A Specific Screening Color Test for Diazepam

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ABSTRACT: A new, highly specific color test for the screening/presumptive identification of diazepam is reported. The test is a variant of the McKibben test for flunitrazepam. Treatment of diazepam with alkaline dimethylsulfoxide produces a reddish color which gradually changes to yellow with passage of time. The color instantly vanishes upon addition of water or attempted extraction with organic solvents, suggesting that the color is due to a transient charge-transfer complex. Somewhat unexpectedly, the test does not produce color with powder scraped from diazepam-containing tablets - in such cases, a chloroform extraction is required. The test is negative for other controlled substances, including other benzodiazepines, and also for various diluents and binders typically present in tablets (62 compounds were tested). The LODs were 20 µg for diazepam extracted from tablets, and 2 µg for diazepam standard. The test is particularly useful for the rapid screening of illicit Lemmon 714 (Quaalude) mimic tablets, which contain diazepam as a substitute for methaqualone.

KEYWORDS: Diazepam, Dimethylsulfoxide, Screening, Color Test, Forensic Chemistry

Introduction

Diazepam (Figure 1), most commonly known by its trade name Valium, is a benzodiazepine and a controlled substance (Schedule IV in the United States [1]). It is a potent sedative - hypnotic (CNS depressant), and is one

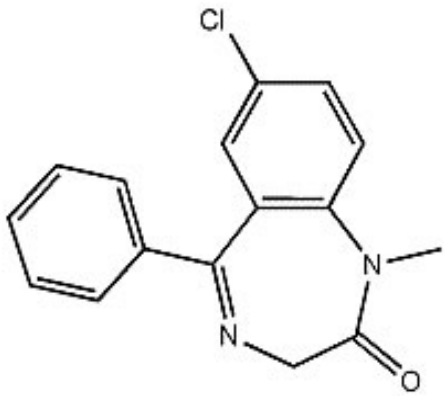

Figure 1. Diazepam (7-Chloro-1-methyl-5-phenyl-1,3-dihydro-2H-1,4-benzodiazepin-2-one); $C_{16}H_{13}ClN_2O$; m.w. (Base) = 284.7; mp = 131.5-134.5^{O}C

of the most prescribed drugs in the world. It is also one of the top five most abused benzodiazepines, and misuse can lead to both psychological dependence and/or physical addiction [2].

In addition to Valium and numerous other licit (prescription) formulations, diazepam is found as an adulterant in heroin and as a substitute in various mimic drugs (most notably as a substitute for methaqualone in Lemmon 714 (Quaalude) mimic tablets [3]). Because its synthesis is challenging, its presence in illicit drug markets is almost universally due to diversion of pharmaceutical stocks.

There are numerous analytical methods for the identification of diazepam in forensic and toxicological laboratories [4,5], including a number of color tests; in general, however, the latter are not sensitive or specific (*vide infra*). Herein, a new presumptive color test for the preliminary screening of diazepam is reported. The test, which is a variant of the McKibben test for flunitrazepam [6], is simple, easy to perform, highly sensitive, and highly specific.

Experimental

Materials: Dimethylsulfoxide (hereafter DMSO) and sodium hydroxide were both acquired from Merck (Whitehouse Station, NJ). The diazepam standard was acquired from Roche Pakistan Ltd. (Lahore). Tablets containing other benzodiazepines and other tested compounds, and standard (pure) materials, were purchased from various pharmaceutical companies (see Table 1). All other chemicals used were of analytical grade or better.

Methods: Test Reagent A was prepared by adding 50 parts of DMSO and one part of 3M sodium hydroxide. [Note: Use of sodium hydroxide solutions below 2M or over 3M was not as effective.] Test Reagent B was prepared by adding solid sodium hydroxide to DMSO and vortexing it for approximately 2 minutes. In the latter case, the supernatant was transferred to another tube and used as the test reagent. In this study, both test reagents were freshly prepared just before use; however, subsequent studies indicated that the reagents were stable for at least 4 days. Both test reagents are colorless (see Photo 1).

Photo 1 - Reagent

Detection of Diazepam: A Valium tablet containing 2 mg of diazepam was ground to a fine powder. A small portion of powder (approximately 0.5 mg) was added to 1 mL of chloroform, and the resulting mixture was vortexed for approximately one minute and then centrifuged at 13,200 rpm for 3 minutes. The supernatant chloroform was isolated and evaporated to dryness, and 3 - 4 drops of the test reagent was added to the remaining residue. An immediate reddish color developed (see Photo 2). If Test Reagent A was used, the color faded to yellow after approximately 1 minute; if Test Reagent B was used, the color persisted for at least 20 minutes before gradually fading to yellow. Repeating the sequence with the diazepam standard gave the same results. Addition of 2 - 3 drops of water to either the reddish or yellow test solutions caused the color to instantly vanish. None of the other compounds tested (Table 2) gave similar results.

Limit of Detection (LOD) from Tablets: The above analysis was repeated on a second Valium tablet, except that five sequential extractions were performed on the same powder, and each extract isolated in separate test tubes. Upon testing, only the first three extracts displayed color; it was therefore assumed that the 2.0 mg of diazepam in the original tablet was quantitatively extracted by three serial extractions. Another tablet was extracted in the

same manner, and the first three extracts were combined to form the "stock solution." Aliquots containing 70, 60, 50, 40, 30, 20, 10, and 5 μg of diazepam were each evaporated to dryness, and tested. The results indicate a detection limit of 20 μg.

