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# Simple and Rapid Method for Simultaneous Determination of Isoniazid and Acetyl Isoniazid in Urine by HPLC

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## Abstract

A high performance liquid chromatographic method for simultaneous determination of isoniazid and acetyl isoniazid in urine was developed. Urine samples were filtered through Whatman number 1 filter paper and diluted filtrate was directly injected into $C_8$ column (250mm). The mobile phase comprised of water and methanol in the ratio of 85:15. The analytes were detected at a wavelength of 274nm. The assay was specific for isoniazid and acetyl isoniazid and linear from 1.25 to 40.0µg/ml for isoniazid and 3.125 to 100µg/ml for acetyl isoniazid. The relative standard deviation of intra- and inter-day assays was lower than 10%. The average recovery of isoniazid and acetyl isoniazid from Urine was 100% and 95% respectively. Due to its simplicity, the assay can be used for determination of isoniazid acetylator status using urine and to check patient adherence to tuberculosis treatment.

**Keywords:** Isoniazid, Acetyl isoniazid, Urine, Acetylator status, HPLC

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## 1. INTRODUCTION

Isoniazid is a critical component of first line antituberculosis therapy. The primary step in its metabolism is acetylation via hepatic N-acetyl transferase to acetyl INH. Isoniazid and acetyl isoniazid levels depend on the concentration of N-acetyl transferase enzyme, which is genetically determined. Thus, there are two phenotypes of this enzyme, namely, slow and rapid acetylators of INH. Rapid acetylators achieve significantly lower plasma concentrations of the drug after a standard dose, which has been shown to adversely impact TB treatment outcomes especially in certain specific situations such as during treatment with once daily regimens, in unsupervised treatment settings, during HIV infection, and in young children [1 – 4]. Success of anti-TB therapy depends on the right dosage and combination of drugs being taken for an adequate length of time, usually 6-8 months. Drug resistant tuberculosis is on the rise due to various factors such as cases where many patients are unable to complete a full course of treatment or are not properly monitored. These factors lead to increased mortality and morbidity as well as acquired drug resistance. To combat the spread of drug-resistant TB and empower physicians and healthcare workers to administer the appropriate drug dosages, it is important to monitor treatment adherence during treatment and also to determine the acetylator status, in order to make appropriate dose adjustments.

Determination of isoniazid and acetyl isoniazid concentrations in urine could help in ascertaining the acetylator status of an individual or to check patient's compliance to tuberculosis treatment. Urine collections are non-invasive and most suited for all groups of patients. There are several published methods for determination of isoniazid and acetyl isoniazid in urine by HPLC. Methods requiring sample pre-treatment followed by extraction into organic solvents and subsequent evaporation of the solvents have been reported [5 - 7]. These methods are laborious and time-consuming. The method described by Von Sassen and others required derivatisation with m-fluorobenzoyl chloride prior to separation on the column [8]. Cumbersome pre-treatment procedures using Sep-Pak cartridges have also been reported [9]. The method described by Jin et al, has employed pre-treatment of samples with vanillin followed by separation in a HPLC column [10]. Some methods have employed fluorescence detectors for quantification of isoniazid and acetyl isoniazid in urine; these methods also require derivatisation into fluorescent compounds [11, 12]. We developed and validated a simple and rapid assay procedure for estimation of isoniazid and acetyl isoniazid in urine.

## 2. METERIALS AND METHODS

### 2.1. Chemicals

Pure isoniazid powder from Sigma Chemical Company, MO, USA, acetyl isoniazid powder from Toronto Chemical Company, ON, Canada and Methanol (HPLC grade) from Hi-Media, India were used. Deionized water was processed through a Milli-Q water purification system (Millipore, USA).

### 2.2. Chromatographic System

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-10ATvp), diode array detector (SPD-M10Avp) and autosampler with built-in system controller (SIL-HTA). ClassVP-LC workstation was used for data collection and acquisition. The analytical column was a $C_8$, 250 x 4.6mm ID, 5µ particle size (Lichrospher 100 RP-18e, Merck, Germany) protected by a compatible guard column.

