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Detection and Assay of Isoniazid Utilizing Isocratic High-Performance Liquid Chromatography

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Detection and Assay of Isoniazid Utilizing Isocratic High-Performance Liquid Chromatography

University Honors Program Thesis

University of Nebraska at Omaha

Submitted by

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April 2018

Faculty Advisor

Dr. Ronald Bartzatt

SENIOR HONORS THESIS ACCEPTANCE

This thesis has been accepted for the faculty of the College of ____Arts and Sciences________ in partial fulfillment of the requirements for completion of the Honors Program of the University of Nebraska at Omaha.

Dr. Ronald Bartzatt

Thesis Adviser

March 26 2018

Date

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Student

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UNIVERSITY OF NEBRASKA AT OMAHA

HONORS THESIS

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STATE: YES
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THESIS: REQUIRED
THESIS ISSUED: PROGRAM

***ABSTRACT OF THESIS*:**

To demonstrate an analysis for isoniazid from aqueous mixtures and solid tablets, utilizing isocratic conditions, with high performance liquid chromatography (HPLC) and UV detection. Isoniazid in solid and liquid samples is assayed utilizing HPLC. Isoniazid is a tuberculostat, that is assayed by high performance liquid chromatography that utilizes a reversed-phase C-18 column. The eluent solvent is 5.3% ethanol, 93.7% water, and 1% acetic acid. Aqueous samples are prepared in distilled water as the solvent. The ultraviolet-visible detection of isoniazid is accomplished at 265 nm, which is the wavelength having the highest sensitivity for isoniazid. Having a column pump pressure at 2100 psig, rise time 0.1, and with flow rate 1.0 mL/minute, the elution of isoniazid occurred at 2.2 minutes. The standard curve that was applied ranged from zero to 0.001460 molar in concentration (y = 50,237,766.7435 x). The model described by the standard curve had a coefficient of determination of $R^2 = 0.9921$, and a very strong positive correlation coefficient (r= 0.9960). Percent recovery of drug ranged from 97.3% to 105%. Utilizing reversed phase column with isocratic solvent conditions is shown here to be an effective methodology for determination of isoniazid.

INTRODUCTION

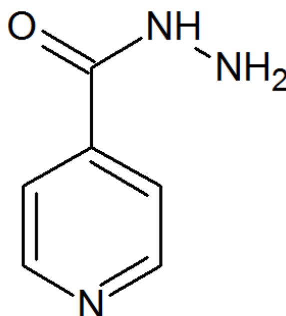
Tuberculosis, (TB, *Mycobacterium tuberculosis*) is a deadly infectious disease caused by various strains of mycobacteria, particularly the *Mycobacterium tuberculosis* in humans. Tuberculosis most often attacks the lungs; however, it can also infect other parts of the body as extra-pulmonary tuberculosis. The bacteria spreads through the air when people carrying the disease cough, sneeze, or spit. Often times in humans, the result is an asymptomatic, latent infection [1]. However, about ten percent of latent infections eventually progresses to active disease, which can kill more than 50% of its victims if not treated [1].

Tuberculosis meningitis is caused by the bacterium that is the causative agent of pulmonary tuberculosis, *Mycobacterium tuberculosis,* and is an infection of the meninges (the dura mater, arachnoid, and pia mater that line the skull and vertebral canal which enclose the brain and spinal cord) [1]. Various risk factors that increase the possibility of central nervous system (CNS) infection include pulmonary tuberculosis and various other disorders that compromise the immune system [1]. *Mycobacterium tuberculosis* can infect the CNS from an origin located from elsewhere in the body [1]. The types of TB infections that involve the central nervous system can be placed into three clinical categories [2]: 1) Meningitis, 2) Intracranial tuberculoma, and 3) Spinal tuberculous arachnoiditis.

Clinical treatment of tuberculosis meningitis requires several tuberculostatic drugs that are administered simultaneously, with treatment lasting up to 12 months in duration [3]. The clinical treatment and control of tuberculosis disease is substantially impaired with the appearance of multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-

TB) [3]. The appropriate use of second-line drugs is required to treat cases of MDR-TB, and to inhibit the proliferation of XDRTB [1, 2, 3].

Tuberculosis is a serious health threat and is thought to cause up to 1.5 million deaths annually [4]. With the appearance of drug-resistant strains of tuberculosis and with HIV (human immunodeficiency virus) coinfection, the incidence of this disease has increased [4]. Almost one-third of the world's population is infected with *Mycobacterium tuberculosis* [5]. Annually, up to 8.8 million patients are newly diagnosed with an active infection of tuberculosis [5, 6]. The current first-line drugs for treatment (isoniazid, rifampin, pyrazinamide, ethambutol) must be taken for up to 6 months to achieve high level of cure rates (>95%) [5, 6].



