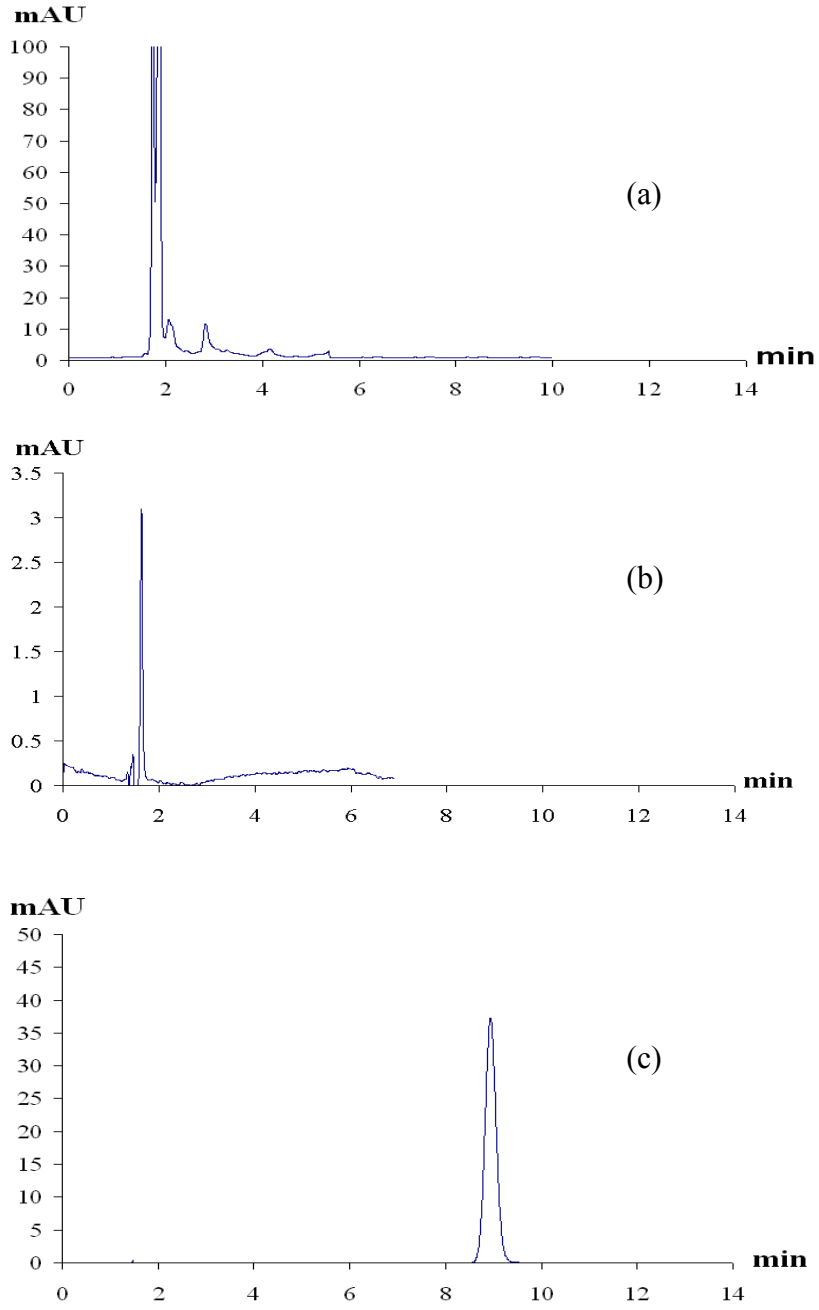


Figure 5.1 Chromatogram of (a) urine, (b) 0.036 µM of INH and (c) 0.41 µM of SIH. Column Agilent ZORBAX Eclipse® XDB 4.6 x 150 mm i.d., 5 µm was used with an isocratic mobile phase system 45:55 (*v/v*) methanol-10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and analyte monitored at 289 nm.

5.2 Selection of aldehyde for derivatization of isoniazid

In this experiment, four aldehydes were selected with cyclic and double bonds: cinnamaldehyde, salicylaldehyde, 2-nitrobenzaldehyde and 4-nitrobenzaldehyde. The hydrazone of the four aldehydes were expected to absorb in the uv-visible region with high molar absorptivity.

5.2.1 UV-Visible spectrum of pure hydrazone

The UV-Visible spectra of INH and the pure hydrazones, CIH, SIH, 2-NIH and 4-NIH, were measured to find the suitable monitoring wavelength. The absorption spectrum of each compound was measured using 45:55 (v/v) methanol -10 mM acetate buffer (pH 4.0, containing 2 mM EDTA) as solvent.

Figure 5.2 shows the absorption spectrum of INH which showed absorption at 250 nm. Figure 5.3 shows the absorption spectrum of (a) 0.021 μM SIH and (b) 0.037 μM 2-NIH, respectively. The wavelength of 289 nm was selected for detection of SIH and 2-NIH. Figure 5.4 shows the spectra of (a) 0.037 μM 4-NIH and (b) 0.010 nM, respectively. The suitable wavelength for these two hydrazone is 325 nm.

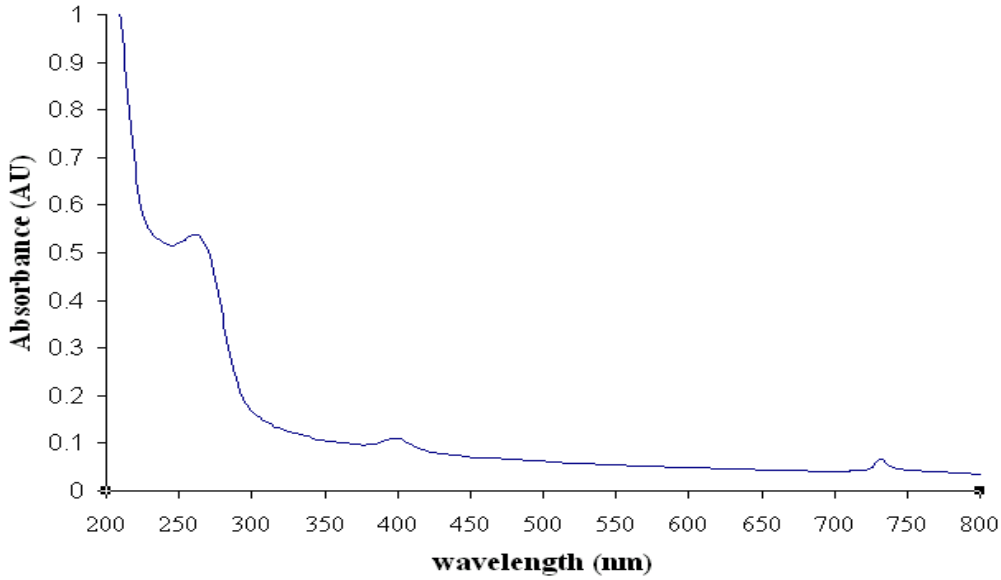


Figure 5.2 Absorption spectrum in the range 200-800 nm of 0.07 mM INH in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0) after subtraction of the solvent spectrum.