The mobile phase consisted of water and methanol mixed in a ratio of 80:20 v/v. Prior to preparation of the mobile phase, water and methanol were degassed separately using a Millipore vacuum pump. The PDA detector was set at a wavelength of 274nm. The chromatogram was run for 6 minutes at a flow rate of 1.2 ml/min at ambient temperature. Unknown concentrations were derived from linear regression analysis of the peak height vs. concentration curve. The linearity was verified using estimates of correlation coefficient (r).

### 2.3. Preparation of standard solution

Stock standards (1 mg/ml) were prepared by dissolving isoniazid and acetyl isoniazid in milli Q water. Drug-free human urine urine was used to prepare the working standards. This urine was filtered using Whatman number 1 filter paper and diluted 1:25 in mill Q water. Working calibration standards of isoniazid and acetyl isoniazid in concentrations ranging from 1.25 to 40µg/ml and 3.125 to 100 µg/ml respectively were prepared using the diluted urine.

### 2.4. Sample preparation

The calibration standard solutions prepared in urine were diluted 1:25 in milli Q water. Filtered and diluted drug-free urine served as blank. Urine samples from healthy human subjects were filtered using Whatman number 1 filter paper, diluted 1:25 in milli Q water. 100 µl of each of calibration standards/blank/test urine was directly injected to the HPLC column.

### 2.5. Accuracy and Linearity

2.6. The accuracy and linearity of isoniazid and acetyl isoniazid standards were evaluated by analyzing a set of standards ranging from 1.25 to 40 µg/ml and 3.125 to 100 µg/ml respectively. The within day and between day variations were determined by processing each

standard concentration in duplicate for six consecutive days.

### 2.6. Precision

In order to evaluate the precision of the method, five urine samples containing isoniazid and acetyl isoniazid were analysed on six consecutive days.

### 2.7. Recovery

For the recovery experiment, known concentrations of isoniazid (1.25 & 10.0 µg/ml) and acetyl isoniazid (3.125 & 25.0 µg/ml) were prepared in drug-free human urine. These were used to spike urine samples containing isoniazid and acetyl isoniazid. The percentage of recovery was calculated by dividing sample differences with the added concentrations. Recovery experiments were carried out on three different occasions.

### 2.8. Specificity

Interference from endogenous compounds was investigated by analysing blank urine samples obtained from six each of male and female subjects. Interference from certain anti-tuberculosis drugs such as rifampicin, pyrazinamide, ethambutol, ethionamide, moxifloxacin and ofloxacin and certain antiretroviral drugs, namely, nevirapine, efavirenz, stavudine, lamivudine, lopinavir, ritonavir and tenofovir at a concentration of 10µg/ml was also evaluated.

## 3. RESULTS AND DISCUSSION

Several HPLC methods have been described to measure isoniazid and acetyl isoniazid levels in urine. Most of the methods require intense sample pre-treatment processing, extraction into organic solvents, post-column derivatisation etc. These methods are cumbersome to perform, laborious and time-consuming [9 – 16]. While some methods have employed fluorescence detectors, most of the methods have used UV detectors. The method described in this paper does not require any sample pre-treatment, but direct injection of diluted urine into the HPLC column. The mobile phase was also quite simple, consisting of only water and methanol. Thus, the present method has obvious advantages of being rapid (run time is only 6 minutes) and not requiring sample pre-treatment or post-column derivatisation, without any loss of analyte.

Under the chromatographic conditions described above, isoniazid and acetyl isoniazid were well separated as seen in the representative chromatograms (Fig 1a, b). The retention times of the acetyl isoniazid and isoniazid were 3.6 and 4.1 minutes respectively. Blank urine samples did not give any peak at the retention times of isoniazid and acetyl isoniazid (Fig 1c). The lowest concentrations of acetyl isoniazid and isoniazid gave discrete peaks at 3.6 and 4.1 minutes respectively (Fig 1a).

Isoniazid is an important component of first-line anti-tuberculosis regimen. It is used in combination with other anti-tuberculosis drugs and also combined with anti-retroviral drugs during the treatment of HIV and tuberculosis co-infected patients. Hence it becomes necessary to rule out interference of anti-tuberculosis and anti-retroviral drugs in the assay of isoniazid and acetyl isoniazid and establish the specificity of the method. No endogenous substances or anti-tuberculosis drugs such as rifampicin, pyrazinamide, ethambutol, ethionamide, moxifloxacin and ofloxacin or antiretroviral drugs such as nevirapine, efavirenz, stavudine, lamivudine, lopinavir, ritonavir and tenofovir interfered with the isoniazid/acetyl isoniazid chromatogram.