The molecular structure of isoniazid having the hydrazide group (-C(=O)NHNH$_2$). The IUPAC name is pyridine-4-carbohydrazide. This drug has a molecular formula $C_6H_7N_3O$ and formula weight 137.14 grams/mole**.** The SMILES (simplified molecular-input line-entry system) notation is O=C(NN)c1ccncc1.

Isoniazid is a first-line tuberculostatic agent that is part of the core of TB treatment regimens. It has been approved as prophylactic therapy for both latent and active tuberculosis infections. It is activated in the body by a bacterial catalase-peroxidase enzyme in *Mycobacterium tuberculosis* called KatG. This prodrug inhibits the synthesis of mycolic acids, which are essential

for the mycobacterial cell wall [7]. It interferes with the metabolism of vitamin B-6 in some patients [8]. For active tuberculosis infections, isoniazid is often used in combination with other tuberculosis medications, in order to prevent the development of drug resistance. Isoniazid was selected for analysis because it is first-line tuberculostatic drug that is a small molecule with properties suitable for penetration into the central nervous system and appropriate for treatment of tuberculosis infection in the central nervous system.

METHODOLOGY

Reagents and Instrumentation

All solvents are analytical grade and obtained from Sigma-Aldrich (St. Louis MO 63178 USA). The isoniazid (isonicotinic acid hydrazide) for use as standards and preparation of samples is from Sigma (PO Box 2060, Milwaukee Wi 53201). For the high-performance liquid chromatography analysis, an Alltech 426 HPLC Pump and Linear UVS 200 detector with reversed-phase isocratic conditions was utilized (Deerfield, Illinois 60015-1899). The HPLC Alltech instrumentation is controlled by computer interface.

Instrument Settings, Preparation of Standards and Test Sample

For analysis by HPLC, a reversed-phase C-18 octadecylsilyl ($C_{18}H_{37}$) bonded phase column packing was utilized. The isoniazid analyte elutes at 2.28 minutes. Detection by ultraviolet detector set to 265 nm, rise time 0.1, range AUFS set to 1.0. The HPLC pump was set to 1135 psig

and one milliliter per minute flow rate. Actual volume injected into the column is about 20 microliters. The dead time of eluting non-retained species is 1.5 minutes and calculated based on relationship, dead time= t_O = volume/flow rate = 1.5 mL/1.0 mL/min = 1.5 minutes.

Sample Types and Samples

Column solvent utilized throughout the project was made for a total volume of 1000 mL, by adding 52.6 mL of 95% ethanol, 937.4 ml of distilled water, and 10 mL of glacial acetic acid (stock at 17.4 molar). Therefore, the working concentrations: 5% ethanol, 0.174 molar acetic acid, and 93.7% water (v/v). Sample solvent used for solubilizing isoniazid in various test samples: distilled water. Stock standard of isoniazid was prepared by dissolving 1.509 grams of isoniazid into 250 mL volumetric flask of distilled water, making a mixture of 6.000 x 10^{-2} molar.

If any sample required clarification prior to HPLC analysis, then where necessary, this is accomplished by Whatman 6900-2502 GD/X 25 Sterile Syringe Filter, 25 mm, 0.2 Micron, PVDF Filtration Medium, with a suitable plastic syringe. Samples for HPLC analysis were in distilled water as solvent, which works exceptionally well for the very water-soluble isoniazid. Ampoule-type samples, used in clinical application, were isoniazid drugs prepared in dilute HCl aqueous solvent with HCl concentration at 1.00 x 10^{-6} molar. Tablet/solid samples consisted of the drug isoniazid prepared in various known percentage of combinations of excipients lactose, cellulose, starch, and/or dextrin. After thorough mixing of isoniazid with excipient(s), the tablets were prepared utilizing a standard Parr Pellet Press (Parr Instrument Company, 211 Fifty Third Street, Moline Illinois 612265 USA). The pressed tablets were then carefully weighed by digital balance, the isoniazid determined by mass percentage present, then followed by solubilizing in distilled

water utilizing 100 mL volumetric flasks. Clarification of solution can be accomplished by syringe filter. Samples were then analyzed after 48 hours of mixing/settling and harvesting of supernatant**.**

Statistical Analysis and Properties Determination

Wherever indicated, the numerical analysis such as correlation of sets of data was performed by PAST version 2.06. Summary statistical analysis was accomplished by Microsoft Excel, and PAST v. 2.06. To test for outliers, the Grubb's test was performed by GraphPad InStat, version 3.00 for Windows 95. Determination of 95% confidence intervals was accomplished by Method Validator version 1.1, and linear regression done by Excel and Method Validator version 1.1. Passing-Bablok regression analysis was accomplished by ACOMED statistic (www.acomed-statistik.de, copyright: Dr. Thomas Keller) and Method Validator version 1.1.