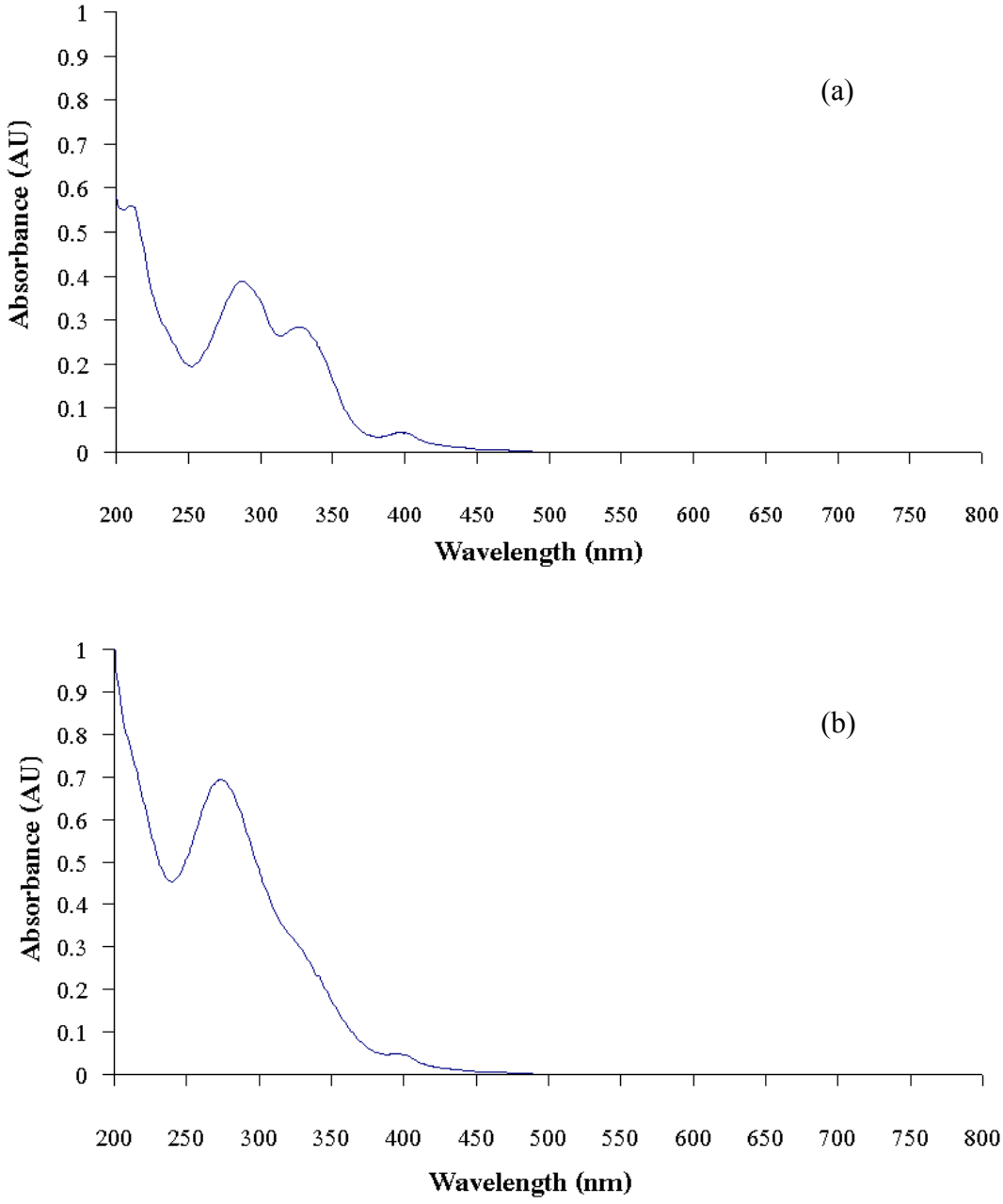


Figure 5.3 Absorption spectrum in the range 200-800 nm of (a) 0.021 μM SIH and (b) 0.037 μM 2-NIH in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0) after subtraction of the solvent spectrum.

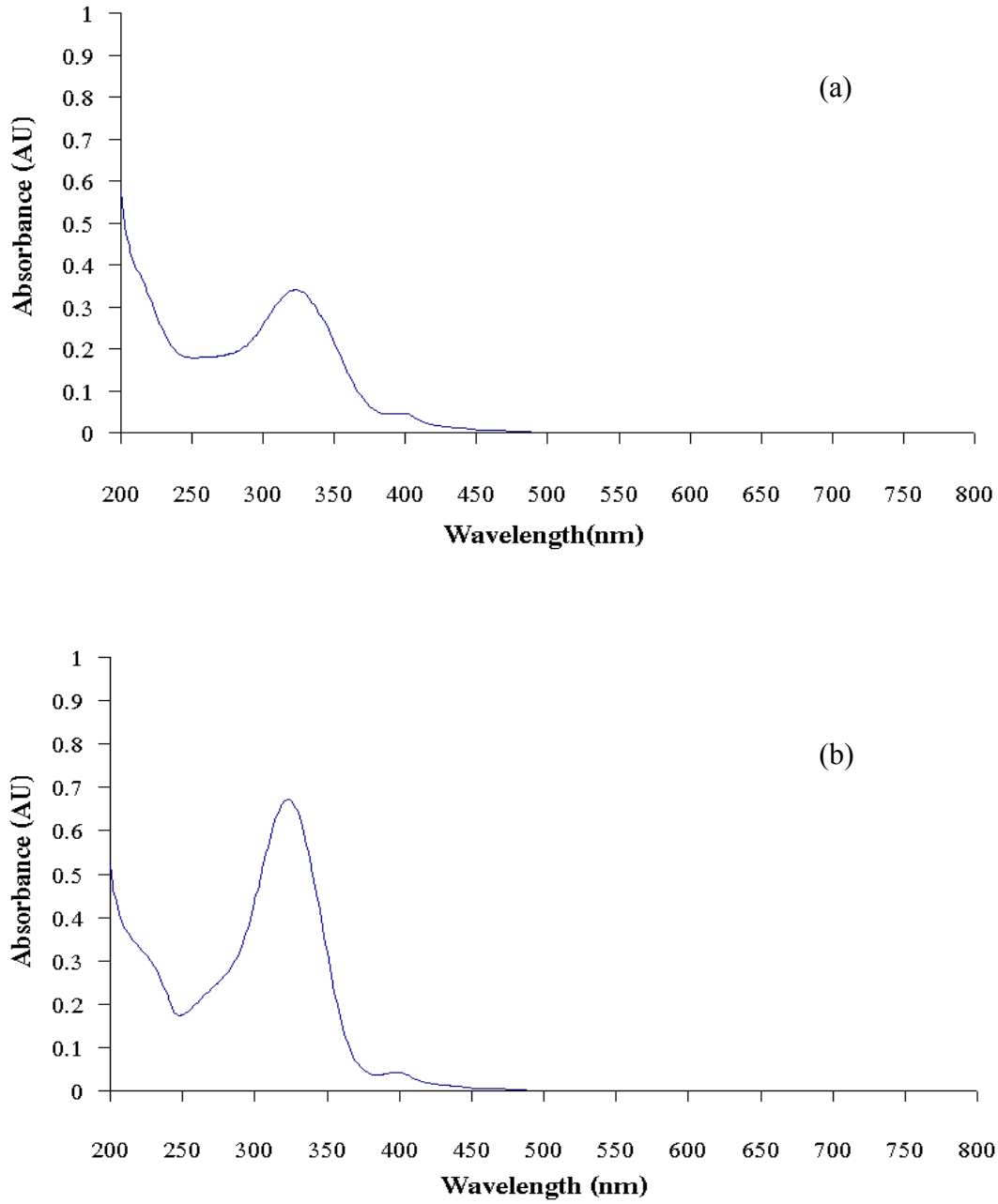


Figure 5.4 Absorption spectrum in the range 200-800 nm of (a) 0.037 µM 4-NIH and (b) 0.010 µM CIH in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0) after subtraction of the solvent spectrum.

Table 5.1 shows the values of molar absorptivity which is calculated from A = εbc. The results show that CIH has the highest molar absorptivity.

Table 5.1 Data of molar absorptivity of the hydrazones

Compound	Molar absorptivity (ε) (M^{-1} cm^{-1})	Wavelength (nm)
SIH	19.0×10^6	289
2-NIH	18.9×10^6	289
4-NIH	9.4×10^6	325
CIH	70.0×10^6	325

5.2.2 Derivatization Parameters

In order to obtain complete derivatization of isoniazid in the urine, excess aldehyde must be employed. Also the concentration of acid, temperature and derivatization time must be optimised. In this section, two aldehydes, SA and CA were selected for study. Preliminary studies showed that nitro-aldehydes require must longer derivatization time.

5.2.2.1 Concentration of acid in derivatization steps

The study of Seifart *et al.* [33] indicated that acidic condition was necessary for the reaction to take place. We varied the concentration of hydrochloric acid between 0.05 mM to 0.3 mM. Figure 5.5 shows the peak area with time. The results are different from Seifart *et al.* [33] who employed 0.3 mM acid. In our experiment the results, show that 0.05 mM HCl gave higher peak area than for 0.3 mM HCl for both of CA and SA.

