Development of a Microcrystal Test
for the Detection of Clonazepam

BY

DANIELLE K. SILLETTI
B.S., Hope College, 2011

THESIS
Submitted as partial fulfillment of the requirements
for the degree of Master’s in Science in Forensic Science
in the Graduate College of the
University of Illinois at Chicago, 2013

Chicago, Illinois

Defense Committee:

Karl Larsen, Chair and Advisor
Adam Negrusz
Gary Laughlin, McCrone Research Institute
ACKNOWLEDGMENTS

I would like to extend a huge thank you to the entire staff at McCrone Research Institute for their constant support and advice. Without them this project would not have been possible. They taught me all I know about microscopy and provided me with the equipment and resources I needed to carry out this research. I’d also like to thank the Illinois State Police drug chemistry section for providing samples and Jim Dunlop at the Michigan State Police for consulting on this project.

I’d also like to thank my professors, Dr. Karl Larsen and Dr. Adam Negrusz, for their support and belief in this research. Their teachings did a lot to strengthen my passion for forensic science and encouraged me to undertake a thesis project in the first place.
TABLE OF CONTENTS

CHAPTER                                PAGE

I.  INTRODUCTION................................................................. 1
    A.  Background.......................................................... 1
        i.  Microcrystal Tests........................................ 1
        ii. Clonazepam.............................................. 2
    B.  Related Literature.............................................. 4
        i.  Tewari 1978.............................................. 4
        ii. Haynes 2000............................................ 4
    C.  Purpose of the Study............................................ 5

II. MATERIALS AND METHODS.............................................. 6
    A.  Clonazepam Samples............................................. 6
    B.  Light Microscopy................................................ 7
    C.  Hot Stage Microscopy.......................................... 7
    D.  Solubility Tests................................................ 8
    E.  Microcrystal Tests.............................................. 8
        i.  Direct Test............................................... 9
        ii. Hanging Drop Test..................................... 9
        iii. Hanging Drop Test – Tewari........................................ 9

III. DISCUSSION............................................................... 11
    A.  Optical Characterization..................................... 11
        i.  Microscopical Melting Point.......................... 11
        ii. Solubility............................................... 12
        iii. Recrystallization....................................... 13
        iv.  Optical Properties..................................... 14
    B.  Microcrystal Tests............................................... 17
        i.  Clonazepam with Platinum Chloride.................... 19
        ii. Clonazepam with Potassium Cadmium Iodide......... 21
        iii. Clonazepam with Potassium Triiodide............... 21
        iv.  Clonazepam with Ammonium Thiocyanate............... 22
        v.  Clonazepam with Sodium Carbonate..................... 22
        vi.  Clonazepam with Potassium Chromate.................. 22
        vii. Clonazepam with Potassium Mercuric Iodide......... 23
        viii. Clonazepam with Lead Iodide........................ 24
        ix.  Clonazepam with Zinc Chloride....................... 25
        x.   Clonazepam with Gold Chloride (H₂PO₄)............ 25
        xi.  Clonazepam with Platinum Chloride and Chloroform... 26
        xii. Clonazepam with Platinum Chloride and Acetone..... 27

IV.  CONCLUSION............................................................. 30

REFERENCES........................................................................ 31

APPENDIX........................................................................... 33
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>VITA</td>
<td>35</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. SOLUBILITY OF CLONAZEPAM IN COMMON SOLVENTS</td>
<td>12</td>
</tr>
<tr>
<td>II. OPTICAL PROPERTIES OF CLONAZEPAM</td>
<td>17</td>
</tr>
<tr>
<td>III. RESULTS OF MICROCRYSTAL TEST ON CLONAZEPAM</td>
<td>18</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. The periods per printed page system used to measure the amount of clonazepam used during testing</td>
<td>6</td>
</tr>
<tr>
<td>2. A photomicrograph of clonazepam in its glass phase</td>
<td>11</td>
</tr>
<tr>
<td>3. Photomicrographs of clonazepam recrystallized in benzyl alcohol</td>
<td>13</td>
</tr>
<tr>
<td>4. Photomicrographs of clonazepam recrystallized in thymol</td>
<td>14</td>
</tr>
<tr>
<td>5. A photomicrograph of clonazepam analytical standard in 1.66 refractive index oil</td>
<td>15</td>
</tr>
<tr>
<td>6. A photomicrograph of a clonazepam crystal showing high order interference colors</td>
<td>15</td>
</tr>
<tr>
<td>7. A photomicrograph series of a clonazepam crystal showing dispersed extinction</td>
<td>16</td>
</tr>
<tr>
<td>8. Photomicrographs of crystals formed from a clonazepam standard and platinum chloride solution (plane-polarized light)</td>
<td>19</td>
</tr>
<tr>
<td>9. Photomicrographs of crystals formed from a clonazepam standard and platinum chloride solution (crossed-polarized light)</td>
<td>20</td>
</tr>
<tr>
<td>10. Photomicrographs of crystals formed from a clonazepam standard and potassium chromate solution</td>
<td>23</td>
</tr>
<tr>
<td>11. A photomicrograph of crystals formed from a clonazepam standard and lead iodide solution</td>
<td>25</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>ED</td>
<td>Emergency Department</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
</tr>
<tr>
<td>H$_3$PO$_4$</td>
<td>Phosphoric Acid</td>
</tr>
<tr>
<td>H$_2$PtCl$_6$</td>
<td>Platinum Chloride</td>
</tr>
<tr>
<td>HAUCl$_4$</td>
<td>Gold Chloride</td>
</tr>
<tr>
<td>K$_2$CdI$_4$</td>
<td>Potassium Cadmium Iodide</td>
</tr>
<tr>
<td>K$_2$CrO$_4$</td>
<td>Potassium Chromate</td>
</tr>
<tr>
<td>K$_3$HgI$_4$</td>
<td>Potassium Mercuric Iodide</td>
</tr>
<tr>
<td>KI$_3$</td>
<td>Potassium Triiodide</td>
</tr>
<tr>
<td>Na$_2$CO$_3$</td>
<td>Sodium Carbonate</td>
</tr>
<tr>
<td>NH$_4$SCN</td>
<td>Ammonium Thiocyanate</td>
</tr>
<tr>
<td>PbI$_2$</td>
<td>Lead Iodide</td>
</tr>
<tr>
<td>PLM</td>
<td>Polarizing Light Microscopy</td>
</tr>
<tr>
<td>PPP</td>
<td>Periods on a Printed Page</td>
</tr>
<tr>
<td>SWGDRUG</td>
<td>Scientific Working Group for the Analysis of Seized Drugs</td>
</tr>
<tr>
<td>ZnCl$_2$</td>
<td>Zinc Chloride</td>
</tr>
</tbody>
</table>
Microcrystal tests are chemical microscopy techniques that date back to the 1800’s. Recently, they have been used as an analytical technique for distinguishing certain classes of drugs in the forensic sciences. Some advantages of microcrystal tests are they’re relatively inexpensive, fairly simple to conduct, and use very little drug sample. While some drugs, such as cocaine and heroin, have well validated microcrystal tests, other classes of drugs, i.e. the benzodiazepines have not been extensively studied for this purpose.