PROJECT DATA AND INTERPRETATION

Isoniazid is an important first-line synthetic tuberculostatic drug, having bactericidal activity to rapidly dividing mycobacteria and bacteriostatic activity if the mycobacteria are slow-growing. It easily dissolved in the test samples of distilled water solvents for standards, as well as aqueous samples for tablet/ solid preparations. The HPLC analysis was accomplished operating under isocratic conditions. During the study, the mobile phase composition remained constant. The biggest advantages of these isocratic conditions includes a column being equilibrated at all times and a system that does not suffer from fast chemical changes [9].

The mobile phase in reversed phase HPLC consists of water as the aqueous solution and an organic modifier. Therefore, for the case of reverse phase HPLC, water is the "weakest" solvent. Because of the polarity of water, it more readily repels hydrophobic analytes into the stationary phase, more so than any other solvent. An organic modifier is added so that a hydrophobic analyte is no longer as so strongly repelled into the stationary phase. The analyte will then elute earlier, making the modifier "chromatographically strong" since it is the cause of the elution speed increase. The organic modifier utilized in this study is the alcohol ethanol, but other common organic modifiers include other alcohols.

To signal the elution of isoniazid from the HPLC instrument, it is essential to determine the wavelength setting for the UV-Vis detector. The wavelength of detection is set to a wavelength where the analyte maximum absorbance occurs in the solvent mixture. To determine isoniazid's maximum wavelength for HPLC detection, mixtures having identical molar concentrations in distilled water were injected using constant column and instrument settings. With only the wavelength of detection being varied, the wavelength of maximum absorbance was identified, which that wavelength selected for this analysis. The wavelengths examined were: 250 nm, 260 nm, 265 nm, 270 nm, 280 nm, and 290 nm. The maximum absorbance for isoniazid occurred at 265 nm.

Initially, it is necessary to show the wavelength in which the maximum absorbance for isoniazid occurs for accurate assay by HPLC. This result is presented in Table 1, with the data plotted in Figure 1. The maximum absorbance is at 265 nm, having a peak area determined to be 34,608.0 uV.Min . This wavelength is the setting for the UV/Vis detector in determining isoniazid. At 290 nm, the wavelength had a peak of 9,618.6 uV.Min. At 280 nm, the peak was 19,805.5; double what it was at the first concentration sample. At 270 nm, it almost doubles again, with a

peak area of 32,719.4 uV.Min. Post approaching the maximum, the wavelength begins to dip back down with 260 nm wavelength having a 31,880.4 uV.Min peak area. The last sample tested gave at 250 nm wavelength with a 21,873.6 uV.Min peak area. The assay of isoniazid will therefore be conducted at 265 nm, the the absorbance maxima for isoniazid under these conditions of the HPLC instrumentation and drug solubilization.

TABLE 1

Wavelength (nm)	Peak Area (uV. Min)
290	9,618.6
280	19,805.5
270	32,719.4
265	34,608.0
260	31,880.4
250	21,873.6