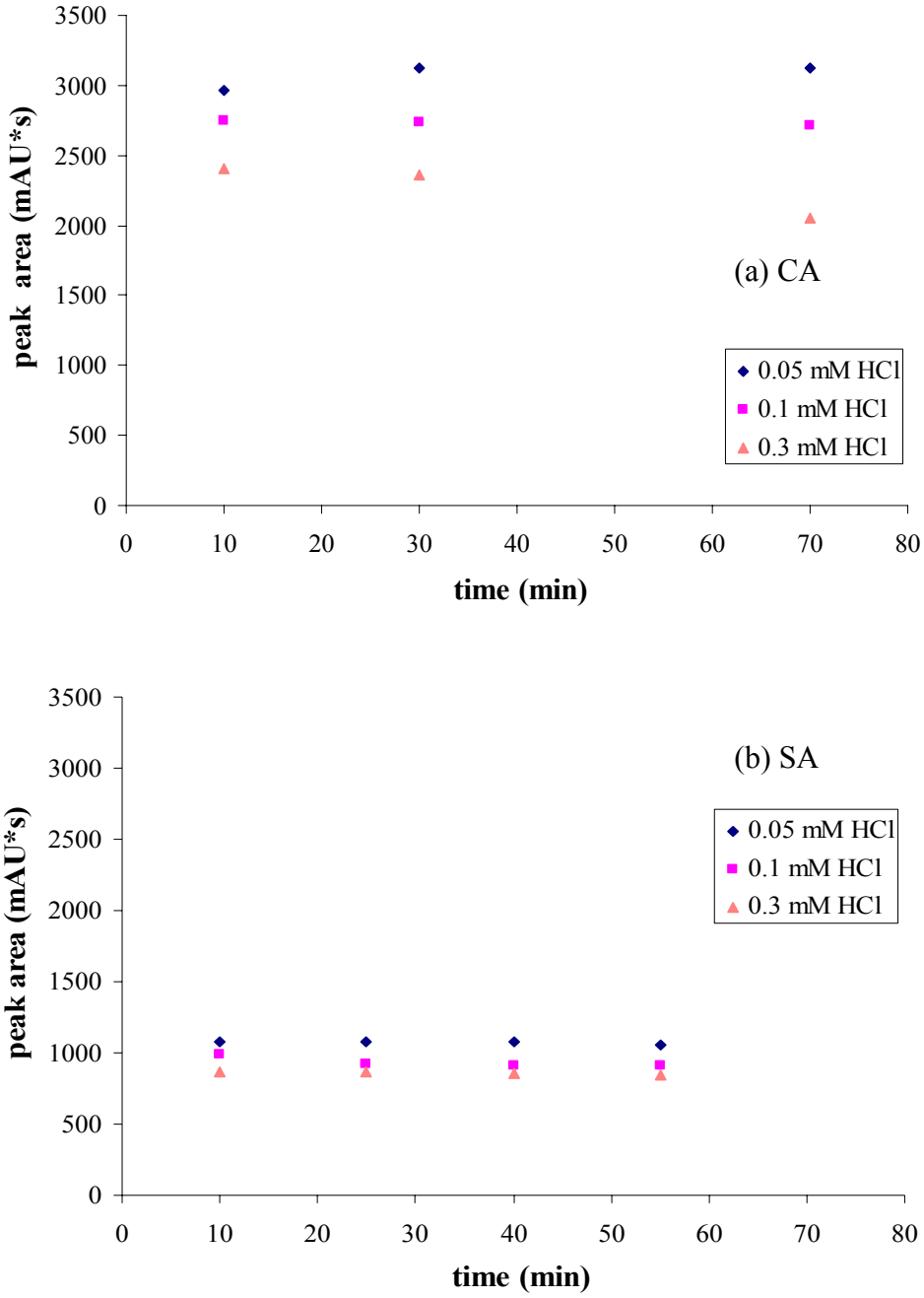


Figure 5.5 Kinetics of peak area with time, for various concentration of acid at room temperature. An endcapped C18 bonded-silica column (Eclipse® XDB-C18, 150 x 4.6 mm i.d., 5 µm) was used with an isocratic mobile phase system (45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0)). Flow rate was 1.0 mL/min and SIH was monitored at 289 nm and CIH at 325 nm.

5.2.2.2 Effect of temperature

To derivatize INH to form the hydrazone a mole ratio between aldehyde:INH of 20:1 and room temperature was selected by Seifart *et al.* [33]. The concentration of INH was 100 µg/mL because this is the highest concentration expected in patient human urine. The reaction was first carried out at room temperature. The results are shown in Figure 5.6.

In the study of the temperature for derivatizations of INH, it was found that the suitable temperature for derivatize INH with CA was 50 ˚C for 30 min. The result is in contrast Seifart which used at room temperature for 10 min. Using SA, suitable temperature was 50˚C for 10 min. Considering between CA and SA, it was found that SA gave the better results because it require only 10 min at 50 ˚C for complete derivatization. Chromatogram of CIH and SIH are shown in Figure 5.7.

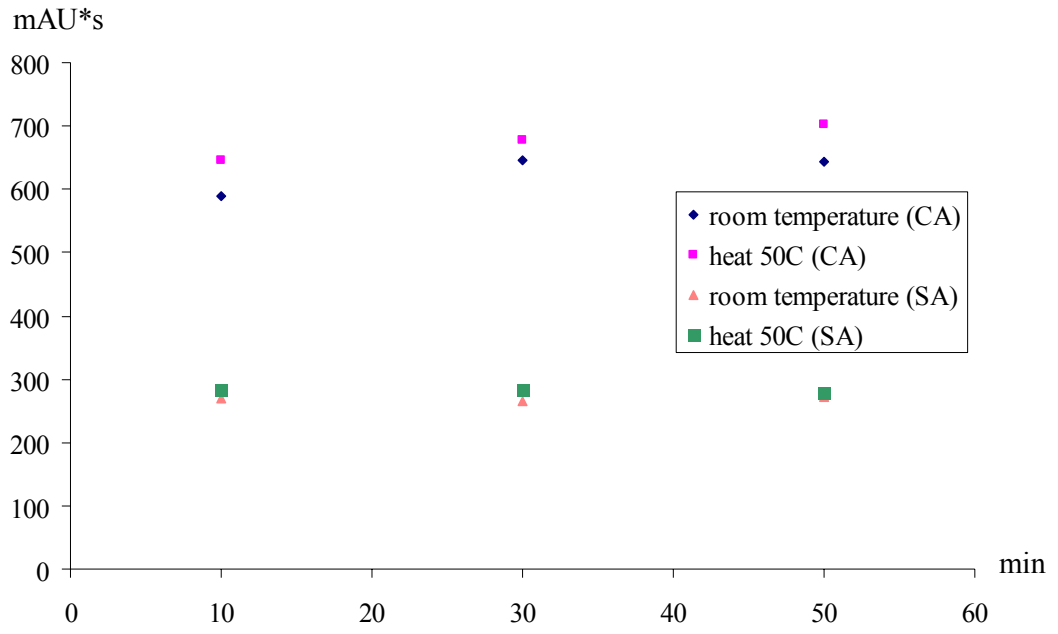


Figure 5.6 Kinetics of peak area of aldehyde (mole ratio 20:1). Conditions are as in Figure 5.5.

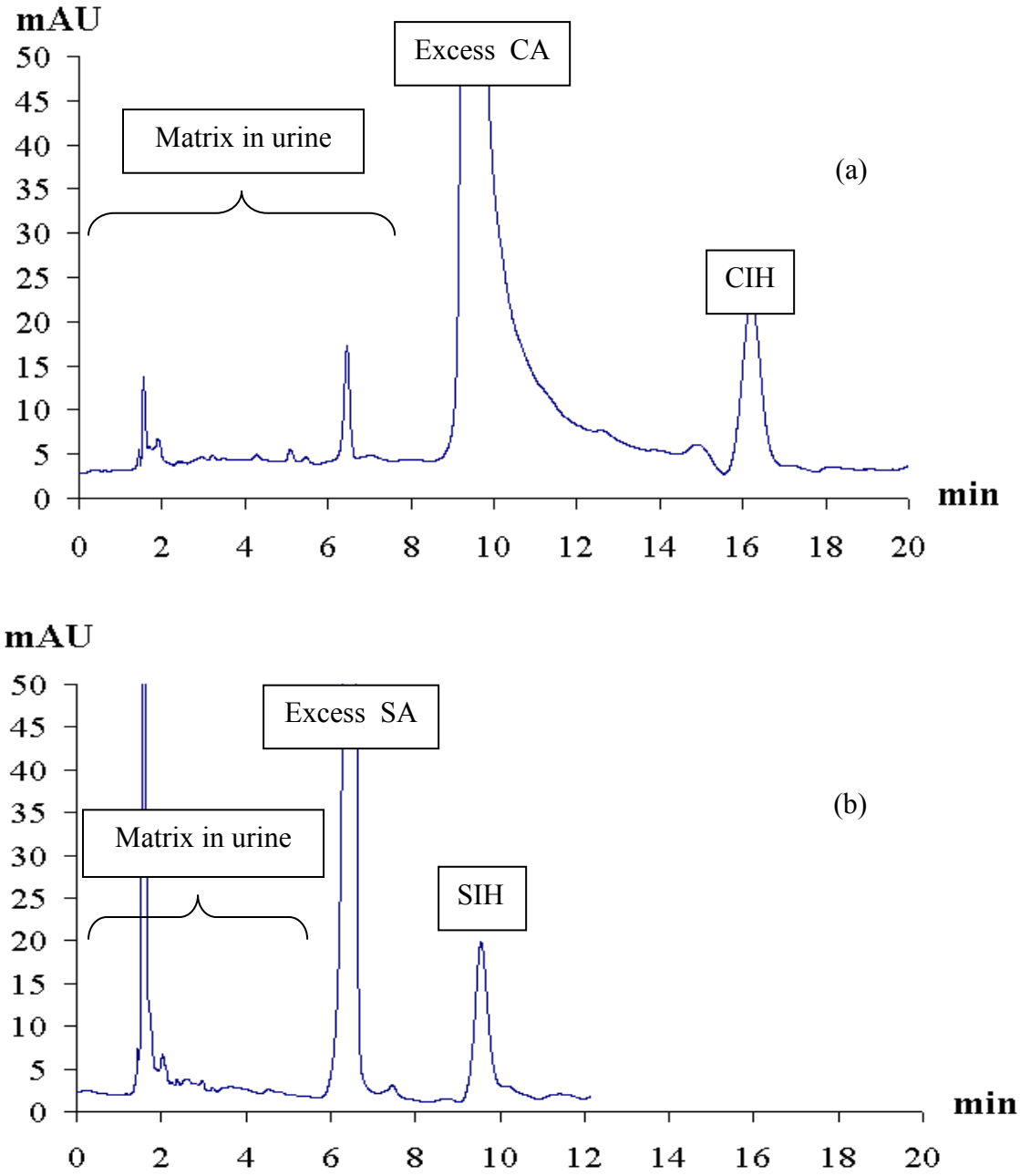


Figure 5.7 Chromatogram of (a) CIH and (b) SIH (mole ratio of aldehyde to INH = 20:1), at room temperature. Conditions are as in Figure 5.6. First peak is excess aldehyde and the second is hydrazone .