Clonazepam is a benzodiazepine drug sold under the trade name Klonopin®. It is known for its sedative-hypnotic properties and has therapeutic uses as an anticonvulsant and anxiolytic. Benzodiazepines have been abused for these sedative effects, but are more popularly known for their use in drug-facilitated sexual assaults. In the past decade emergency department visits involving the abuse and misuse of benzodiazepines have increased, indicating the benefit of validated microcrystal tests for their detection.

A microcrystal test was developed that is able to detect clonazepam in its pharmaceutical preparations at all of its therapeutic dosages. The test involves the addition of acetone and a 10% platinum chloride solution to microgram quantities of clonazepam. The presence of clonazepam is indicated by the formation of rosettes made of colorless, blunt-ended rods. No other drug is known to exhibit the same reaction with these reagents making the test a good candidate for use in forensic lab settings. In addition, it follows a simple procedure, uses minute quantities of the drug sample, and involves reagents that are common and involved in other known drug microcrystal tests.
I. INTRODUCTION

A. Background

i. Microcrystal Tests

Microcrystal tests are chemical precipitation tests that rely on the formation of distinctly shaped crystals to identify drugs of interest (Fulton, 1969; Siegel, 1988; Wielbo and Tebbett, 1992). These chemical microscopy techniques date back to the 1800’s (Fulton, 1969; Wielbo and Tebbett, 1992). In general, the tests involve fully or partially dissolving a minute amount of sample in a solvent on a microscope slide. A test reagent is then added to the sample drop and usually within a few minutes distinct crystals will form. These microcrystals are examined and characterized under the microscope to identify the compound in the sample (Chamot and Mason, 1989; Clarke, 1969; Fulton, 1969). There are many different variations of this method, however, and these differences can be exploited to help enhance the sensitivity and specificity of the tests (Chamot and Mason, 1989). Some advantages of microcrystal tests are that they’re relatively inexpensive, fairly simple to conduct and use very little drug sample. There are disadvantages to this technique, however, including the need for comparison standards, and a clean sample (Jickells and Negrusz, 2008; Wielbo and Tebbett, 1992).

In the forensic science community microcrystal tests are primarily used as preliminary or screening tests, meaning they help to identify a class of drug but are rarely considered by themselves to be conclusive for drug identification (Jickells and Negrusz, 2008; Siegel, 1988). Although their use has been diminishing they still have an important place in the drug chemist’s arsenal. In fact, they are still thought to be the best way to
distinguish the $d$- and $l$- isomers of amphetamine and cocaine (Siegel, 1988). In their 2011 report, the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) recommended microcrystalline tests as a valid technique for drug identification when used in conjunction with an additional and distinct analytical technique such as mass spectrometry or infrared spectroscopy (SWGDRUG, 2011).

i. **Clonazepam**

Clonazepam is a benzodiazepine drug with the chemical formula $\text{C}_{15}\text{H}_{10}\text{ClN}_{3}\text{O}_{3}$ and the structure shown below (SIGMA, 2012).