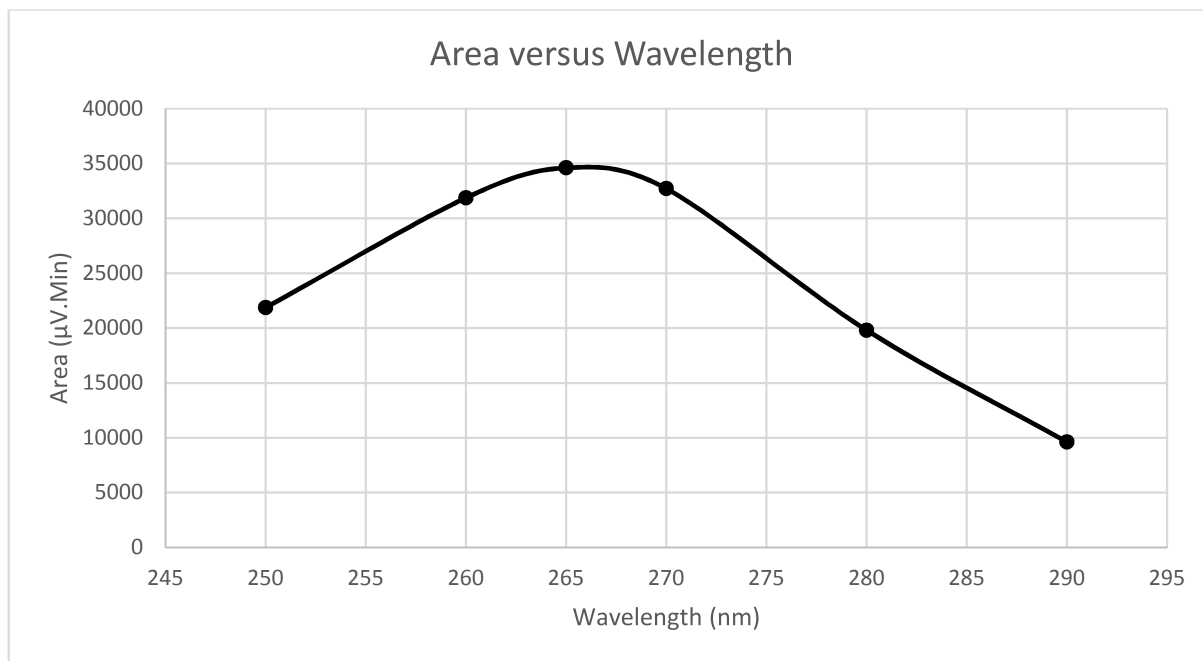
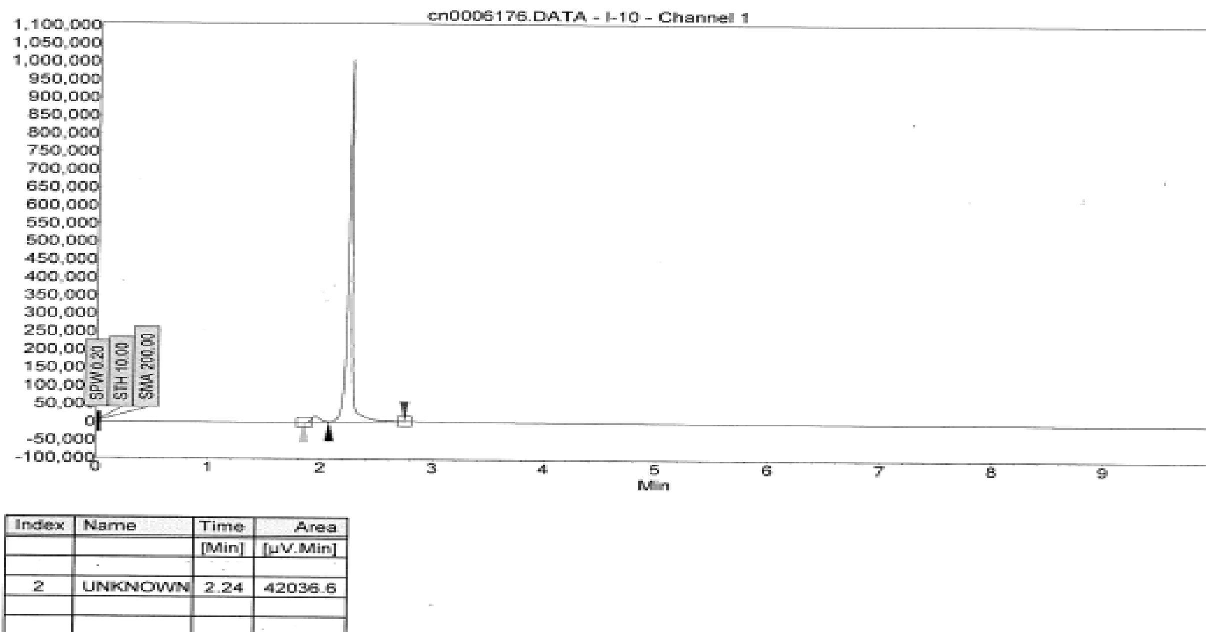


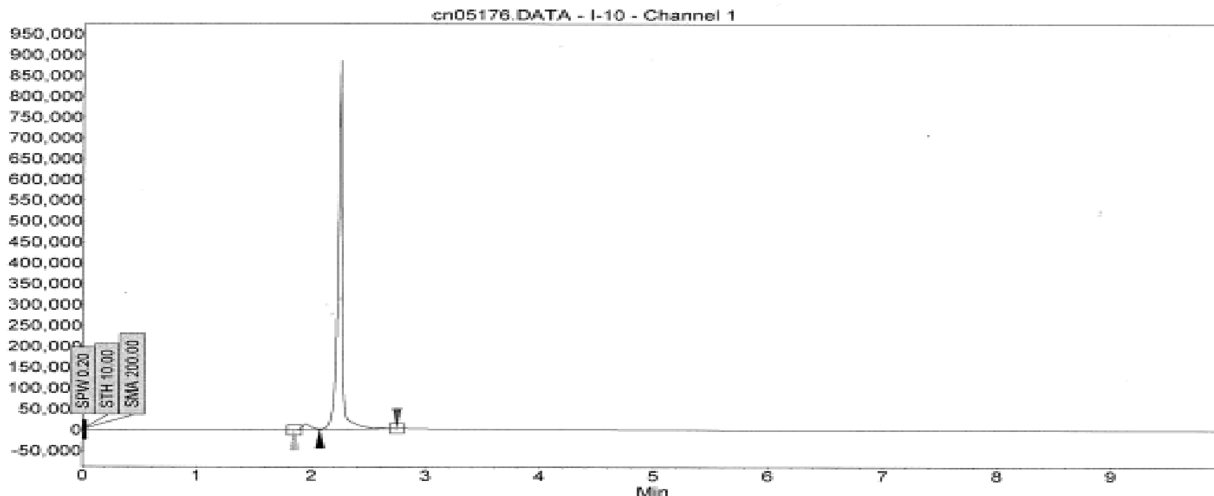
Figure 1. Comparison of peak area to wavelength in nanometers for six sample injections. The wavelengths on x-axis and their corresponding peak areas on y-axis are plotted. The highest peak area shown is at 265 nm, with a value of 34,608.0 uV. Min. The curve projects roughly a bell shaped structure. Concentration of all samples are at 6.000 x 10^{-4} molar and volume of injections at 20 microliters.