5.2.3 Conclusion

It was found that salicylaldehyde is the best reagent to derivatize INH to form hydrazone. It reacts faster and has shorter retention time than cinnamaldehyde. This result contrasted with the work of Seifart *et al.* [33], where the reaction time of only 10 min at room temperature was employed. Also the study did not show any data for other reaction time or temperature. Therefore salicylaldehyde was selected as the derivatizing reagent at mole ratio of aldehyde:INH of 20:1 with heating at 50 ºC for 10 min, as the find derivatizing condition.

5.3 Optimization of mobile phase composition for analysis of SIH

In the previous experiments to select the suitable aldehyde, an isocratic mobile phase system of 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0) was used. In this section, mobile phase compositions were studied to achieve a satisfactory separation between excess salicylaldehyde and hydrazone in urine samples.

The composition of methanol was increased from 45 and 50%. As shown, in Figure 5.8 increasing the ratio of organic solvent in the mobile phase decreased the retention time of the hydrazone. Although using high ratio of methanol (see Figure 5.8 (b)) gave shorter retention time for the low concentration of 2 μg/mL, the SIH peak overlapped with excess SA peak. Consequently, the optimal mobile phase for analysis of isoniazid in the form of SIH is 45:55 (v/v) methanol – acetate buffer containing 2 mM EDTA (pH 4.0) (see in Figure 5.8 (a)).

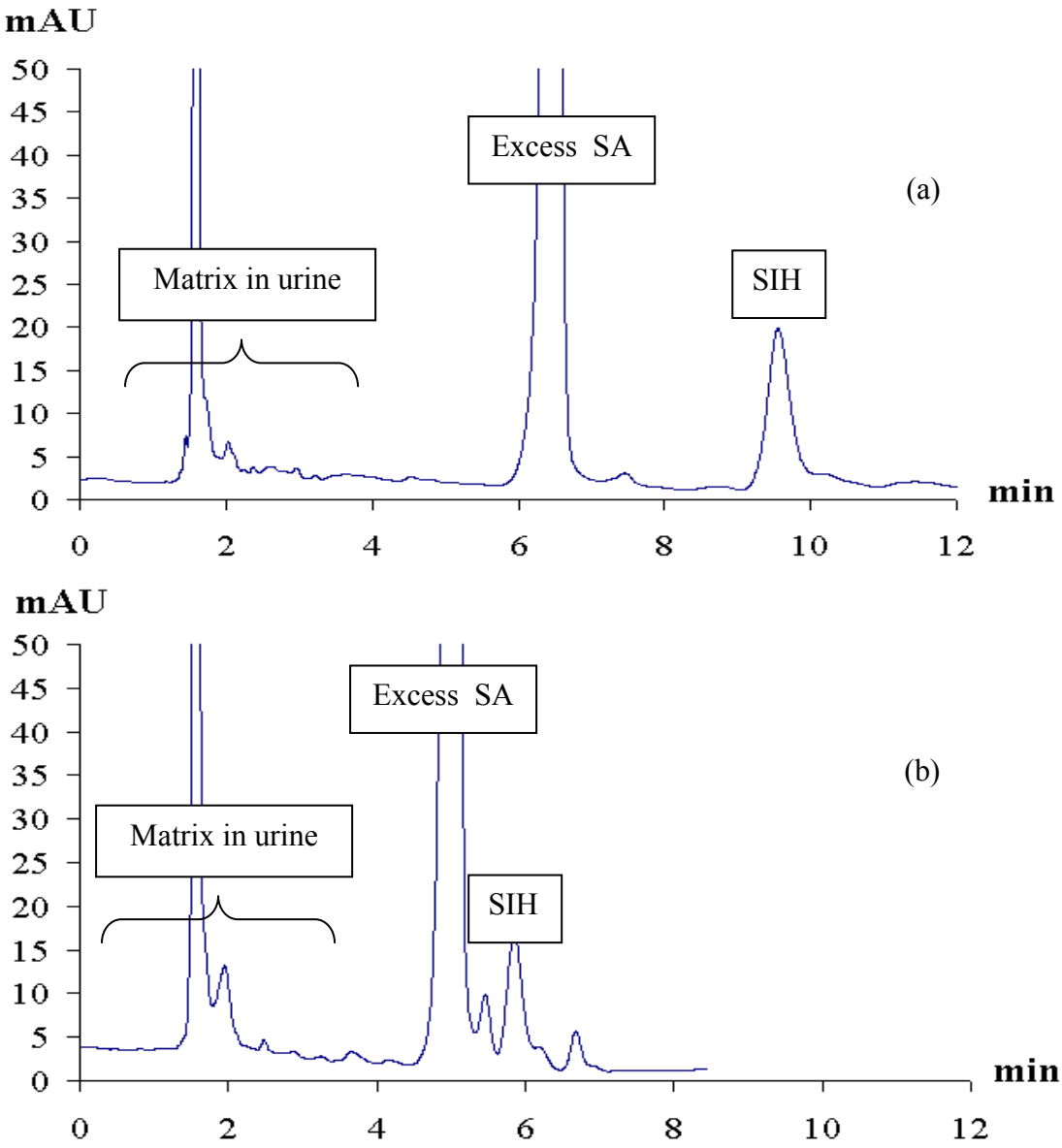


Figure 5.8 HPLC chromatograms of 2 µg/mL INH, in spiked urine,using isocractic elution at various ratios of methanol to 10 mM acetate buffer containing 2 mM EDTA (pH 4.0) (a) 45:55,*v/v* and (b) 50:50, *v/v*. Other conditions are as in Figure 5.5. First peak is an excess salicylaldehyde (SA) and the second is hydrazone (SIH).

5.4 Analytical Performance

Method validation ensures that the method performance is suitable for intended use. Various validation parameters were evaluated for HPLC method using the optimized chromatographic condition. Results for linearity, precision, sensitivity, detection limit and recovery are given in the following sections.

5.4.1 Linearity

The linear dependence of peak area for absorbance at 289 nm with isoniazid concentration was evaluated for spiked normal human urine samples using the procedure described in Section 4.9.1. The spiked spike urine samples were at five different concentrations of isoniazid in the range 2-100 µg/mL. The data were analyzed by least-squares linear regression and the calibration line is presented in Figure 5.9.

Excellent linearity was obtained over the entire concentration range and correlation coefficients (r^2) was greater than 0.999.

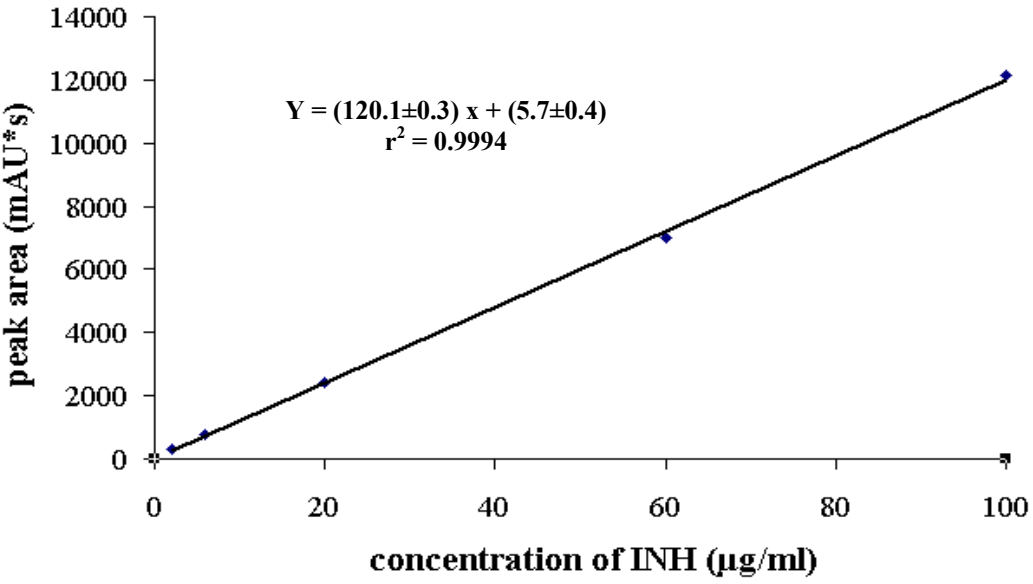


Figure 5.9 The calibration curve of standard isoniazid solution spiked in human urine in the range of 2-100 µg/mL. The chromatographic conditions are as in Figure 5.5.

5.4.2 Precision

Table 5.2 shows, the within-day precision of determination, as indicated by the relative standard deviation (RSD) value, for spiked urine samples. The between-day RSD values obtained for urine was between 0.36-3.84% over the entire concentration range 2-100 μg/mL. The results showed that the proposed HPLC method has satisfactory precision for the analysis of isoniazid in human urine samples.