It was first synthesized in 1969 and is currently made and marketed in the U.S. by Hoffman-LaRoche, Inc. under the trade name Klonopin®. Clonazepam is considered a Schedule 4 drug under the Controlled Substances Act of 1970 which means it has a moderate risk of abuse, but is approved for medical use. It is considered a licit drug of abuse, which means that it is manufactured legally but its possession and supply is restricted by the law because of its potential harmful effects on its user. Clonazepam is almost always found and taken in oral tablet form. It is known for its sedative-hypnotic properties and has therapeutic uses as an anticonvulsant and anxiolytic drug (Jickells and Negrusz, 2008; Pagliaro and Pagliaro, 2009).
Benzodiazepines have been abused for their sedative effects or euphoric-inducing properties, but are more popularly known for their use in drug-facilitated sexual assaults. Benzodiazepines are central nervous system (CNS) depressants. They act as agonists at benzodiazepine receptors (BZD-1 and BZD-2) in the CNS, particularly in the cerebral cortex and limbic system. When bound to these receptors, benzodiazepines facilitate and increase the inhibitory action of the neurotransmitter gamma-aminobutyric acid (GABA) resulting in sedation and other effects. (Longo and Johnson, 2000; Jickells and Negrusz, 2008; Pagliaro and Pagliaro, 2009). In acutely toxic doses, clonazepam can result in dizziness, confusion, drowsiness, and cognitive and psychomotor impairment. Although benzodiazepines can cause somnolence or coma at overdose levels, they rarely result in death.

Misuse and/or abuse of benzodiazepines in the United States have been on the rise in the past decade. Benzodiazepines have been misused/abused over other hypnotics, such as barbiturates, because they have fewer side effects and less potential to cause death (Longo and Johnson, 2000; Pagliaro and Pagliaro, 2009). Currently clonazepam is one of the top three most abused benzodiazepines in North America (Pagliaro and Pagliaro, 2009) and was ranked 51st for total number of prescriptions written in 2012 (Bartholow, 2013). Clonazepam specifically is considered to have a high abuse liability because it is a strong benzodiazepine and has a relatively short onset of action. According to the 2010 Drug Abuse Working Network report, emergency department (ED) visits involving benzodiazepine abuse and misuse increased by 139 percent between 2004 and 2010 (US Government, 2012). Among pharmaceuticals, the abuse and misuse of
benzodiazepines were involved in the highest rate of ED visits for persons aged 20 and under and the second highest rate of ED visits for persons aged 21 and over.

B. Related Literature

i. Tewari 1978

In 1978, S. Tewari conducted a studied entitled “Studies on the identification of 1.4-benzodiazepine drugs by micro-crystal tests”. In this study, Tewari looked at a number of different benzodiazepines and identified reagents that resulted in positive crystal formation when combined with the drugs in a microcrystal test. He used the same method, a hanging drop test, for all of his microcrystal tests so that the test drops wouldn’t evaporate over the 24 hour testing period. If crystals formed within 24 hours the test was considered positive and the crystal shape was recorded in a diagram. The limit of detection for these microcrystal tests and the time in which it took crystals to form were also reported. He observed that a variety of different reagents, when added to clonazepam, led to the formation of 4-sided tablets.

This study provided majority of the reagents that were used in this study and their recipes. It also gave a published procedure for a method that reportedly had led to crystal formation when clonazepam was combined with the proper reagent. The results of this study as they compare to the results of Tewari’s study are reported in Section III.

ii. Haynes 2000

In his 2000 thesis, titled “On the Development of Microcrystal Tests for some 1,4-Benzodiazepines”, William Haynes reported reagents that proved successful in identifying various benzodiazepines though microcrystal tests. Haynes used the same
direct test method for all of microcrystal tests and reported the results after the drug and reagent had been combined for 60 minutes. If crystals formed within 60 minutes the test was considered positive and a photomicrograph of the crystals was obtained. The limit of detection for these microcrystal tests was reported along with the results of a blind study conducted to verify his results. Haynes found that when a 5% platinum chloride solution was added to clonazepam rosettes, sheafs, and fans of needles formed,

This thesis provided a crucial reagent that was used in this study and its recipe. It also helped with the development of the method that was used in some of the successful microcrystal test trials. The results of this study as they compare to the results of Haynes’s study are reported in Section III.

C. **Purpose of the Study**

While some drugs, such as cocaine and heroin, have well validated microcrystal tests, other classes of drugs, i.e. the benzodiazepines have not been extensively studied for this purpose. Since the abuse/misuse of benzodiazepines has been increased over the last decade so has the need to fully understand the physical and chemical properties of these drugs for detection. Although microcrystal tests tend to be overlooked in favor of more complex instrumentation, they remain a valid and important method of detection. The goal of this project, therefore, was to optically characterize and develop a microcrystal test that can be used to detect clonazepam.
II. MATERIALS AND METHODS

A. Clonazepam Samples

Both analytical standards and pharmaceutical preparations of clonazepam were used for this study. The analytical standard was a loose powder of pure clonazepam and was manufactured by Sigma-Aldrich®. This sample was donated by the Illinois State Police Crime lab and therefore was validated using an ATI Mattson Quantum Infrared Microscope™. The pharmaceutical preparations studied were a 2 mg Klonopin® tablet (from Hoffman-LaRoche Inc.) and generic 2 mg, 1 mg, and 0.5 mg tablets. A generic 0.25 mg orally disintegrating tablet was also used. To get a representative sample of the contents of the pharmaceutical preparations, the tablets were cut in half and portions of the inner cross section of the tablet were removed with a razor blade.

The amount of clonazepam sample used in the tests was measured in periods on a printed page (PPP). This means that the amount of sample used was enough to cover “x amount” of periods typed on a page of paper, such as the one found at the end of this sentence (Figure 1).

The periods were written in size 10 Times New Roman font.

1 PPP  2 PPP  3 PPP  4 PPP  5 PPP

Figure 1. The PPP system used to measure the amount of clonazepam used during testing.
This system was employed to make the tests reported in this paper easier to reproduce and to help address the problem of ambiguous concentrations used in previously published microcrystal test methods.

