An Internal Validation of the Janovsky Color Test and Testing the Platinum Chloride Microcrystalline Method for Benzodiazepines

Allison Swigart*, B.S.[1]; Kathleen Schell, M.S. ABC-DA[2]; Lyndsay Cone, M.S. ABC-DA[2]; Rebecca Barlag[3], Ph.D; Lauren Richards Waugh, Ph.D.[1]

[1]Marshall University Forensic Science Center, 1401 Forensic Science Drive, Huntington, WV 25701

[2] 121 E. Tyron Rd. Raleigh, NC 27603

[3] 319 Chemistry Building Athens, OH 45701

Abstract

Benzodiazepines contain a benzene ring attached to a seven membered ring containing two nitrogens. Pharmaceutical benzodiazepines are schedule IV controlled substances. Benzodiazepines fall into two categories, pharmaceutical and designer benzodiazepines (schedule I). The Janovsky color test gives a purple color change in the presence of benzodiazepines and the platinum chloride microcrystalline test gives a crystal structure of needles forming rosettes.
Results: The Janovsky color test was found to be precise over a 10-day period and reproducible by other forensic practitioners. The limit of detection (LOD) was ~500 - 750 parts diluent to one part benzodiazepine. In the robustness study: 3 drops of solution A and 1 drop of solution B yielded a purple color, ~2 mins can go by when adding both reagents and between adding both reagents in the spot well before the color change is affected. The color change was more prominent with increased sample and more concentrated with a clumped sample. Ruggedness study: no solvent effect in cleaning agents. Commonly encountered controlled substances did not hinder the Janovsky color test. When using the microcrystalline test 4 out of the 13 benzodiazepines yielded rosettes, the LOD was ~250 -500 parts diluent to one part benzodiazepine. Controlled substance mixtures interfered with crystal formation. Mock case samples were accurately identified, however due to the inconsistency of the microcrystalline test, the Janovsky color test is recommended for implementation in the NCSCL analytical scheme.

Introduction

Benzodiazepines cause a sedative effect by interacting with the GABA receptor in the body[1]. Benzodiazepines grew in popularity in the 1960's through the 1970's, though recently designer benzodiazepines have been encountered[1]. Designer benzodiazepines are clandestinely prepared, potentially more dangerous than pharmaceutical benzodiazepines, and are considered schedule I controlled substances due to a lack of quality control in dosage, preparation, and because they exhibit a high potential for abuse with no accepted medical use[2]. The Scientific Working Group for Seized Drug (SWGDrug) analysis as well as Organization of Scientific Area Committees (OSAC) at minimum requires a preliminary test and a confirmatory test in tandem for the analysis of controlled substances[3]. Currently the North Carolina State Crime Laboratory (NCSCL) does not have a preliminary test for benzodiazepines. Benzodiazepines, especially designer, have become more prevalent in casework. Crime laboratories need uncomplicated, efficient, sensitive, selective, reliable, reproducible, robust, rugged, and accurate tests for benzodiazepines. The goal is to validate the Janovsky color test for benzodiazepines, develop a platinum chloride microcrystalline test for benzodiazepines, and perform a comparison between the two methods. This includes the testing of mock case samples. The Janovsky reaction for benzodiazepines occurs through the creation of a carbanion intermediate and a chromophore product, known as the Meisenheimer complex[4]. The Janovsky color test is known to produce a purple color change in the presence of benzodiazepines[5]. Microcrystalline tests use Polarized Light Microscopy, reagents, and preparation methods for the indication of controlled substances[6]. Microcrystalline tests are more selective than color tests, conversely, they are more cumbersome, and require more resources than color tests. Some benzodiazepines are known to produce distinct crystal formations, needles forming rosettes in a platinum chloride microcrystalline test[6].

Materials

Certified Benzodiazepine Standards (Obtained from various manufacturers):
Alprazolam, clonazolam, delorazepam, diazepam, estazolam, etizolam, flualprazolam, flubromazolam, flunitrazepam, oxazepam, and phenazepam.
Secondary Standards (Obtained from various sources):
Alprazolam, diazepam, diazepam base, lorazepam, phenazepam, and triazolam.
Diluents:
Benzocaine base, caffeine, corn starch, creatine, $DMSO_2$, guaifenesin, inositol, mannitol, phenacetin, sodium bicarbonate, and sucrose.
Other Controlled Substances:
Cocaine base, cocaine HCl, fentanyl, heroin, and methamphetamine.

- 1,3-dintrobenzene
- Potassium hydroxide
- Chloroplatinic acid hexahydrate
- Hydrochloric acid
- Phosphoric acid
- Absolute ethanol
- Methanol
- Acetone
- DI water
- Analytical balance
- Polarized Light Microscope
- Gas Chromatography Mass Spectrometer instrument (confirmation)

Methods

Janvosky Color Test
Solution A: 2% m-dinitrobenzene solution with 99.5+% ethanol as the solvent[5]
Solution B: 5N potassium hydroxide solution[5]
Made a negative control well for every color test, performed a 10-day precision study, a reproducibility study, limit of detection study, robustness study, a ruggedness study, and a controlled substance study.

Platinum Chloride Microcrystalline Test
5% w/v chloroplatinic acid hexahydrate solution[6]. Main slide preparations included, HCl hang drop, reagent hang drop, and dissolving in acetone[6]. Benzodiazepines were tested, along with common diluents, limit of detection was determined. A controlled substances study was done. At the end mock case samples were run for both tests and confirmed via GC-MS.