Table 5.2 Method precision for chromatographic analysis of isoniazid spiked in human urine[a]

Concentration (μg/mL)	RSD (%) (n=6)	
	Within-day	Between-day
2	2.06	3.84
6	0.64	1.09
20	0.97	1.59
60	0.05	1.65
100	0.33	0.36

[a]Standard isoniazid solution were spiked in normal human samples at each concentration level. Two spiked samples at each concentration level were determined with three replicate injections.

5.4.3 Limit of detection (LOD)

The value of limit of detection (LOD) indicated that the isoniazid can be determined down to the 1.30 μg/mL for urine. The method for calculation of the LOD is shown in Appendix C.

5.5 Application to urine samples

Spot human urine samples from patients who are taking isoniazid drug were collected at Ramathibodi Hospital and stored at –20 °C. Before analysis, the samples were thawed at room temperature and cleaned up using the procedure described in Section 4.10.

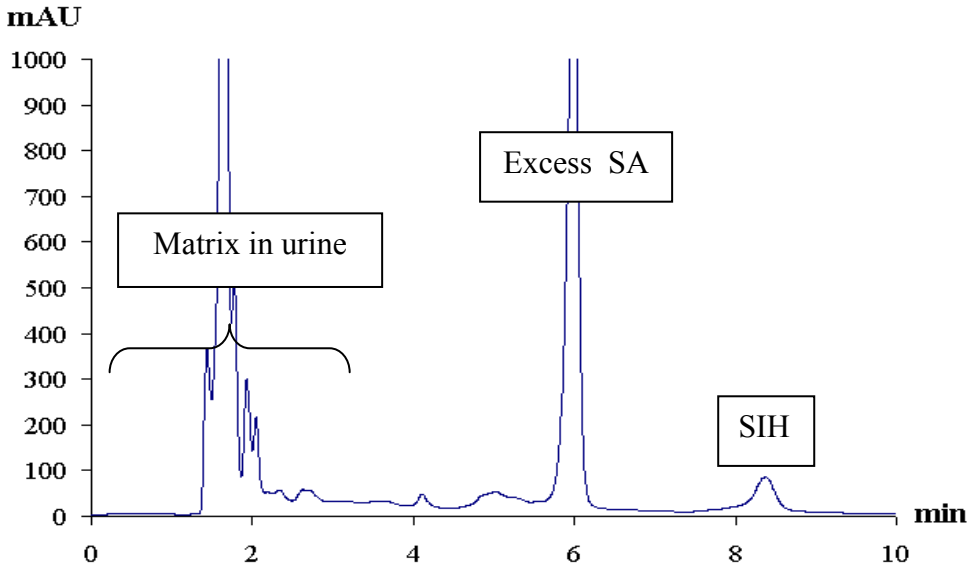


Figure 5.10 Chromatogram of a patient urine sample A derivatized, with 10.9 mM SA. An endcapped C18 bonded-silica column (Eclipse® XDB-C18, 150 x 4.6 mm i.d., 5 μm) was used with isocratic mobile phase system (45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0)). Flow rate was 1.0 mL/min and absorbance monitored at 289 nm.

The results show that this method can be used to measure INH in form of salicylaldehyde isonicotinoyl hydrazone (SIH) with no interference from the matrices in urine. The results of INH concentration in four patients urine are shown in Table 5.3.

Table 5.3 Data of patient urine samples

Urine	Isoniazid Concentration (µg/mL)	RSD% (n=6)*
Patient urine A	5.0	1.23
Patient urine B	59.6	1.02
Patient urine C	14.3	0.84
Patient urine D	29.1	1.29

* (2 aliquots with 3 replicate injections)

5.5.1 Analytical Recovery

Recovery study was performed by analyzing real human urine samples spiked with a known amount of isoniazid standard within the working range. Recovery for the added isoniazid was calculated as given in Section 4.1. The mean value is shown in Table 5.4. The average recovery is in the range 103.5-105.3%. Therefore, this method shows good accuracy.

Table 5.4 Analytical recovery for determination of isoniazid spiked in urine samples

Sample	Isoniazid concentration (µg/mL)			Recovery (%) (n=6)*
	Present	Added	Found	
A	5.0±1.2	6	11.2±1.0	**101.8±0.8**
B	59.6±1.0	6	65.9±0.3	**105.3±0.4**
C	14.3±0.8	6	20.4±1.2	**100.7±0.5**
D	29.1±1.3	6	35.3±1.0	**103.5±0.4**

* (2 aliquots with 3 replicate injections)

5.5.2 Hydrolysis

A hydrolysis procedure is used to convert the N-acetyl isoniazid metabolite to isoniazid. Before analysis, the samples were thawed at room temperature and cleaned up using the procedure described in Section 4.12.

The hydrolysis was carried out for 4 patient urine samples that had been stored at -20 ºC for more than one day (Section 5.4.3) and blank urine spiked at 6 µg/mL of INH. The results are shown in Table 5.5.

Table 5.5 Hydrolysis of metabolites in urine

Urine	Before hydrolysis (µg/mL)	After hydrolysis (µg/mL)
Spiked urine	6.0	0.5
Patient urine A	5.0	5.5
Patient urine B	59.6	153.2
Patient urine C	14.3	63.0
Patient urine D	29.1	62.8

Data in Table 5.5 show that hydrolysis of spiked normal urine (6 µg/mL INH) leads to loss of INH in urine. The hydrolyzed patient urine samples gave increased levels of INH. Thus the real urine samples contained the metabolite, N-acetylisoniazid. However, an accurate estimate of the metabolite is not possible without pure standard sample of N-acetylisoniazid.

Therefore, the hydrolysis method can not be used to determine free concentration of the metabolite of INH because some INH is destroyed during the hydrolysis step.

5.5.3 Stability

Long-term stability study was performed by investigating the effect of storage time of INH spiked in human urine. Three aliquots at low, medium and high concentrations of INH were stored at -20 ºC (Section 4.13). The results are shown in Figure 5.11 (a) for spiked INH. The result show that the compounds stored at -20 ºC decomposed after one week storage. Furthermore, we also studied the stability of patient human urine (Figure 5.11 (b)). The results are the same as for spiked human urine. Therefore, patient human urine samples should not be stored for more than one week at -20 ºC before analysis which is agreement with the study of Seifart *et al.* [33].