B. **Light Microscopy**

All of the sample characterization and crystal tests were examined with a Nikon Optiphot polarized light microscope at 100X, 200X, and/or 400X total magnification. Plane-polarized light was most commonly used in the examinations, but crossed-polarized light with and without a first order red compensator plate (~530 nm) were also employed. Photomicrographs were taken using an Olympus DP-70 camera mounted on a Nikon Optiphot polarizing light microscope. Scale bars were added to the pictures using DP Controller software (Olympus Corporation, 2001), but no other enhancements or alterations to the photos were made. The colors seen in some of the photomicrographs are true representations of the colors seen through the microscope and were not artificially added.

C. **Hot Stage Microscopy**

Microscopical melting point determination was conducted on a Mettler FP-5 hot stage affixed to an Olympus BH-2 polarizing light microscope. The clonazepam analytical standard was heated at a rate of 3°C/min until the temperature of the stage was within 10°C of the literature reported melting point (Sigma, 2012; Merck Index, 2001). The sample was subsequently heated at a rate of 1°C/min until all of the clonazepam in the field of view had melted. This test was repeated three times and the range of these values is reported as the melting point range of clonazepam in this paper. The melting point values were then corrected for small discrepancies in the instrument using the following calibration curve equation: \( y = 0.9748x + 3.3201 \); where \( x \) is the experimental melting point and \( y \) is the actual melting point.
D. **Solubility Tests**

A small amount of clonazepam (~2 PPP) was placed on a clean microscope slide. While being viewed under a microscope, 10 µL of a solvent was added to the sample. The solvents used were water, acetone, ethanol, chloroform, methanol, xylene, benzene, thymol, and benzyl alcohol.

E. **Microcrystal Tests**

The first reagents selected for this testing were those that had been previously reported in the literature to have had successful reactions with clonazepam or other benzodiazepines. These reagents were platinum chloride (Clarke 1969; Haynes 2000), potassium cadmium iodide (Tewari 1978), potassium triiodide (Clarke 1969; Tewari 1978), ammonium thiocyanate (Haynes 2000; Tewari 1978), potassium chromate (Tewari 1978), potassium mercuric iodide (Tewari 1978), sodium carbonate (Clarke 1969; Haynes 2000; Tewari 1978), lead iodide (Tewari 1978), and zinc chloride (Tewari 1978). Additional reagents were chosen for testing that are currently used for drug testing in forensic laboratories, relatively safe, inexpensive, and easy to obtain or produce.

The reagents were screened with 5 PPP of the clonazepam standard. If the tests were negative, the reagent was not tested on any pharmaceutical preparations of clonazepam. A test was considered negative if no crystals formed after 20 minutes had elapsed from the time the reagent was added to the sample. If the screening tests with the clonazepam standard gave positive results, increasingly lower doses of the pharmaceutical preparations were tested until negative results were obtained. The following types of tests were used in this study, however not...
all types were used for every test reagent. The specific type(s) of test(s) used with each reagent will be reported in Section III.

i. **Direct Test**

The direct test involved dropping 5 μL of the test reagent directly onto the clonazepam on a glass microscope slide. The reaction was covered with a glass coverslip and viewed under the microscope. With some tests, the clonazepam was partially dissolved in a separate reagent before the test reagent was added. This additional step will be noted in Section III when the results of each reagent trial are described in detail. Negative controls were run by adding 5 μL of the test reagent to a blank portion of the microscope slide and viewing it under the microscope. The direct test is also known as Method IB according to Chamot and Mason (1931).

ii. **Hanging Drop Test**

The hanging drop test involved preparing the clonazepam on a glass coverslip and adding 5 μL of the test reagent to the clonazepam. The coverslip was subsequently inverted and placed on a glass ring sitting on a glass microscope slide. This small reaction chamber was then viewed under the microscope. Negative controls were run by adding 5 μL of the test reagent to a coverslip, inverting it over the reaction chamber, and viewing under the microscope.

iii. **Hanging Drop Test –Tewari**

This hanging drop test procedure is the same as that utilized in a paper by Tewari (2000). The method is similar to the hanging drop test described above except that 5 μL of 50% ethanol was added to the clonazepam before the test reagent was introduced. Negative controls were run by combining 5 μL each of the test reagent and 50% ethanol.
solution on a coverslip, inverting it over the reaction chamber, and viewing it under the microscope.
III. DISCUSSION

A. Optical Characterization

i. Microscopical Melting Point

The microscopical melting point range for the clonazepam standard was found to be 237.9°C-238.2°C. This experimental melting point for the clonazepam was on the higher end of the 236.5°C-238.5°C temperature range reported by Sigma (2012) and the Merck Index (2001). This discrepancy could result from the scale at which this test was performed. This paper reports a microscopical melting point for clonazepam, i.e. the melting point taken using microgram quantities of a sample on a hot stage attached to a microscope. If the sources mentioned above used a different method to determine melting point and/or used different quantities of the substance, small changes in the reported melting point could occur.

When clonazepam melts it cools to form a metastable glass phase (Figure 2).