Example chromatograms of isoniazid elution are presented in Figures 2a-d below. The peak in each of the chromatograms is very well defined and sharp. This was a consistent attribute for the isoniazid analysis performed during the study. Elution time of the isoniazid was consistent. These sharply defined peaks were utilized for obtaining values for the peak areas and concentrations. These peaks appearing in the chromatograms provided quick and trouble-free selection by the instrumental software. The sensitive ultraviolet light detector would have revealed any contamination or degradation of the analyte isoniazid, if it had occurred during analysis. No evidence of contamination or degradation of analyte was observed during the course of the study.



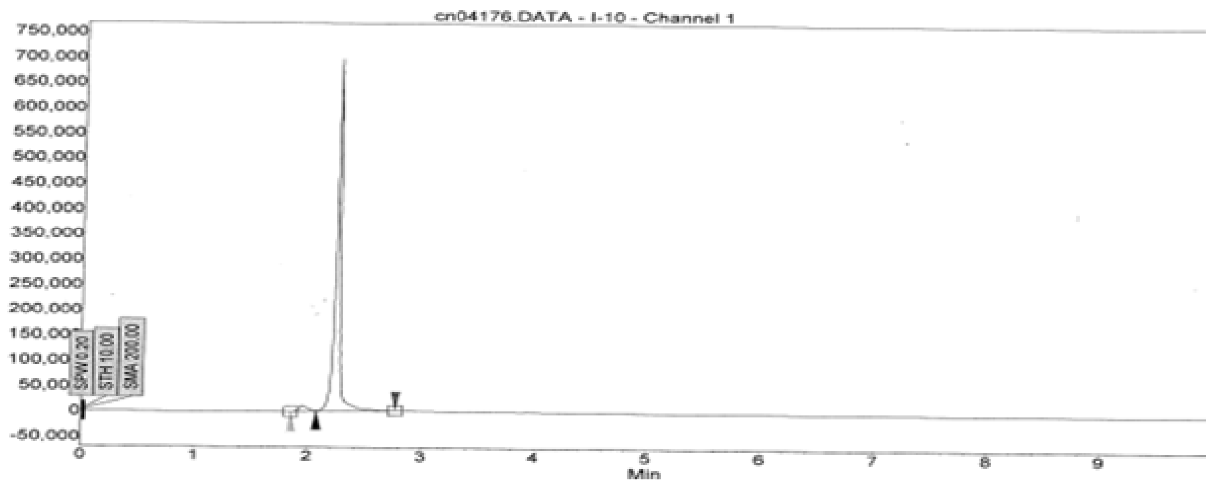
Index	Name	Time	Area
		[Min]	[µV.Min]
2	UNKNOWN	2.24	42036.6

Figure 2a. Chromatogram of isoniazid elution from HPLC. Isoniazid concentration is at 8.312×10^{-3} molar. Elution of isoniazid peak occurring with a peak area of 42,036.6 uV.Min.



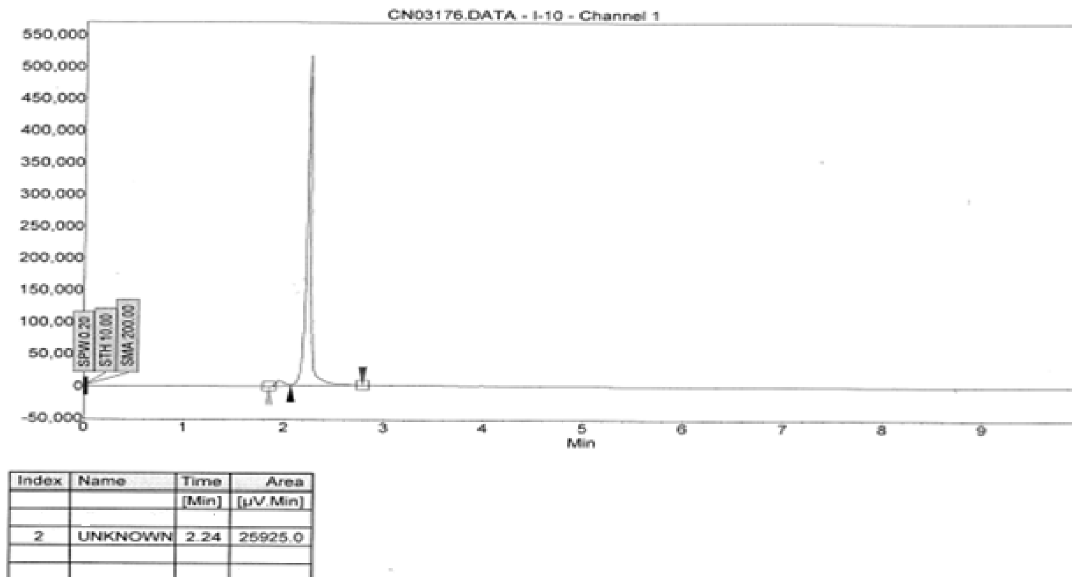
Index	Name	Time	Area
		[Min]	[µV.Min]
2	UNKNOWN	2.24	36803.7