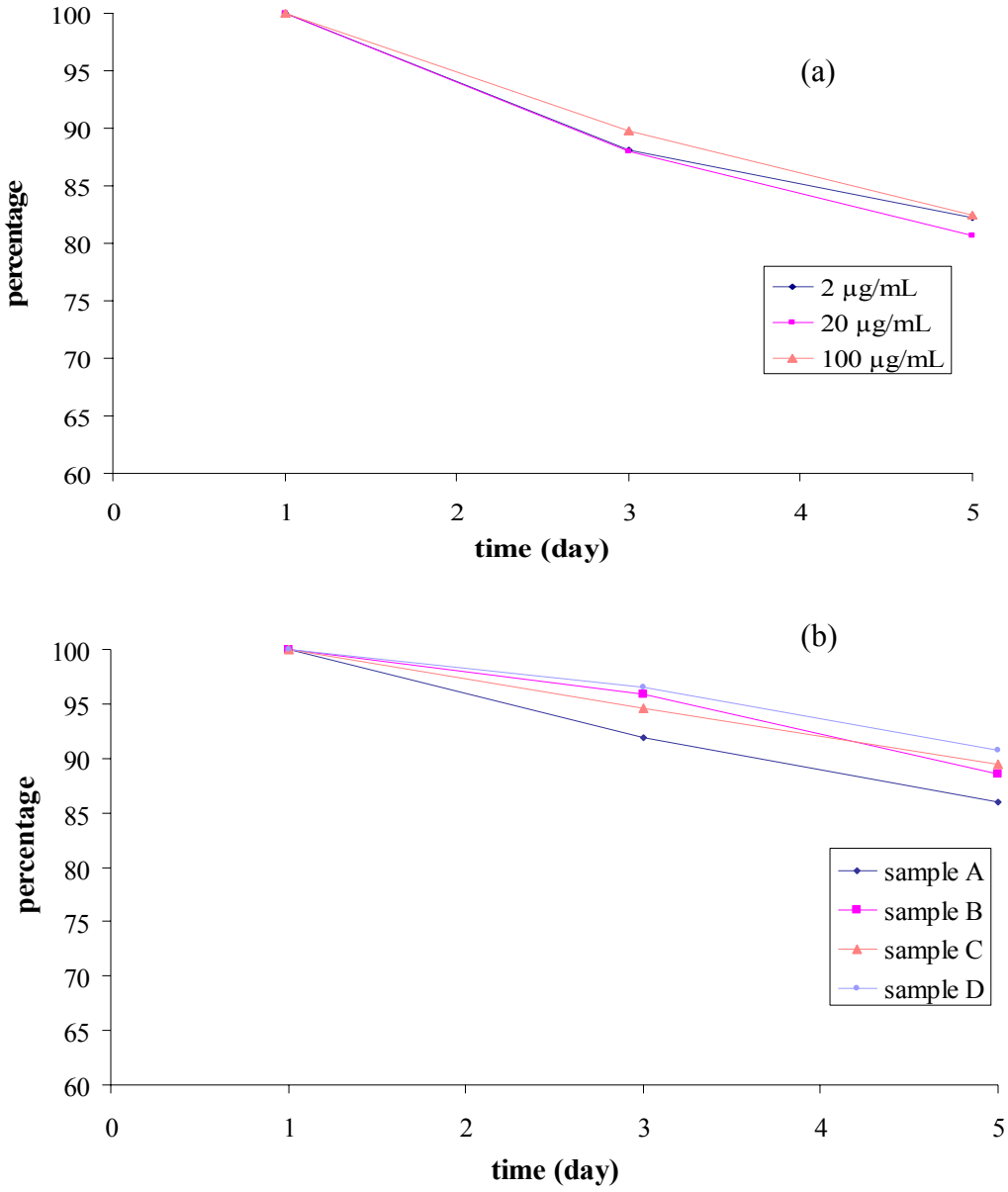


Figure 5.11 The stability profile for storage at -20 ºC of (a) INH spiked in human urine, presented as the percentage decrease. (a) spiked urine samples at concentration 2 (♦), 20 (■) and 100 (▲) µg/mL, respectively; (b) patient urine sample A (♦),patient urine sample B (■),patient urine sample C (▲) and patient urine sample D (●).

CHAPTER VI
CONCLUSION

The objective of this work was the development of a simple, reliable and accurate HPLC method for determination of INH in form of hydrazone, with direct UV detection.

The first part of this work involves selection of the best aldehyde from the four aldehydes, salicylaldehyde (SA), 2-nitrobenzaldehyde (2-NBA), 4-nitrobenzaldehyde (4-NBA) and cinnamaldehyde (CA), for derivatization with isoniazid. The results showed that salicylaldehyde is the best compound to form hydrazone as the detection compound, at ratio of aldehyde:INH of 20:1 with heating at 50 ºC for 10 minute, and monitoring at 289 nm. Mobile phase condition was 45:55 (*v/v*) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0) at flow rate of 1.0 ml/min.

Analytical performance for HPLC method was evaluated for linearity, accuracy and precision. Calibration curves were linear with correlation coefficients (r^2) greater than 0.999 in concentration range from 2 to 100 µg/mL INH. This method was reproducible for within-day and between-day measurements. Limit of detection based on three times of y-residuals plus the calculated intercept from regression line was 1.30 µg/mL for INH.

The developed HPLC method was applied to the determination of INH in 4 patient urine samples, which were found to have INH in the range 5.04-59.55 µg/mL. The recovery of spiked INH was in the range of 103.5-105.3%.

The analysis of the metabolite of isoniazid, N-acetylisoniazid, needs further study of the hydrolysis step or modification of the HPLC procedure for direct detection. The latter method would require a pure N-acetylisoniazid standard, which is not commercially available.

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APPENDICES

APPENDIX A
CHROMATOGRAMS OF PURE HYDRAZONES

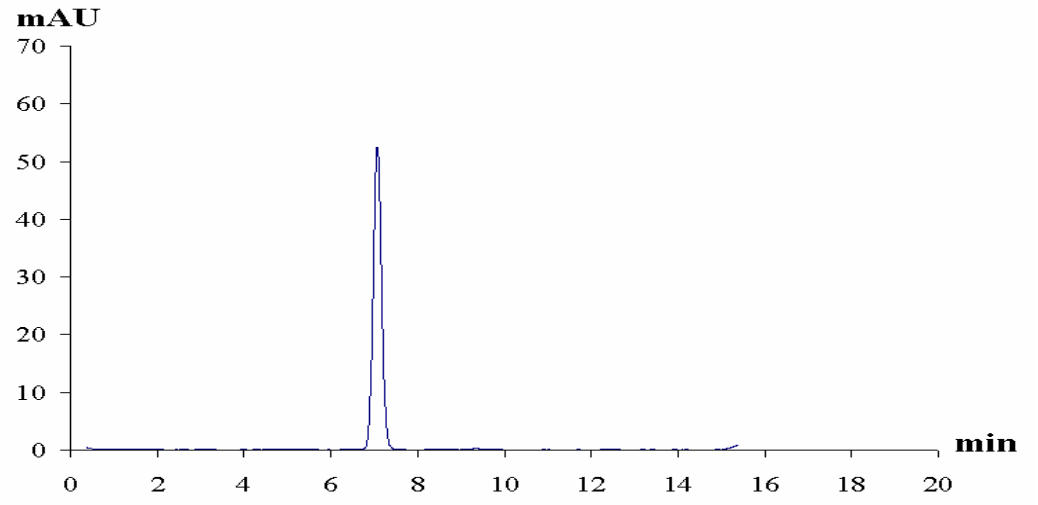

Figure A1 Chromatogram of 10 µg/mL benzaldehyde isonicotinoyl hydrazone (BIH) in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 325 nm.

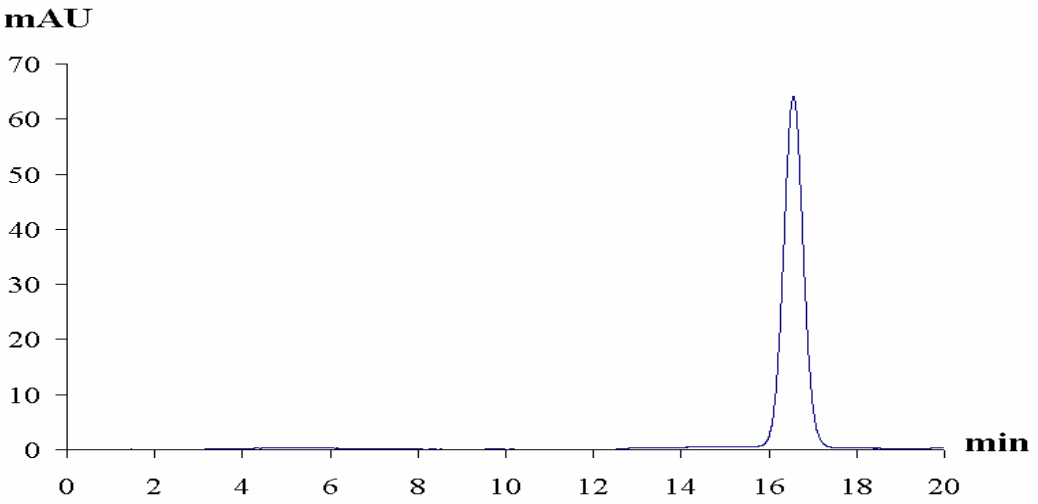

Figure A2 Chromatogram of 10 µg/mL cinnamaldehyde isonicotinoyl hydrazone (CIH) in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 325 nm.

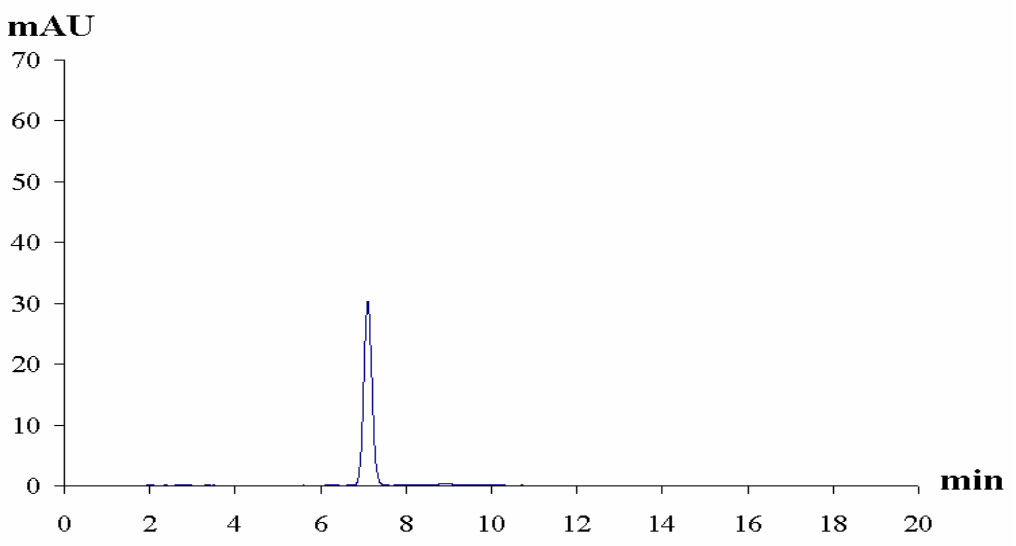

Figure A3 Chromatogram of 10 µg/mL 2-nitrobenzaldehyde isonicotinoyl hydrazone (2-NIH) in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored 325 nm.