Figure 2. A photomicrograph of clonazepam in its glass phase. Taken in plane-polarized light at 100X magnification.
The temperature at which clonazepam goes from a glass to a viscous liquid is 151°C, below this temperature mobility is stopped. This is the glass transition temperature ($T_G$) for clonazepam.

ii. **Solubility**

The solubility of clonazepam in various common solvents was determined. Clonazepam is practically insoluble in water, benzene, and xylene and only slightly soluble in ethanol and methanol. Clonazepam is soluble in acetone, chloroform, benzyl alcohol, and thymol. These results are consistent with those reported by the Merck Index (2001). Upon mounting the clonazepam in Cargille refractive index oil for PLM examination it was found that the sample is not soluble in the 1.66 oil. The solubility tests results are summarized in Table I.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Acetone</th>
<th>Benzene</th>
<th>Benzyl Alcohol</th>
<th>Chloroform</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>Soluble</td>
<td>Insoluble</td>
<td>Soluble</td>
<td>Soluble</td>
<td>Slightly soluble</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Methanol</th>
<th>Thymol</th>
<th>Water</th>
<th>Xylene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubility</td>
<td>Slightly Soluble</td>
<td>Soluble</td>
<td>Insoluble</td>
<td>Insoluble</td>
</tr>
</tbody>
</table>
iii. **Recrystallization**

Clonazepam was successfully recrystallized in both benzyl alcohol and thymol by following a procedure described by Water C. McCrone in his PLM textbook (1984). A drop of solvent was saturated with clonazepam so that a small amount remained undissolved. A coverslip was placed over this slide and the preparation was gently heated over an alcohol lamp until crystals could be seen. Crystals formed in both solvents within 5-10 minutes of heating. In benzyl alcohol, clonazepam forms colorless, six-sided prisms (Figure 3). In thymol, clonazepam exhibits a rhombohedral habit (Figure 4).

![Figure 3A](image)

![Figure 3B](image)

![Figure 3C](image)

Figure 3. Photomicrographs of clonazepam recrystallized in benzyl alcohol. Taken in plane-polarized light at 400X magnification.

Although clonazepam exhibits more than one crystal habit it is probable they are different forms of the same drug rather than polymorphs because they were crystallized using different solvents. It can’t be ruled out, however, that the different forms are
actually polymorphs of clonazepam. To determine this more detailed analysis would have had to be conducted that was outside the scope of this research.

Figure 4A

Figure 4B

Figure 4. Photomicrographs of clonazepam recrystallized in thymol. Taken in plane-polarized light at 400X magnification.

iv. **Optical Properties**

In microgram quantities pure clonazepam is a fine white powder, but on a microscopic level the individual crystals are colorless and transparent (Figure 5). The crystals range from approximately 10-80µm in size and are not pleochroic. Under crossed-polarized light the crystals display high level interference colors and this, in conjunction with the crystal size, leads to high birefringence (Figure 6).
Figure 5. A photomicrograph of clonazepam analytical standard in 1.66 refractive index oil. Taken in plane-polarized light at 400X magnification.

Figure 6. A photomicrograph of a clonazepam crystal showing high order interference colors. Taken in crossed-polarized light at 400X magnification.

Clonazepam recrystallized in benzyl alcohol exhibits both dispersed (Figure 7) and oblique extinction patterns placing it in the triclinic crystal system. Clonazepam recrystallized in thymol exhibits dispersed extinction. A summary of the optical properties of clonazepam is shown in Table II. The pharmaceutical tablets of clonazepam
also contain a variety of other ingredients which can include: corn starch, lactose monohydrate, magnesium stearate, microcrystalline cellulose, colloidal silicon dioxide, crospovidone, mannitol, sodium stearyl fumarate, artificial flavoring, xylitol, and various dyes (Sifton, 1994; drugs.com, 2011). Some of these compounds can be identified by observation under the microscope without much difficulty. The clonazepam crystals are very tough to distinguish visually from the other components in the tablets. This is not surprising since the clonazepam itself comprises only ~ 1% of the tablets’ mass in its highest dosage, i.e. the 2 mg tablet.

![Figure 7. A photomicrograph series of a clonazepam crystal showing dispersed extinction. Taken in crossed-polarized light at 400X magnification.](image)
TABLE II
OPTICAL PROPERTIES OF CLONAZEPAM

<table>
<thead>
<tr>
<th>Size</th>
<th>Color (Absorption)</th>
<th>Pleochroism</th>
<th>Crystal System</th>
<th>Birefringence</th>
<th>Extinction Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-80µm</td>
<td>Colorless</td>
<td>None</td>
<td>Triclinic</td>
<td>High</td>
<td>Dispersed, Oblique</td>
</tr>
</tbody>
</table>

B. Microcrystal Tests

The results of the microcrystal tests performed on the clonazepam standard and pharmaceutical preparations are listed in Table III. Five of the reagents-solvents, when added to the clonazepam standard, resulted in the development of microcrystals. Only three of these reagents caused crystals to develop when added to the clonazepam pharmaceutical preparations. Details on the method used for each test have been previously stated in Section II of this paper. Only the type of test utilized will be listed in these sections along with a description of the results and photomicrographs of crystals formed when applicable. The imaging condition for each photomicrograph can be found in its accompanying figure caption. The recipes for the microcrystal test reagents can be found in the Appendix.
<table>
<thead>
<tr>
<th>Reagents/Drugs</th>
<th>Clonazepam Standard</th>
<th>Clonazepam 2 mg Tablet</th>
<th>Clonazepam 1 mg Tablet</th>
<th>Clonazepam 0.5 mg Tablet</th>
<th>Clonazepam 0.25 mg Wafer</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂PtCl₆</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K₂CdI₄</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KI₃</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH₄SCN</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K₂CrO₄</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K₂HgI₄</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PbI₂</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂PtCl₆ + (H₃PO₄)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H₂PtCl₆ + Acetone</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H₂PtCl₆ + Chloroform</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) : crystal formation observed
(-) : no crystal formation observed after 20 minutes
i. **Clonazepam with Platinum Chloride**

