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## Spectrophotometric and colorimetric methodology to detect and quantify hydrazide based chemotherapeutic drugs

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### ABSTRACT

Pharmaceuticals having the hydrazide functional group are an important division within anti-tuberculosis, anti-parasitic, anti-cancer, and anti-radiation chemotherapeutics. This work presents a colorimetric methodology to detect and quantify hydrazide based pharmaceuticals. Processes for rapid spot test, qualitative colorimetric assay, and spectrophotometric quantitative assay are presented. The reagent 2,6-dichloroquinone-4-chloroimine (Gibb's reagent) is utilized to accomplish the various analytical objectives. The rapid spot test and qualitative colorimetric assay enables a detection of hydrazide drugs to a level of 0.0037 grams per milliliter. Quantitative spectrophotometric detection of hydrazide drugs is sensitive to a level of 128.2 micrograms per milliliter. The molar absorptivity ( $\epsilon$ ) is calculated to be 25.32 Liters/(mole $\times$ cm) at 970nm. Inorganic salts and organic compounds were found not to interfere with colorimetric detection of hydrazide drugs, and this includes NaBr, NaCl, MgSO<sub>4</sub>, 2-naphthol, benzoin, p-aminobenzoic acid various other compounds. Reagents necessary for this methodological approach are readily available. The colorimetric response of these tests is readily identified by visual inspection. These methods then are highly sensitive and provide a manner to determine this important type of drugs from environmental as well as biological origins.

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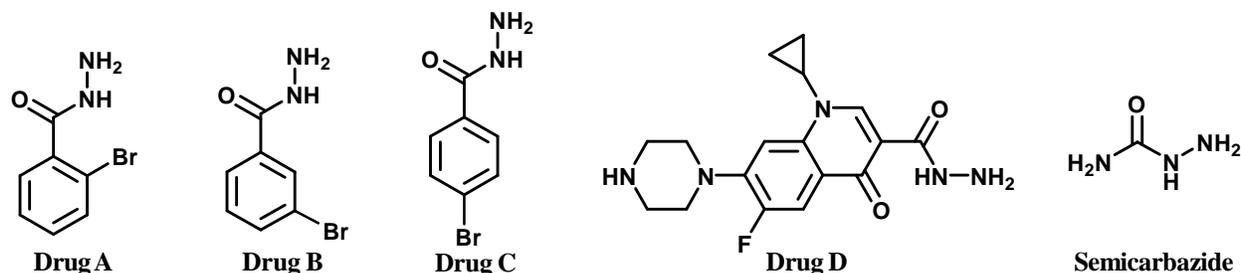
### KEYWORDS

Hydrazide;  
Isoniazid;  
Colorimetric;  
Gibb's reaction.

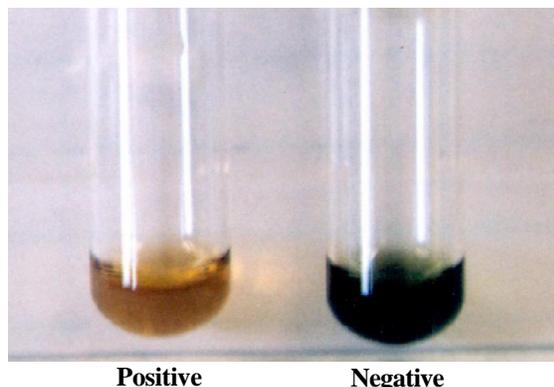
### INTRODUCTION

Hydrazide based pharmaceuticals (have the hydrazide functional group located on the molecular structure) are an important group of diversified chemotherapeutics. Examples of this important category of drugs come from medical areas of anti-tuberculosis, anti-parasitic,

anti-cancer, and anti-radiation clinical therapeutics. One example that is credited as saving many lives is isoniazid, a first-line medication/treatment for the bactericidal/bacteriostatic inhibition of tuberculosis<sup>[1-3]</sup>. Acyl hydrazides have shown substantial potential for important anti-trypanosomal chemotherapeutic agents including the treatment for parasitic protist Trypanosoma



**Figure 1 :** Molecular structures of pharmaceuticals utilized in this work are presented having hydrazide functional groups. The hydrazide functional group is indicated by inset rectangle. Drugs A, B, C, and D were synthesized utilizing microwave methodology while semicarbazide was used in this study as the spectrophotometric standard.