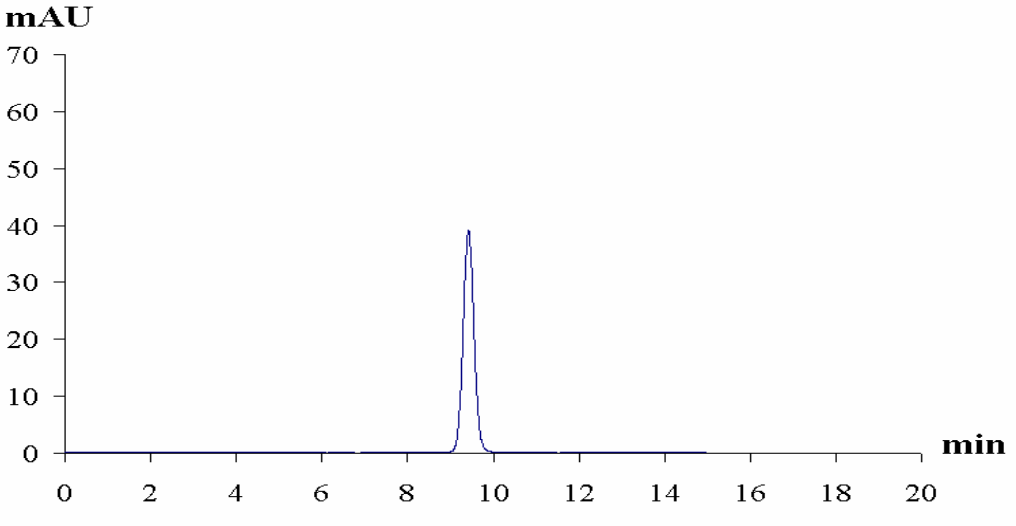

Figure A4 Chromatogram of 10 µg/mL 4-nitrobenzaldehyde isonicotinoyl hydrazone (4-NIH) in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 325 nm.

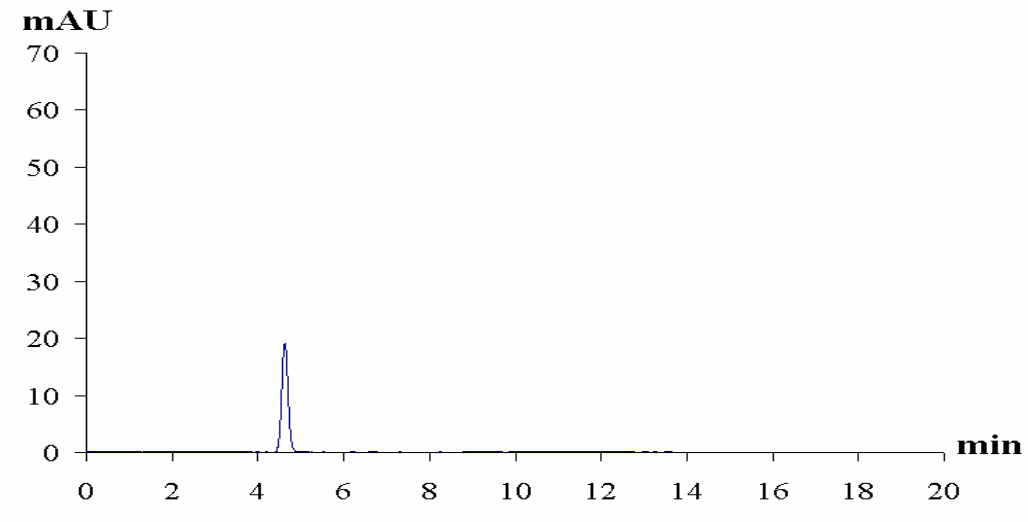

Figure A5 Chromatogram of 10 µg/mL pyridoxal isonicotinoyl hydrazone (PIH) in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 325 nm.

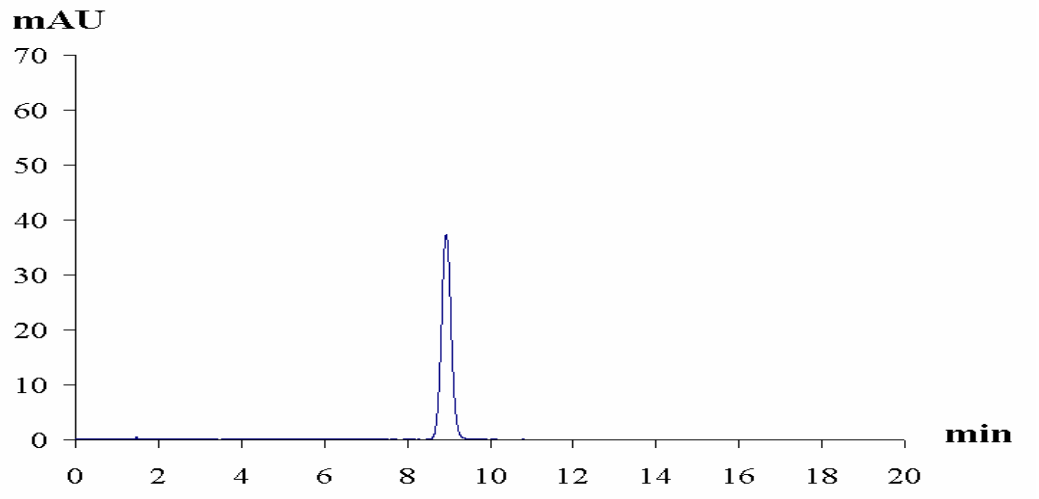

Figure A6 Chromatogram of 10 µg/mL salicylaldehyde isonicotinoyl hydrazone (SIH) in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 325 nm.

APPENDIX B

CHROMATOGRAMS OF URINE SAMPLES

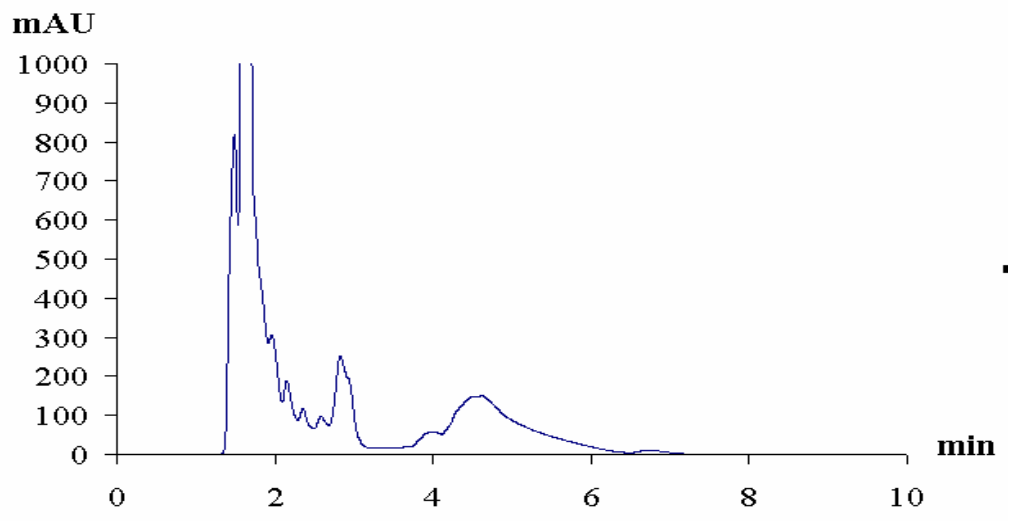

Figure B1 Chromatogram of a patient urine sample A in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 289 nm.

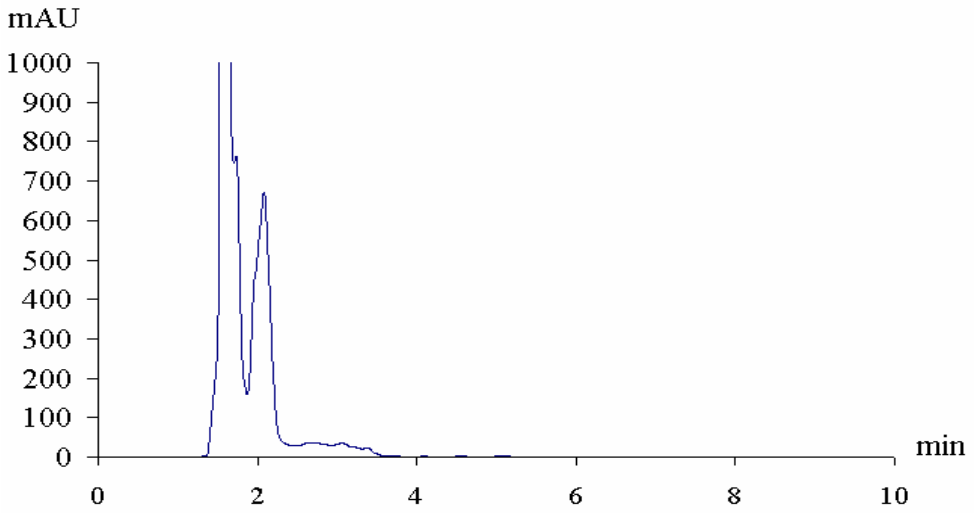

Figure B2 Chromatogram of a patient urine sample B in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 289 nm.