In the Haynes thesis (2000), platinum chloride resulted in crystal formation when added to both the clonazepam standard and pharmaceutical preparation. The method used was a direct test in which a 5% platinum chloride aqueous solution was added to clonazepam in .1N hydrochloric acid. Upon replicating this test with 5 PPP of the clonazepam analytical standard, no crystals formed. The test was unable to be replicated as described by Haynes. When the test was modified, by omitting the hydrochloric acid, crystals formed with as low as 1 PPP of the clonazepam standard. Within 1-2 minutes of the addition of the platinum chloride rosettes and sheaths formed in the sample (Figure 8). These rosettes were made up of blunt-ended rods with positive signs of elongation (Figure 9).

![Figure 8A](image)

![Figure 8B](image)
Figure 8. Photomicrographs of crystals formed from a clonazepam standard and platinum chloride solution. Taken in plane-polarized light at 400X magnification.

Individually these rods are somewhat cigar-shaped, being tapered at the two terminating ends and widening slightly in the middle. When tested on the 2 mg tablet the platinum chloride resulted in positive detection of clonazepam down to 1 PPP. The crystals formed with the pharmaceutical preparation were the same as those observed with the clonazepam standard. This test was conducted on 5 PPP of the 1 mg clonazepam tablet but no crystals had formed after 20 minutes. The direct test, without hydrochloric acid, was repeated with a 10% platinum chloride aqueous solution to see if a stronger reagent might stimulate crystal formation in lower dosages of clonazepam. The same negative results were observed with both concentrations of the platinum chloride; no crystals formed with the clonazepam tablets at dosages lower than 2 mg. When the 10% platinum
chloride solution was utilized for the microcrystal test the crystals formed quicker and were more abundant than with the 5% platinum chloride solution.

Although the platinum chloride solution reliably worked with clonazepam to form well characterized and identifiable crystals, many times the dosages that come into lab as casework are lower than 2 mg (Dunlop, 2013, personal communication). The fact that this reagent only works with the standard clonazepam and 2 mg tablets makes it impractical for use in forensic controlled substances testing.

**ii. Clonazepam with Potassium Cadmium Iodide**

Tewari (1978) described positive results upon adding potassium cadmium iodide to clonazepam. The method utilized for this test was the hanging drop method described in that paper (and mentioned earlier in section II of this paper). The results from Tewari were unable to be reproduced; after 20 minutes, crystals still had not formed on the test slide using 5 PPP of the analytical standard. The test was repeated without the dissolution of clonazepam in the 50% ethanol solution but was negative after 20 minutes.

**iii. Clonazepam with Potassium Triiodide**

Potassium Triiodide was tested as a reagent because of its reported success with clonazepam by Tewari (1978) and other benzodiazepines by Clarke (1969) and Haynes (2000). These results were not able to be reproduced after 20 minutes using 5 PPP of the clonazepam standard and the Tewari hanging drop method. After 15 minutes some crystal formation was seen, however it was not a result of the reagent interacting with the clonazepam. The crystals appeared in both the test sample and the negative control indicating that they were caused by evaporation of the reagent and not the presence of
clonazepam. Repeating the test without dissolving the clonazepam in 50% ethanol still gave negative results.

iv. **Clonazepam with Ammonium Thiocyanate**

In the paper by Tewari (1978), the addition of a 5% ammonium thiocyanate solution to clonazepam caused crystals to form. When repeating this hanging drop test for this study only negative results were obtained with 5 PPP of the analytical standard after 20 minutes. The hanging drop test was repeated with a 10% ammonium thiocyanate solution to see if a stronger reagent might stimulate crystal formation. After 20 minutes, however, crystals still had not formed, and the test was considered negative.

v. **Clonazepam with Sodium Carbonate**

Sodium carbonate was another reagent used by Clarke (1969), Haynes (2000), and Tewari (1978) that showed positive results with a number of benzodiazepines. Although Tewari reported a positive reaction, the crystals took over 24 hours to form and would not have been considered a positive result by the requirements in this study. Nonetheless it was tested with 5 PPP of the standard clonazepam using the Tewari hanging drop and direct methods. Neither test showed any crystal formation after 20 minutes.

vi. **Clonazepam with Potassium Chromate**

In the Tewari (1978) paper, potassium chromate combined with clonazepam in a Tewari hanging drop test caused the formation of four-sided tablets. When this test was replicated with 5 PPP of clonazepam standard, crystals formed within 6-10 min. The crystals were colorless, six-sided tablets approximately 5-10 µm in size (Figure 9). It is not known why these crystals exhibited a different shape than those reported by Tewari.
No crystal formation was observed when potassium chromate was added to 5 PPP of the 2 mg clonazepam tablet. The inability of the reagent to cause crystals to form with the pharmaceutical preparation of clonazepam makes potassium chromate unsuitable for use in forensic controlled substances testing.