Figure 2b. Chromatogram of isoniazid elution from HPLC. Isoniazid concentration is at 7.278 x 10^{-4} molar with a peak area of 36,803.7 uV.Min.



Index	Name	Time	Area
		[Min]	[µV.Min]
2	UNKNOWN	2.25	30871.8

Figure 2c. Chromatogram of isoniazid elution from HPLC. Isoniazid concentration is at 6.150 x 10^{-4} molar. Peak showing isoniazid eluting with a calculated peak area of 30,871.8 uV. Min.



Index	Name	Time [Min]	Area [µV.Min]
2	UNKNOWN	2.24	25925.0

Figure 2d. Chromatogram of isoniazid elution by HPLC. Isoniazid concentration is at amount 5.126 x 10^{-4} molar. Peak showing isoniazid eluting with a calculated peak area of 25,925.0 uV. Min.

A standard curve was elucidated for the determination of isoniazid concentration through the HPLC assay of aqueous samples, ampoule type samples, and solid tablet based samples that are utilized for oral administration. Prepared standard solutions that were injected and assayed produced values that are shown in Table 2. These values being the basis for determination of concentration of isoniazid in the various mixtures analyzed in terms of molarity. The values show consistent increase as the concentration of isoniazid is increasing. The two dimensional plot of molarity (x-axis) with respective areas (uV.Min) from generated chromatograms (y-axis), is presented in Figure 3. The result came out to be highly linear, with a coefficient of determination of R^2 = 0.9921, and a Pearson correlation coefficient of 0.9960. The Pearson r correlation indicating an extremely high positive inter-relationship of concentration to area of peak. The range of detection ranges from 0.000 molar to 1.460 x 10^{-3} molar. The equation of the line was y = 50,237,766.7435x. The coefficient of determination becomes R^2 = 0.9921, indicating that 99.21%

the proportion of the variance in the dependent variable that is predictable from the independent variables [10].

TABLE 2

Molarity	Area
0	0
0	0
.000015	2008.5
.000030	3258.2
.000150	8182.7
.000300	16315.1
.000530	31070.1
.000600	31779.3
.000900	45757.8
.001069	51534.0
.001146	60419.8
.001460	69703.1