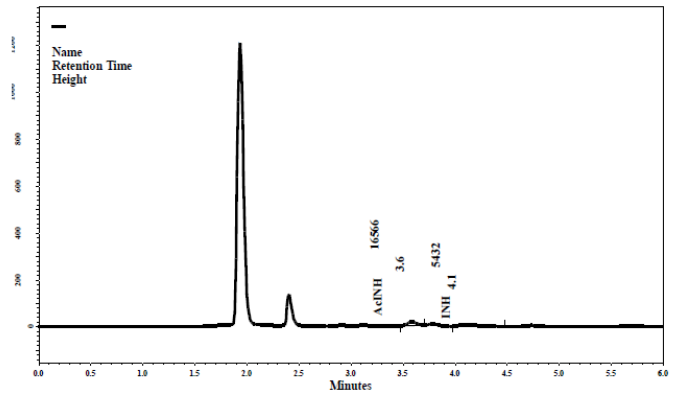


**Figure 1a:** Chromatogram of isoniazid and acetyl isoniazid standards in urine at concentrations of 1.25 and 3.125 µg/ml respectively.

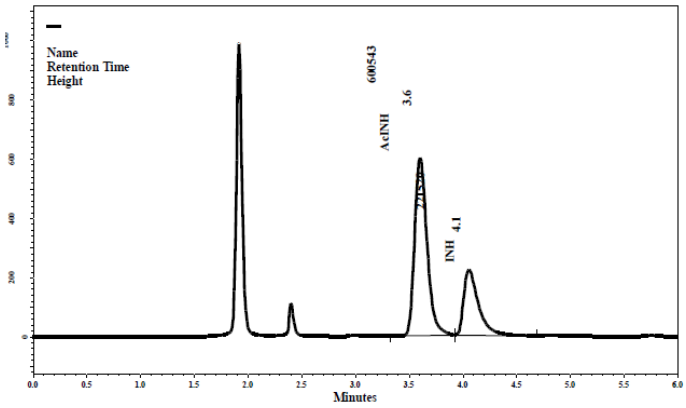


**Figure 1b:** Chromatogram of isoniazid and acetyl isoniazid standards in urine at concentrations of 40.0 and 100.0 µg/ml respectively

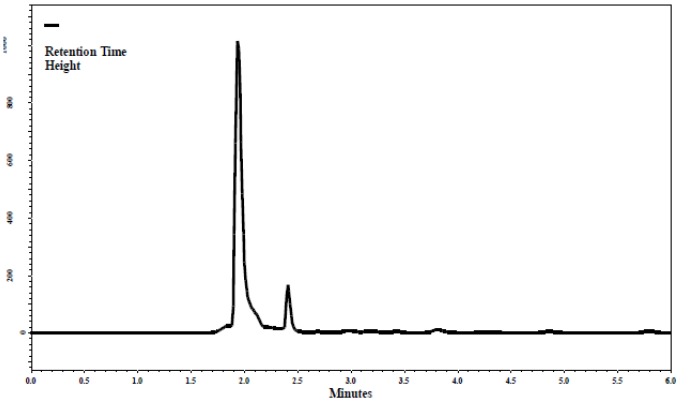


**Figure 1c:** Chromatogram of blank urine

In the present method, isoniazid and acetyl isoniazid concentrations ranging from 1.25 - 40.0 µg/ml and 3.125 – 100.0 µg/ml respectively were checked for linearity. The lowest concentrations of isoniazid and acetyl isoniazid gave peak heights approximately 5000 and 16000 respectively. Since sensitivity is not an issue with urine estimations, we set the lowest concentration at 1.25 µg/ml for isoniazid and 3.125µg/ml for acetyl isoniazid. In view of the heights that we obtained, it is possible to go down further, as low as 0.1µg/ml and 0.025µg/ml for isoniazid and acetyl isoniazid respectively. However, this would be seldom required, since urine samples require several-fold dilution before subjecting to analysis.