Figure 10A

Figure 10B

Figure 10. Photomicrographs of crystals formed from a clonazepam standard and potassium chromate solution. Taken in plane-polarized light at 400X magnification.

vii. **Clonazepam with Potassium Mercuric Iodide**

Potassium mercuric iodide was reported by Tewari (1978) to cause crystal formation when combined with clonazepam. A Tewari hanging drop test using this reagent was conducted with 5 PPP of clonazepam standard. No crystals were observed after 20 minutes.
viii. **Clonazepam with Lead Iodide**

Tewari (1978) observed the formation of four-sided tablets when lead iodide was combined with clonazepam in the Tewari hanging drop test. This test was replicated with 5 PPP of clonazepam standard and within 5-8 min crystals formed. The crystals were colorless, six-sided tablets between 20-30 µm in size (Figure 10). Upon first forming the crystals were quite small, but after letting the reaction continue for a few minutes they increased in size. These crystals were not exactly the same as those reported by Tewari, having six sides instead of four, and it is unknown why this result was inconsistent with previous findings. Crystals were detected using this test with as little as 1 PPP of the clonazepam standard. No crystals formed, however, when the test was repeated with 5 PPP of the 2 mg clonazepam tablet. Since no crystals form upon combining lead iodide and the pharmaceutical preparation of clonazepam, lead iodide is not suitable for use in forensic drug testing.

The crystals that formed with lead iodide appeared to have the same habit as those that formed with potassium chromate, but there was a size difference between them. The crystals from lead iodide were generally larger, 20-30 µm in size, than the crystals from potassium chromate which ranged from 5-10 µm.

Lead iodide proved to be somewhat difficult as a reagent because the double salt can precipitate out of this solution. These double salts are colorless, blunt-ended rods that seem to appear as the reagent ages or if the reagent hasn’t been made well. This is problematic because it could potentially cause a false positive in a microcrystal test by a
user unfamiliar with the reagent. This example shows how vital it is to microcrystal tests that negative controls be run along with the test samples to avoid false conclusions.

Figure 11. A photomicrograph of crystals formed from a clonazepam standard and lead iodide solution. Taken in plane-polarized light at 400X magnification.

ix. **Clonazepam with Zinc Chloride**

In the paper by Tewari (1978) zinc chloride resulted in crystal formation when added to clonazepam in a Tewari hanging drop test. This test was replicated with 5 PPP of clonazepam standard but the results were not reproducible. After 20 minutes, no crystals had formed in the test solution.

x. **Clonazepam with Gold Chloride (H3PO4)**

Gold chloride (H3PO4) was chosen as a test reagent because it is used for the detection of amphetamines and cocaine in microcrystal tests and, therefore, is more likely
to be found in forensic laboratories. A direct test was performed with the reagent and 5
PPP of the clonazepam analytical standard, but after 20 minutes no crystals had formed.

xi. **Clonazepam with Platinum Chloride and Chloroform**

As a result of the semi-positive results of platinum chloride with the pharmaceutical clonazepam, and the failure of the other reagents in this study to be successful, it was decided that this reagent should be investigated further. A 10% platinum chloride solution was used for these trials because it exhibited a more positive response with the clonazepam. That is, the crystals formed with the more concentrated reagent were better formed and more abundant in the test drop. In contrast to the direct method used in the trials previously reported on in this paper, this test involved dissolving the clonazepam in chloroform before adding the platinum chloride test reagent. This extra step was added because it has been suggested that dissolving the drug being tested for in a solvent gives the reagent better vehicle in which to interact with the drug (Siegel, 1988).

Within 1-3 minutes of the addition of the platinum chloride to the analytical standard, rosettes and sheaths formed in the sample (Figure 8). These rosettes were made up of blunt-ended rods with positive signs of elongation (Figure 9). The rods individually are cigar-shaped, being wider at the middle and thinning at the two ends. Crystals formed with as low as 1 PPP of the clonazepam standard. Successful crystal formation was also noted with the 2 mg clonazepam tablet. The crystals formed with the pharmaceutical preparation were the same as those observed with the analytical standard. The platinum chloride/chloroform combination resulted in positive detection of clonazepam within 3-5
minutes at levels as low as 1 PPP of the 2 mg tablet. Crystals successfully formed with this reagent-solvent combination, with all of the dosages of the pharmaceutical tablets. The limit of detection for the various clonazepam tablets are as follows: crystals formed with 3 PPP of the 1 mg tablet in 1-3 minutes, 5 PPP of the 0.5 mg tablet in 3-5 minutes, and 5 PPP of the 0.25 mg tablet in 3-5 minutes.

Since distinct crystals formed reliably upon combining platinum chloride and chloroform with both the analytical standard and all pharmaceutical preparations of clonazepam, this reagent-solvent combination would suitable for use in forensic drug testing.

xii. **Clonazepam with Platinum Chloride and Acetone**

Since clonazepam is reported to be more soluble in acetone than chloroform (Merck & Co., 2001), it was thought that better results might be obtained by dissolving clonazepam in this solvent before adding the test reagent. A 10% platinum chloride solution was used as the test reagent due to its strong positive reaction with the 2 mg clonazepam tablet and standard. Within 1-2 minutes of the addition of the platinum chloride to 5 PPP of the analytical standard, rosettes and sheaths formed in the sample (Figure 8). These rosettes were made up of blunt-ended rods with positive signs of elongation (Figure 9). Individually these rods are cigar-shaped, being tapered at the two terminating ends and widening slightly in the middle. It should be noted that for this test to be positive, full rosettes must be present. Individual cigar-shaped rods do not positively indicate the presence of clonazepam in a sample. Crystals formed with as low as 1 PPP of the clonazepam standard. When tested on the 2 mg tablet the platinum
chloride/acetone resulted in positive detection of clonazepam down to 1 PPP. The crystals formed with the pharmaceutical preparation were the same as those observed with the clonazepam standard. Using this reagent-solvent combination, crystals successfully formed with all of the dosages of the pharmaceutical tablets. The limit of detection for the various clonazepam tablets are as follows: crystals formed with 3 PPP of the 1 mg tablet in 1-3 minutes, 5 PPP of the 0.5 mg tablet in 1-3 minutes, and 5 PPP of the 0.25 mg tablet in 5-8 minutes.