Formation of Standard Curve for Determination of Sample Concentrations

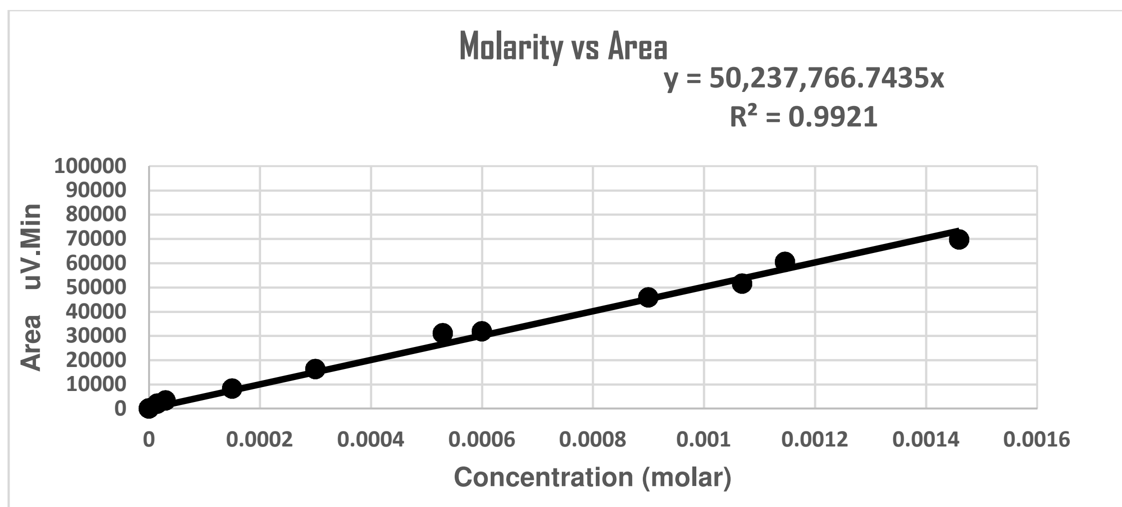


Figure 3. The standard curve for determination of concentration of drug in mixtures analyzed in terms of molar amount. The equation line is y = 50,237,766.7435x with a coefficient of determination of R^2 = 0.9921 (99.21% is variance in the model). The Pearson r correlation coefficient for these concentrations is r = 0.9960 (very strong positive correlation).

For comparing the instrumental parameters utilized in this study for the assay of isoniazid, two other medicinal compounds, quinine sulfate and inositol were also injected. The elution observed are seen in the following chromatograms (see Figures 4 and 5. Therefore, the instrument parameters utilized for isoniazid determination may be appropriate and efficient for other drugs.

Chromatograms of Selected Agents under Conditions Specified

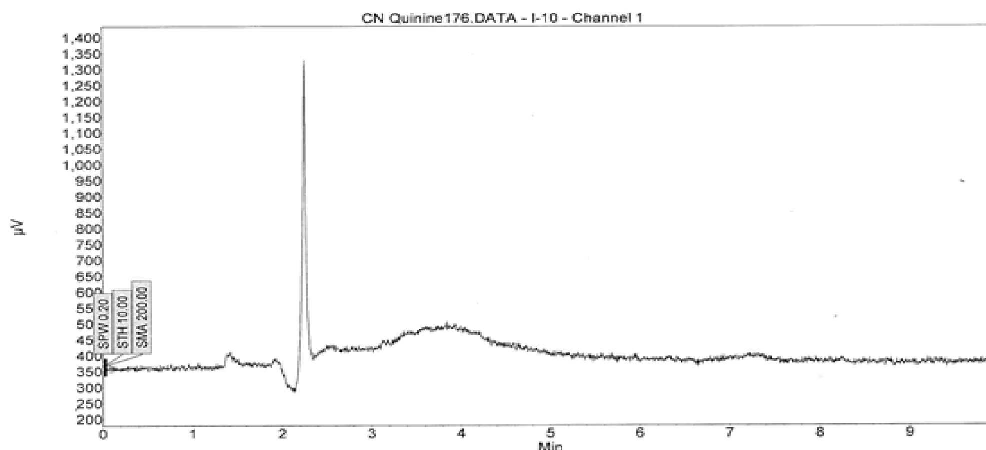


Figure 4. Chromatogram of quinine sulfate at 4.342×10^{-4} molar (molar mass = 782.96 grams/mole). HPLC conditions include detection at 265 nm, flow rate 1.00 mL/minute, rise time 0.1, and range AUFS 1.0. Solvent is 94% water, 5 % ethanol, and 1% acetic acid.

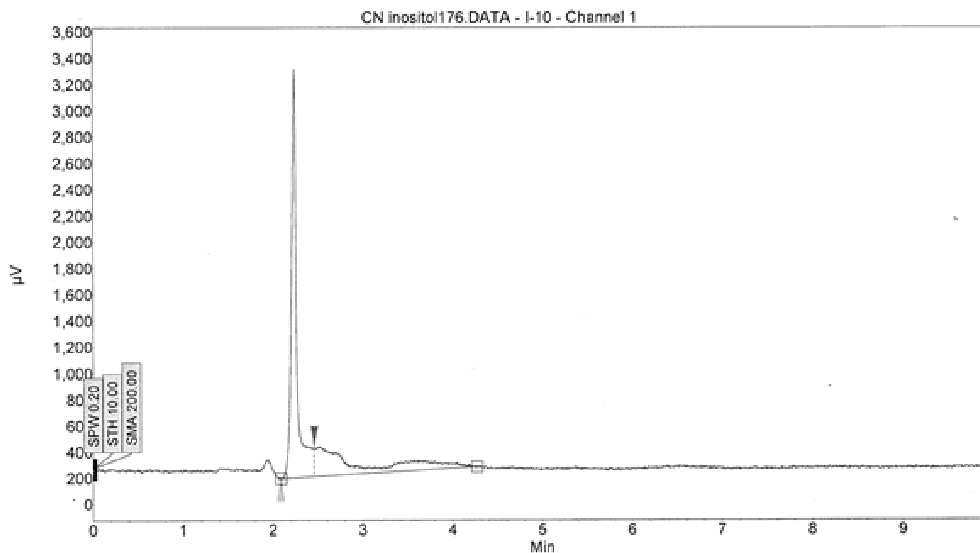


Figure 5. Chromatogram of inositol at 1.998×10^{-3} molar (molar mass = 180.16 grams/mole). HPLC conditions include detection at 265 nm, flow rate 1.00 mL/minute, rise time 0.1, and range AUFS 1.0. Solvent is 94 % water, 5 % ethanol, and 1 % acetic acid.