**Figure 2 :** A rapid spot test result demonstrating the use of Gibb's reagent detection of hydrazide functional groups. Left tube is "positive" reaction for the presence of hydrazide drug (2 milligrams), right side is negative colorimetric response (no hydrazide drug).

brucei or African Sleeping Sickness<sup>[4]</sup>. Previous studies of hydrazide derivatives of wild garlic result in compounds that could function as effective anti-genotoxic chemotherapeutics for inhibition of clastogenic (mutagenic breakages of chromosomes) aberrations in vivo<sup>[5]</sup>. Another considerable potential for novel hydrazide base chemotherapeutics is in the area of cancer treatment. Significant cytotoxic activity against ovarian cancer is noted for hydrazide based compounds<sup>[6]</sup>. In addition, hydrazide based drugs have shown remarkable anticancer activity toward hormone-dependent and hormone-independent human cancer cells<sup>[7]</sup>. Various hydrazide based drugs have demonstrated cytotoxicity against drug-resistant cancer cell lines in combination therapy<sup>[8]</sup>.

Other studies have shown that salicylhydrazide compounds demonstrate potent anti-cancer activity against human cancer cell lines derived from different origins<sup>[9]</sup>. The diversified efficacy of hydrazide based drugs forecasts continued application in clinical arenas. Hydrazides have been studied as potential anti-

radiation drugs<sup>[10]</sup>. A benzoic acid hydrazide transferring tagged drug was utilized as target-carrier for studies of human breast cancer<sup>[11]</sup>. Novel medications that are hydrazide based are being studied for clinical treatment of excessive fluid retention<sup>[12]</sup>. In addition, sulfur based hydrazides have been studied for clinical nutritional modulation and control<sup>[13]</sup>. These applications strongly support the necessity of having accurate and sensitive means to detect and quantify hydrazide based chemotherapeutics. This work present a rapid spot test, qualitative colorimetric assay, and a sensitive spectrophotometric methodology to ascertain hydrazide based drugs.

## EXPERIMENTAL

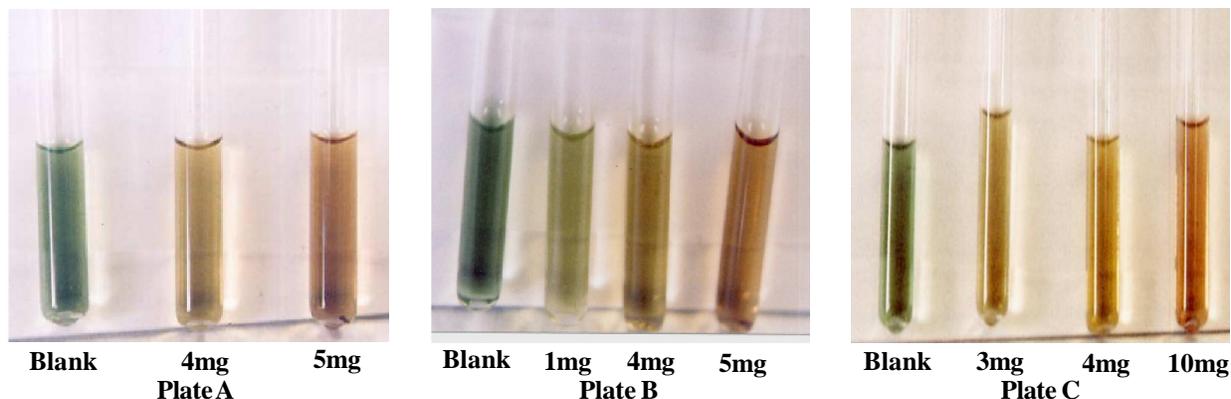
### Reagents and instruments

All reagents utilized for this study were supplied by Sigma-Aldrich (P.O.Box 2060, Milwaukee WI, USA). Gibb's reagent (2,6-dichloroquinone-4-chloroimine) was acquired through Sigma-Aldrich and was stored dry until use at  $-10^{\circ}\text{C}$ . Organic solvents such as acetone was stored in airtight containers over molecular sieves to remove water. All spectrophotometric measurements were made by Milton Roy Spectronic 21D using UV/Visible glass cuvettes.