The ability of this reagent-solvent combination to reproducibly form distinct crystals with all dosages of the clonazepam pharmaceutical preparations makes platinum chloride and acetone suitable for use in forensic controlled substances testing.

Platinum chloride has been recommended for use as reagent in other microcrystal tests such as those for morphine, ephedrine, mescaline, and cocaine and many of its derivatives (Fulton, 1969). No other drug is known to exhibit the same reaction, namely produce the same crystals, with the platinum chloride and acetone or chloroform combination. This is a very important quality because it allows for specificity of this microcrystal test and gives it the potential to identify not just a class of drug but the drug itself.

Although both acetone and chloroform were successful solvents for use in the detection of clonazepam through microcrystal tests, the rosettes that are made with acetone tended to be more completely formed and more abundant than those formed with
chloroform. This makes interpreting the results of the test easier for the analyst, and could decrease the chance of false negatives. It is likely that this discrepancy results from the increased solubility of clonazepam in acetone. Due to the differing results of these tests, the use of platinum chloride and acetone is recommended to be used preferentially over platinum chloride and chloroform.
IV. CONCLUSION

Microcrystal tests were developed that are able to detect clonazepam in its pharmaceutical preparations at all of its therapeutic dosages. These tests involve the addition of acetone or chloroform and a 10% platinum chloride solution to microgram quantities of clonazepam. The presence of clonazepam is indicated by the formation of rosettes made of colorless, blunt-ended rods. While both of these solvents work reliably to produce characteristic crystals in a microcrystal test with clonazepam, acetone is recommend and preferable. The rosettes that are made with acetone tend to be more completely formed and more abundant than those formed with chloroform. Although platinum chloride is recommended for use as reagent in other microcrystal tests, no other drug is known to exhibit the same reaction with these reagent-solvent combinations making these tests good candidate for use in forensic lab settings. In addition, it follows a simple procedure, uses minute quantities of the drug sample, and involves reagents that are common and involved in other known drug microcrystal tests.
REFERENCES


REFERENCES (continued)


APPENDIX

RECIPE FOR MICROCRYSTAL TEST REAGENTS

1. **Platinum Chloride (5%)**:  
   1 g of platinum chloride in 20 mL of water

2. **Platinum Chloride (10%)**:  
   0.25 g of platinum chloride in 2.5 mL of water

3. **Potassium Cadmium Iodide**:  
   1 g cadmium iodide + 2 g potassium iodide in 20 mL of water

4. **Potassium Triiodide**:  
   0.2 g of iodine + 10 g potassium iodide in 20 mL of water

5. **Ammonium Thiocyanate (5%)**:  
   1 g of ammonium thiocyanate in 20 mL of water

6. **Ammonium Thiocyanate (10%)**:  
   2 g of ammonium thiocyanate in 20 mL of water

7. **Sodium Carbonate (5%)**:  
   1 g of sodium carbonate in 20 mL of water

8. **Potassium Chromate**:  
   1 g of potassium chromate in 20 mL of water

9. **Potassium Mercuric Iodide**:  
   0.3 g mercuric chloride + 1 g potassium iodide in 20 mL of water

10. **Lead Iodide**:  
    Saturated lead iodide in 30% acetic acid adjusted to pH 6 with 2N acetic acid
11. Zinc Chloride:

1 mL of zinc chloride and dilute to 20 mL with water

12. Gold Chloride (H$_3$PO$_4$):

0.5 g gold chloride in 10 mL phosphoric acid
VITA

NAME: Danielle Kay Silletti

EDUCATION: B.S., Biology, Hope College, Holland, Michigan, 2011

M.S., Forensic Science, University of Illinois at Chicago, Chicago, Illinois, 2013

EXPERIENCE: McCrone Research Institute, Chicago, Illinois, 2012-2013

Federal Bureau of Investigation, Counterterrorism and Forensic Science Research Unit, Quantico, Virginia, 2010, 2011-2012

Department of Chemistry, Chemistry Research Lab, Hope College; Holland, Michigan, 2010-2011

Department of Biology, Biology Research Lab, Hope College, Holland, Michigan, 2010-2011

HONORS: Graduate College Fellowship, University of Illinois at Chicago, Chicago, Illinois, 2012-2013.

Department of Pharmacy Scholarship, University of Illinois at Chicago, Chicago, Illinois, 2013.

Sigma Xi Research Award, Hope College, Holland, Michigan, 2011

American Society of Crime Lab Directors Scholarship, Hope College, Holland, Michigan, 2010-2011

PROFESSIONAL MEMBERSHIP: State Microscopical Society of Illinois

American Association of Forensic Scientists

Phi Beta Kappa honor society