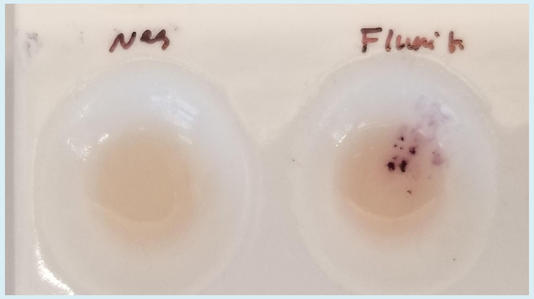

Figure 1: Janovsky color test negative and positive control.

Results

Table 1: Janovsky Color Test Results Summary

Janovsky Color Test	Substance Type	% Positive
Precision Study	Benzodiazepines	100
Precision Study	Diluents	0
Reproducibility	Benzodiazepines with diluents	100
Controlled Substances	Cocaine, fentanyl, heroin, and methamphetamine	20

Table 2: Microcrystalline Test Results Summary

Microcrystalline Test	Substance Type	% Positive
Testing Benzodiazepines	Benzodiazepines	30.7
Testing Diluents	Diluents	0*
Controlled Substances	Cocaine, fentanyl, heroin, and methamphetamine	0*

* = Some crystal formations were observed, but the morphology was not consistent with the rosettes indicative of benzodiazepines.

Table 3: Mock Case Samples Janovsky Color Test Results

Janovsky Test	Result	Positive/negative preliminary
A1	No color change	Negative
A2	Purple	Positive
A3	Purple	Positive

Table 4: Mock Case Samples Microcrystalline Test Results

Microcrystalline Test	Result	Positive/negative preliminary
B1	Needles forming rosettes	Positive
B2	Dendrites	Negative
B3	No distinct crystals formed	Negative

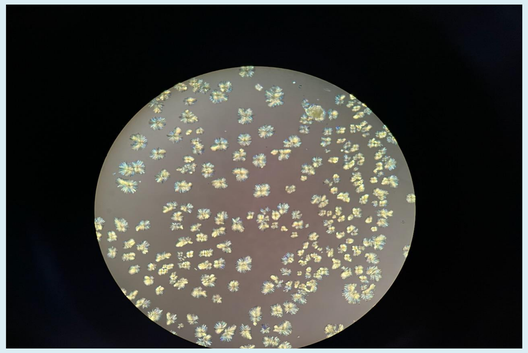

Figure 2: Platinum chloride microcrystalline test, phenazepam crystal formations.

Discussion and Conclusions

The Janovsky color test is easy, efficient, reproducible, sensitive, robust, and rugged. It was not hindered by other common controlled substances. While there are safety concerns with utilizing m-dinitrobenzene, the Janovsky color test was validated and recommended for implementation in the analytical scheme of the NCSCL. The platinum chloride microcrystalline test, while a category B method, was not as reproducible as the Janovsky color test and did not work on a wide variety of benzodiazepines. An analyst must be skilled in polarized light microscopy for proper use of the platinum chloride microcrystalline test. There are also many slide/reagent preparation techniques for microcrystalline tests, making them complex. The platinum chloride microcrystalline test was also hindered when other common controlled substances were also present with the benzodiazepine.
Future directions for a microcrystalline test for benzodiazepines are potentially preparing the reagent differently, such as in acid, or preparing a reagent other than platinic chloride. There are also different slide preparation techniques that impact microcrystalline tests, dissolving samples in different solvents as well as hang drops and sample preparation techniques that can have an impact. Keeping up with the latest microcrystalline works from McCrone Institute would be very helpful for future research in microcrystalline testing that can be implemented into the analytical scheme of crime laboratories. Additionally, the Janovsky color test could be used to test for the indication of other controlled substances in which a color test is not yet implemented, such as synthetic cathinones[7]. The Janovsky color test is thought to produce different color chromophores for different controlled substances[8].

References

1. Edinoff AN, Nix CA, Hollier J, Sagrera CE, Delacroix BM, Abubakar T, et al. Benzodiazepines: Uses, Dangers, and Clinical Considerations. Neurology International. 2021;13(4):594-607. doi: https://doi.org/10.3390%2Fneurolint13040059.
2. Brunetti P, Giorgetti R, Tagliabracci A, Huestis MA, Busardo FP. Designer Benzodiazepines: A Review of Toxicology and Public Health Risks. Pharmaceuticals. 2021;14(6):560. doi: https://doi.org/10.3390%2Fph14060560.
3. Scientific Working Group for the Analysis of Seized Drugs. Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) Recommendations. United States Department of Justice Drug Enforcement Administration; version 8.1 2022.
4. Kovar K., Laudszun M. Scientific and technical notes: chemistry and reaction mechanisms of rapid tests for drugs of abuse and precursors chemicals. Federal Republic of Germany: United Nations; 1989 Feb. v. 89-51669.
5. District of Columbia Department of Forensic Sciences. FSC10 Procedure for Chemical Spot Tests Janovsky Test. District of Columbia Department of Forensic Sciences. Revision 5 2018.
6. Brinsko KM, Golemis D, King MB, Laughlin GJ, Sparenga SB. A Modern Compendium of Microcrystal Tests for Illicit Drugs and Diverted Pharmaceuticals. McCrone Research Institute. Revision 3 2018.
7. Laboratory and Scientific Section United Nations Office on Drugs and Crime. Recommended methods for the Identification and Analysis of Synthetic Cathinones in Seized Materials (Revised and updated). United Nations Office on Drugs and Crime; 2020.
8. Protest Kit. Results. https://protestkit.eu/results/. Accessed 20 July 2023.

Acknowledgements

The presenting author would like to thank NCSCL for hosting internship, especially the Drug Chemistry Section. Thank you to Lyndsay Cone for her mentoring. Other thanks to research committee for their hard work and my peers and faculty in the Marshall University Forensic Science Graduate Program.