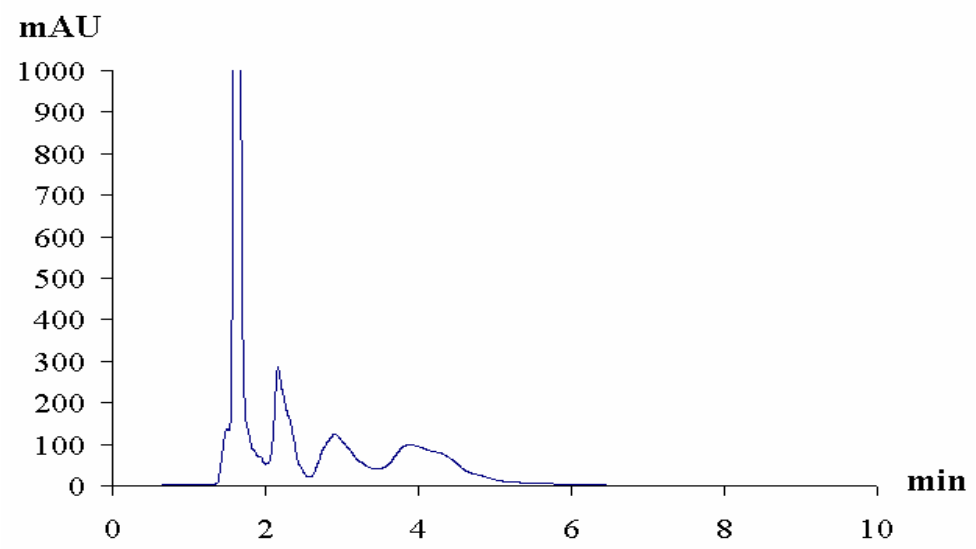

Figure B3 Chromatogram of a patient urine sample C in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 289 nm.

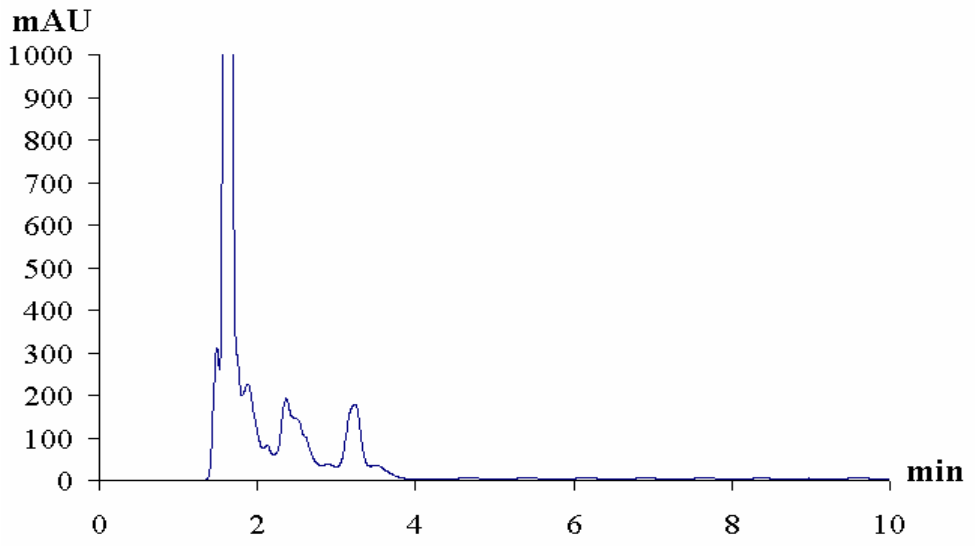

Figure B4 Chromatogram of a patient urine sample D in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 289 nm.

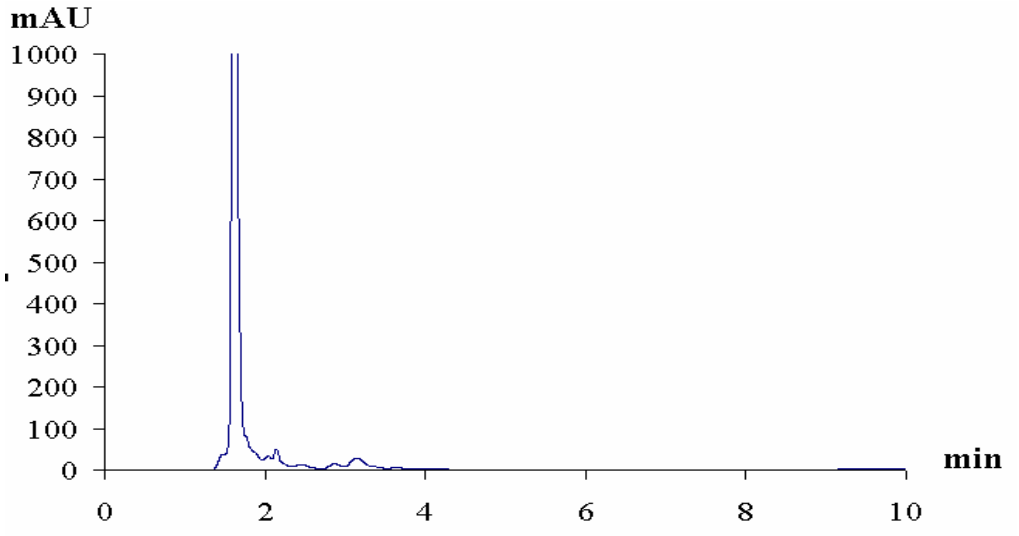

Figure B5 Chromatogram of a normal urine in 45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0). Flow rate was 1.0 mL/min and monitored at 289 nm.

APPENDIX C
DETERMINATION OF LIMIT OF DETECTION

The limit of detection is the lowest concentration of an analyte that an analytical process can reliably detect. There are many methods proposed for determination of the detection limit. In this work used the method as proposed by Miller J.N. and Miller J.C. [45] which detection limit is equal to the calculated intercept from regression line, *a*, plus three times standard deviation of y-residuals, $s_{y/x}$.

$$\text{Detection limit} = a + 3s_{y/x}$$

Where as $$s_{y/x} = \sqrt{\frac{\sum_i (y_i - \hat{y})^2}{n-2}} \qquad \text{(C1)}$$

Note : $s_{y/x}$ is estimate the random errors in the *y*-direction.

$y_i - \hat{y}$ is the point on the calculated regression line corresponding to the individual *x*-values

$n-2$ is degrees of freedom in linear regression calculations

The calculation for isoniazid analysis is shown as follow:

Linear regression equation (y = bx +a)	**$S_{y/x}$**	**LOD (µg/mL)**
y = 120.1x +5.7	52.2	1.30

Using the definition as mention above, the value of *y* for the limit of detection is 5.7 + 3(52.2), i.e. 162.2. From the regression equation, the limit of detection is calculated to be 1.30 µg/mL. The chromatogram of isoniazid at the limit of detection is shown in Figure C1.

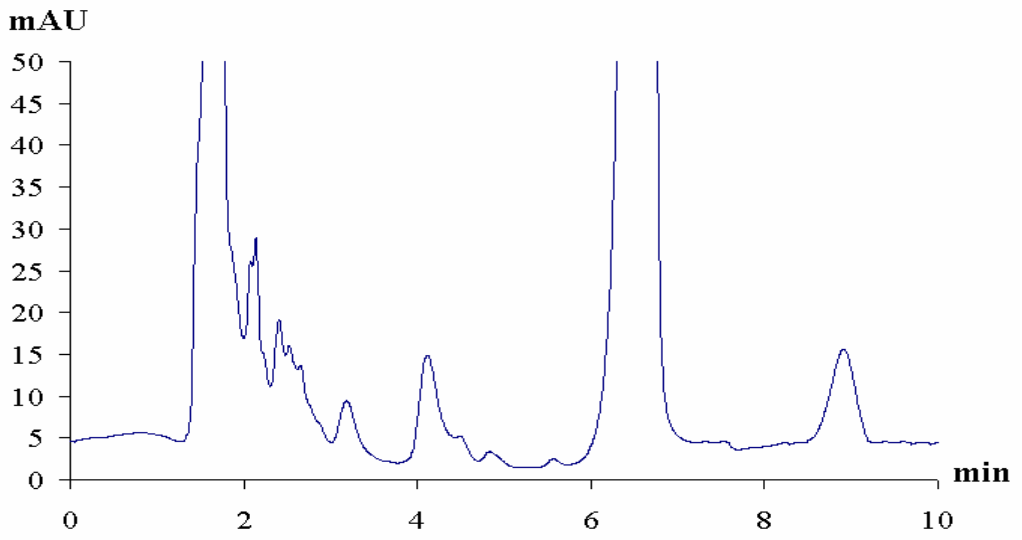

Figure C1 The chromatogram of isoniazid solution spiked in human urine at 1.30 µg/mL. An endcapped C18 bonded-silica column (Eclipse® XDB-C18, 150 x 4.6 mm i.d., 5 µm) was used with an isocractic mobile phase system (45:55 (v/v) methanol – 10 mM acetate buffer containing 2 mM EDTA (pH 4.0)). Flow rate was 1.0 mL/min and monitored at 289 nm.

BIOGRAPHY

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