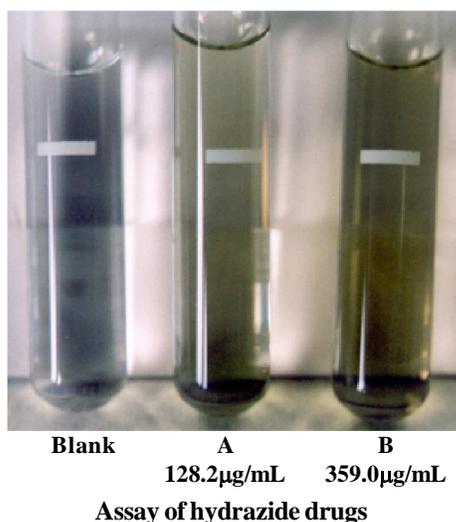
### Colorimetric assay methodology

The mixture containing Gibb's reagent 2,6-dichloroquinone-4-chloroimine is made immediately before use, kept at room temperature, and made fresh before each application. An amount of 14 milligrams 2,6-dichloroquinone-4-chloroimine is dissolved into 14.0 milliliters of acetone. Into a reaction tube is placed 100 microliters of water, 100 microliters acetone, followed by 60 microliters of prepared Gibb's reagent

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**Figure 3 :** The determination of hydrazide class of pharmaceuticals can be accomplished by colorimetric response method which provides a qualitative value of concentration. Shown here are results of this determination from Plate A: Blank-no drug, Test sample at 4 milligrams, and known control at 5 milligrams. By comparison to the 5 milligram control, the test sample can be estimated at about 4 milligrams. Similarly Plate B: Blank-no drug, with known controls at 1 milligram and 5 milligram permit estimation of unknown sample at 4 milligrams. Similarly Plate C: Blank-no drug, known controls at 3 milligrams and 10 milligrams drug permit assay of test sample at 4 milligrams.



**Figure 4 :** For spectrophotometric assay of hydrazide drugs the reaction of Gibb's reagent with hydrazide is quantitative. Shown is the colorimetric response for the Blank (Gibb's reagent plus  $\text{NH}_4\text{OH}$ ). This method is highly sensitive, demonstrated by example samples determined to be (A) 128.2 micrograms per milliliter and (B) 359.0 micrograms per milliliter. mixture, and finally 10 microliters of 6 molar  $\text{NH}_4\text{OH}$  prepared in water. Color reaction begins immediately and is followed by comparison of test samples to appropriate controls of known hydrazide group concentration. For this study, semicarbazide·HCl was utilized and demonstrated consistent and strong color response.

### Rapid spot test

Into 200 microliters of prepared Gibb's reagent (see above for preparation) is added an equal volume of test drug dissolved into acetone. The addition of 20

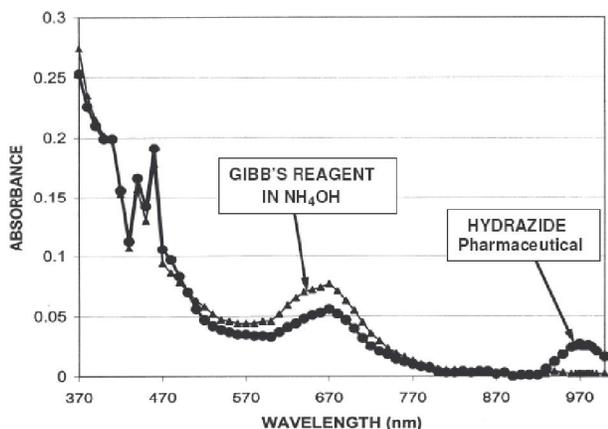
microliters of 6 molar  $\text{NH}_4\text{OH}$  will produce the positive brownish-yellow or a negative dark green-yellow color response.