The linearity and reproducibility of the various standards used for constructing calibration graphs for urine isoniazid and acetyl isoniazid were tested on six consecutive days. The urine isoniazid standard concentrations ranged from 1.25 to 40.0µg/ml and that for acetyl isoniazid from 3.125 to 100.0µg/ml a linear relationship was observed between peak height and concentrations over these ranges with mean correlation coefficients of 0.9999 and 1.0000 for isoniazid and acetyl isoniazid respectively. . The linearity and reproducibility of the various standards used for constructing calibration graphs for urine isoniazid and acetyl isoniazid are given in Table I. The within-day and between-day relative standard deviation (RSD) for isoniazid standards ranged from 0.6 to 5.1% and 0.8 to 5.2% respectively. The corresponding RSD ranges for acetyl isoniazid were 1.7 to 3.7% and 1.3 to 5.3%.

| Mean peak height ± SD (RSD%) | | | | | |
|---|---|---|---|---|---|
| **Isoniazid** | | | **Acetyl isoniazid** | | |
| Standard conc (µg/ml) | Within day (n = 6) | Between day (n = 6) | Standard conc (µg/ml) | Within day (n = 6) | Between day (n = 6) |
| 1.25 | 5159 ± 168 (3.3) | 5114 ± 176 (3.4) | 3.125 | 16804 ± 525 (3.1) | 16300 ± 595 (3.6) |
| 2.5 | 11489 ± 582 (5.1) | 11176 ± 580 (5.2) | 6.25 | 34225 ± 577 (1.7) | 65081 ± 578 (1.6) |
| 5.0 | 25450 ± 506 (2.0) | 25327 ± 473 (1.9) | 12.5 | 71361 ± 1660 (2.3) | 71050 ± 2293 (3.2) |
| 10.0 | 53947 ± 336 (0.6) | 53150 ± 451 (0.8) | 25.0 | 150705 ± 2868 (1.9) | 147230 ± 5541 (3.8) |
| 20.0 | 110544 ± 1794 (1.6) | 108853 ± 1340 (1.2) | 50.0 | 306979 ± 9352 (3.0) | 310360 ± 16395 (5.3) |
| 40.0 | 224502 ± 3974 (1.8) | 217712 ± 4441 (2.0) | 100.0 | 607826 ± 8460 (1.4) | 626248 ± 8315 (1.3) |

**Table I:** Linearity and reproducibility of urine isoniazid and acetyl isoniazid

The reproducibility of the method was further evaluated by analysing six urine samples containing different concentrations of isoniazid and acetyl isoniazid. The RSD for these samples ranged from 2.7 to 7.0% for isoniazid and 2.6 to 7.2% for acetyl isoniazid (Table II). The % variations from the actual concentrations ranged from 99 to 101% for isoniazid and 90 to 102% for acetyl isoniazid. The method reliably eliminated interfering material from urine, yielding recoveries ranging from 97 to 103% fro isoniazid and 95 to 99% for acetyl isoniazid.

| Sample No. | Isoniazid (µg/ml) | Acetyl isoniazid (µg/ml) |
|---|---|---|
| I | 9.5 ± 0.3 (2.7) | 85.7 ± 5.2 (6.0) |
| II | 13.0 ± 0.8 (6.3) | 10.5 ± 0.7 (6.3) |
| III | 11.5 ± 0.8 (6.9) | 74.4 ± 4.8 (6.5) |
| IV | 122.5 ± 8.6 (7.0) | 131.7 ± 7.4 (5.6) |
| V | 2.6 ± 0.1 (3.4) | 80.3 ± 5.8 (7.2) |

**Table II:** Precision of urine isoniazid and acetyl isoniazid assay
Values are Mean ± SD (RSD%) of six individual estimations

## 4. CONCLUSION

In conclusion, a sensitive, specific and validated method for quantitative determination of isoniazid and acetyl isoniazid in urine is described. This simple, rapid, accurate and reproducible method does not require any sample pre-treatment. The chromatogram yields well-resolved peaks for isoniazid and acetyl isoniazid with good intra- and inter-day precision. The simplicity of this method makes it highly suitable for determination of isoniazid and acetyl isoniazid in tuberculosis patients, which can be used to determine acetylator status or for monitoring compliance to treatment.

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