Analyte recovery was consistent and precise in this study. The recovery rate for isoniazid utilizing HPLC is presented in Table 3. The calculated molar values are determined from the molarity of the stock solution dissolved in aqueous solution from the HPLC standard curve analysis. The six samples all had a recovery percentage range of 97.3-105%. The Grubb's test indicated no outliers were present. Percent recovery of drug samples, comparing calculated molarity concentration to values from HPLC standard curve analysis resulted in average percent recovery at 100.2%. The standard deviation s = 3.43, with a variance of $s^2 = 11.756$. The calculated molar values in comparison to HPLC measured molar values shows an excellent relationship by Passing-Bablok regression (slope = 0.9725, y-axis intercept = 0.000). The calculated 95% confidence interval for the y-axis intercept includes the value of zero (0.00 to 0.00) and the 95% confidence interval for the slope does include 1 (0.9488to 1.000). Therefore, there are no constant differences between the calculated values of concentration and the HPLC measured values, so these values can be used interchangeably. Therefore, the calculated molar values are representative of the HPLC measured molar values. Passing – Bablok regression analysis allows comparison of two measurement methods for method comparison studies [11].

TABLE 3

DISSOLVED SAMPLE OF ISONIAZID, PERCENT RECOVERY

Sample	**Concentration by Calculated Value**	**Concentration by HPLC Standard Curve Analysis**	**Percent Recovery**
1	8.583×10^{-4}	8.348×10^{-4}	97.3
2	1.108×10^{-3}	1.101×10^{-3}	99.4
3	9.515×10^{-4}	9.993×10^{-4}	105
4	1.101×10^{-3}	1.080×10^{-3}	98.1
5	1.133×10^{-3}	1.178×10^{-3}	104
6	1.062×10^{-3}	1.034×10^{-3}	97.4

The percent recovery of isoniazid from ampoule type storage of this drug that is intended for clinical usage was also measured, with the outcome presented in Table 4. Average percent recovery is 100.67% with Standard deviation s = 0.577, in addition, having a variance of s^2 = 0.333. Analysis of Table 4 calculated molar values, when in comparison to HPLC measured molar values does show an excellent relationship by Passing-Bablok regression (slope = 0.9725, y-axis intercept = 0.000). With the 95% confidence interval for the y-axis intercept including zero (0.00 to 0.00) and the 95% confidence interval for the slope including 1 (0.9488to 1.000). Therefore, again, there are no constant differences between the calculated and HPLC measured values, and these values can be used interchangeably. Therefore, the calculated molar values are representative of the HPLC measured molar values [11].

TABLE 4

AMPOULE SAMPLE OF ISONIAZID, PERCENT RECOVERY

Sample	Concentration by Calculated Value	Concentration by HPLC Standard Curve Analysis	Percent Recovery
1	6.450×10^{-4}	6.536×10^{-4}	101
2	7.800×10^{-4}	7.835×10^{-4}	100
3	8.850×10^{-4}	8.926×10^{-4}	101

Clearly, isoniazid is a very important antibacterial agent for the treatment of pulmonary tuberculosis and extra-pulmonary tuberculosis. Methods to assay isoniazid will be highly useful for patient compliance verification, quality control in industrial production, and clinical verification of pharmaceutical content. The methodology that is demonstrated in this study is sensitive, efficient, consistent, and accurate. The use of HPLC for assay of drugs is a powerful and versatile approach to achieve high levels of sensitivity and precision. HPLC is effective for isoniazid determination.

CONCLUSION

This study demonstrates the efficacy of isocratic determination of isoniazid – an important drug for the treatment of tuberculosis. The study shows that isoniazid can be assayed from aqueous mixtures as well as from solid origins. Detection is consistent, accurate, and over a broad range of concentrations. The standard curve detected amounts from 0 molar to 0.001460 molar. The coefficient of determination obtained as $R^2 = 0.9921$ explanations for 99.21% of the variance in the dependent variable. Percent recovery of analyte is very high. The standard curve is linear and has an extremely high positive correlation with peak areas derived from HPLC analysis.

The instrumental analysis of pharmaceutical products meant for human consumption is a vital function of analytical chemistry. The methodologies have become tools for identifying substitutes in products by manufacturers and contaminants. The types of instruments allow investigators a broad range of choices, giving the analysis of products by HPLC to continue to be a successful area of application, due to its high sensitivity of detection. This methodology will be useful for clinical monitoring and industrial manufacturing of this important drug, allowing confidence in the quality of available pharmaceutical products in medical facilities.

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