### Assay by spectrophotometer

The spectrophotometric assay of hydrazide pharmaceuticals is accomplished at wavelength 970nm using UV/Visible glass cuvettes with a Spectronic 21D instrument. Test samples to be assayed by spectrometer are first dissolved into acetone previously dried over molecular sieves. A "blank" is prepared using acetone dried over molecular sieves. The pharmaceutical drug samples are dissolved into acetone prior to solubilizing into the reaction mixture. Preparation of the Gibb's reagent is similar to that of colorimetric assay (see above). All standards (utilizing semicarbazide·HCl) and pharmaceuticals are prepared similarly. Into three milliliters of acetone is placed an amount of 500 microliters acetone solution of previously dissolved pharmaceutical drug, followed by 350 microliters of Gibb's reagent mixture, and 50 microliters of 6 molar  $\text{NH}_4\text{OH}$ . Fifteen minutes is allowed for optimum colorimetric production, then read standards and test samples at 970nm using dried acetone for adjusting for zero absorbance.

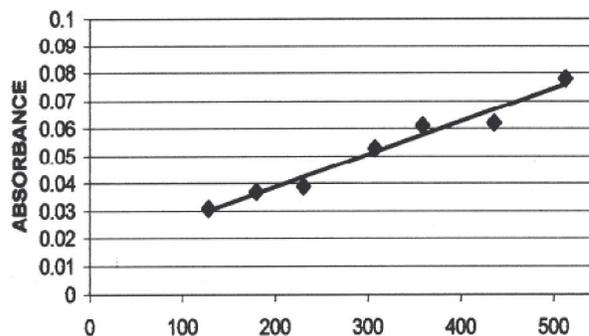
## RESULTS & DISCUSSION

Hydrazide based compounds constitute a major category of clinical drugs utilized to treat cancer, tuberculosis, parasitic infection, liquid retention, and nutritional



**Gibbs reagent with  $\text{NH}_4\text{OH}$  and hydrazide pharmaceutical**  
**Figure 5 :** The absorbance spectra for Gibb's reagent in  $\text{NH}_4\text{OH}$  and hydrazide complex formed upon reaction with Gibb's reagent is shown from 370nm to 1000nm. The absorbance maxima at 970nm from the hydrazide complex is used for spectrophotometric determination of pharmaceuticals.

health. Having the hydrazide ( $-\text{C}(=\text{O})\text{NHNH}_2$ ) functional group facilitates a drug structure having a diverse biological activity. Methods to determine the presence and amounts of these drugs would be valuable for clinical settings as well as industrial and health care facilities. Gibb's reagent (2,6-dichloroquinone-4-chloroimine) will react with, and assay, many para substituted phenolic based compounds<sup>[14-16]</sup>. Because of the tremendous importance of hydrazide based anti-tuberculosis pharmaceuticals (such as the first-line drug isoniazid) for treating and prevention of tuberculosis an efficient methodology for detection and assay would be beneficial. A rapid spot test, colorimetric assay, and spectrophotometric determination of hydrazide based anti-tuberculosis agents is presented in this work. Several hydrazide compounds were synthesized by a microwave methodology demonstrated previously<sup>[17]</sup>, presented in figure 1, and were utilized in this work to establish the efficiency of hydrazide drug determination by utilizing Gibb's reagent 2,6-dichloroquinone-4-chloroimine. Drugs A, B, C, and D (Figure 1) each have a single hydrazide functional group present (indicated by inset rectangle). Drugs A, B, C, and D have been shown in previous work to be effective inhibitors of Tuberculosis proliferation<sup>[17]</sup>. In addition to the hydrazide moiety these four drugs have at least one aromatic ring. The structure of drugs A, B, and C have a bromine substituent on the aromatic ring which is shifted from ortho, to meta, and para position, respectively.



**Determination of hydrazide drugs**

**Figure 6 :** A standard curve for hydrazide based pharmaceuticals shows accuracy and sensitivity to 115 micrograms per milliliter.

The parent structure of drug D is ciprofloxacin which is a second generation fluoroquinolone broad-spectrum antibacterial that inhibits bacterial growth by interfering with enzymes necessary for DNA unwind and duplication<sup>[18]</sup>. In addition to these four drugs the compound semicarbazide was utilized to establish a sensitive spectrophotometric absorbance assay. All reagents are made fresh before use. Many inorganic and organic compounds were shown to be non-interfering for these colorimetric methods. These compounds are presented in TABLE 1, and include NaBr, NaCl,  $\text{MgSO}_4$ ,  $\text{NaH}_2\text{PO}_4$ , salicylic acid, itaconic acid, benzoin, adipic acid, trans-cinnamic acid, DL-mandelic acid, and p-aminobenzoic acid as representative assemblage. For quick identification of hydrazide based pharmaceuticals a colorimetric presumptive test or rapid spot test has several assets. Notably this type of test are viewed as: 1) rapid, 2) simple, 3) facile interpretation, and 4) mobile. An example of a rapid spot test for hydrazide based drugs is presented in figure 2. The 'negative' event is a very dark green colorized mixture that is highly differentiated from a 'positive' event that can be described as a yellow-brown or gold outcome. These only two possible results are contrasted and easily distinguishable. This example shows results for only 2 milligrams of drug. The assay for hydrazide class of pharmaceuticals can be accomplished by a colorimetric response method which provides a qualitative value of concentration. Several examples of this style is presented in figure 3. Shown are outcome of this qualitative assay in Plates A, B, and C. Outcome for Plate A: Blank-no

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TABLE 1 : Compounds not interfering with assay.

Compounds not interfering with chemical identification of hydrazide functional group
Sodium bromide
Sodium chloride
Sodium phosphate, monobasic
Magnesium sulfate
Adipic acid
Trans-cinnamic acid
DL-mandelic acid
Salicylic acid
2-Naphthol
Itaconic acid
Benzoin
p-Aminobenzoic acid

drug (negative control), a Test sample at 4 milligrams, and a known positive control at 5 milligrams. By inspection using the 5 milligram control, the test sample can be estimated at about 4 milligrams. Similarly for Plate B: Blank-no drug (negative control), positive controls at 1 milligram and 5 milligram permit estimation of the unknown sample at 4 milligrams. Similarly with Plate C: Blank-no drug (negative control), known controls at 3 milligrams and 10 milligrams drug permit assay of the test sample at 4 milligrams. Likewise to the rapid spot test this approach is simple yet produces outcomes that are highly distinguishable. A spectrophotometric assay for hydrazide drugs also demonstrates that the chemical reaction of Gibb's reagent with hydrazide functional groups is quantitative. Shown in figure 4 is the colorimetric outcome for the Blank (Gibb's reagent plus  $\text{NH}_4\text{OH}$ ) as well as two example standards of known concentration. This approach is highly sensitive, demonstrated for standard (A) at 128.2 micrograms per milliliter and standard (B) which is at 359.0 micrograms per milliliter. For quantitative spectrophotometric assay it is recommended to enable the analysis at a wavelength in which the analyte of interest establishes maximum absorbance contrasted to any foreign interference. The reaction of Gibb's reagent with  $\text{NH}_4\text{OH}$  generates a dark blue mixture that expresses absorbance through a broad range of wavelength and is significant through a large portion of this range. The absorbance spectrum presented in figure 5 demonstrates the comparison of Gibb's reagent interaction with  $\text{NH}_4\text{OH}$  and the hydrazide functional group of hydrazide based pharma-

ceutics. In the range from 370nm to 1000nm the Gibb's reagent plus  $\text{NH}_4\text{OH}$  produces a dark blue mixture that conflicts with the outcome of the Gibb's reagent plus hydrazide group. Only in the wavelength range from 930nm to 1000nm does the Gibb's reagent plus hydrazide drug generate an overwhelming absorbance compared to the Gibb's reagent plus  $\text{NH}_4\text{OH}$  side reaction. Therefore the wavelength of 970nm is utilized for the spectrophotometric quantitative analysis. The final molar absorptivity ( $\epsilon$ ) is calculated to be 25.32 Liters/(mole $\times$ cm) at 970nm. A representative standard curve is visualized in figure 6. Where the standard curve for hydrazide based pharmaceuticals shows accuracy and sensitivity to 115 micrograms per milliliter. Linearity is extremely high rendering a Pearson correlation constant  $r$  of 0.9841. The equation of this particular line becomes:  $y = 0.0001x + 0.015$ . The percent recovery for pharmaceutical analyte is extremely high from 95% to 105%. The  $R^2$ , coefficient of determination for this data is 0.9685, indicating that 96.85% of all variance in the data is accounted for by the equation. Clearly the identification of hydrazide based pharmaceuticals can be done accurately and consistently by reaction with Gibb's reagent. The methodologies presented in this study will find considerable efficacy for environmental related applications where it is necessary to identify and quantify drugs that bear the hydrazide functional group.

### ACKNOWLEDGEMENTS

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