LOD from Diazepam Standard: Stock solutions containing 50, 30, 20, 10, 5, 3, 2, and 1 μg of reference grade diazepam were prepared in acetone. The solutions were evaporated to dryness, and the remaining residue was tested as detailed above. The reddish color was noted down to 2 μg - one tenth the LOD for diazepam extracted from tablets. The order of magnitude difference is thought to be due to the sensitivity of the test to co-extracted impurities from the tablets (i.e., that are not present in the standard).

Analysis of Other Substrates: All other compounds were either analyzed as standards (pure), or were extracted from tablets using water or an organic solvent (see Table 1).

Results and Discussion

Previous Studies

As noted in the Introduction, a number of color tests have been reported for the presumptive identification of diazepam; however, most are neither specific or sensitive, and others were not tested against other benzodiazepines or other controlled substances. Formaldehyde + H_2SO_4 [7], Zimmerman's reagent (*meta*-dinitrobenzene + benzyltrimethylammonium hydroxide [8]), and the Janovsky reagent (*meta*-dinitrobenzene + KOH [9]) have been used as general screens for benzodiazepines. Of the three reagents, Zimmerman's reagent is the most specific, producing violet/purple colors with keto-benzodiazepine derivatives such as diazepam, fludiazepam, and flurazepam. The Wagner's test (acidic KI_3) gives a brown solution with a brown-black precipitate; however, similar results are obtained for many alkaloids, including cocaine hydrochloride [10,11]. In a commercial test kit (ingredients proprietary), a presumptive test was designed to identify the presence of diazepam, flunitrazepam, or ketamine [12]. After breaking and agitation of the two ampoules in the kit, a pale lavender color will develop for either diazepam and flunitrazepam, and a darker color for ketamine; it is unknown how this kit performs with other benzodiazepines or other controlled substances. Diazepam gives an intense red color product with the addition of picric acid (2,4,6-trinitrophenol), 3,5-dinitrobenzoic acid, or 3,4-dinitrobenzoic acid [13]; however, no other benzodiazepines or drugs were studied. Bromocresol green has been used to produce an orange colored ion-association complex with diazepam [14]; again, no other benzodiazepines or drugs were studied. Treatment of diazepam, bromazepam, and clonazepam with methanolic potassium hydroxide produces a yellow color, which could be analyzed by spectrophotometry [15]; however, a similar coloration is obtained for almost any benzodiazepine.

Alkaline Dimethylsulfoxide

McKibben was the first to report the use of alkaline DMSO for color testing, for screening of flunitrazepam [6]; interestingly, alkaline dimethylformamide (DMF) also worked. There were three variants of the test; in the first, the sample and the reagent were placed in a flint glass/soda lime test tube and heated at 100^{O}C, giving a deep purple color within four minutes. In the second, the sample, reagent, and either barium oxide, barium hydroxide, or finely ground flint glass/soda lime glass were placed in a regular test tube and heated at 100^{O}C, again giving a deep purple color within four minutes. In the third, the sample was dissolved in either DMSO or DMF and a small amount of solid sodium hydroxide added, resulting in immediate formation of a red-purple color (different than that observed in the first two variants). In all three cases, (cautious) addition of concentrated hydrochloric acid resulted in an immediate canary-yellow color.

McKibben tested over 100 different drugs, including diazepam, and determined that the tests were highly specific for flunitrazepam. Diazepam gave no color in either of the heated variants, but gave a dark orange color in the

third (unheated) variant. In all, 30 of the compounds tested by McKibben using the third (unheated) variant gave colors, including greens, yellows, and oranges.

In the current study, markedly different results were obtained, with only diazepam (reddish, see Photo 2), flunitrazepam (purple, see Photo 3), flurazepam (yellow), nitrazepam (yellow), and temazepam (green) giving colors (Table 2); however, fewer compounds (62) were tested, and the tested substrates included many non-drug compounds. Nonetheless, none of the other compounds that were tested displayed a reddish color, and the purple color displayed by flunitrazepam was similar but distinct from the reddish color produced by diazepam. Somewhat surprisingly, and in direct contrast to the McKibben reagents, the test failed to produce any color when DMF was substituted for DMSO. [Note: The colors observed with flurazepam, nitrazepam, and temazepam were not further investigated in this study; however, based on the compounds tested, the green color observed for temazepam also appears to be unique, and may constitute another definitive test. McKibben also observed a light green color for temazepam (Test Variant 3) - but noted similar colors for alprazolam, estazolam, lorazepam, and scopolamine.]

In order to further differentiate diazepam and flunitrazepam, their respective positive test solutions were each further treated (cautiously!) with 2 drops of concentrated hydrochloric acid. The reddish solution from diazepam instantly produced a faint yellow solution (see Photo 4), while the purple solution from flunitrazepam instantly produced a distinct orange solution (see Photo 5). Thus, although we feel that the initial colors allow for differentiation of diazepam from flunitrazepam, if desired this second step would rigorously confirm the identification.

The only difference between the McKibben Test/Variant 3 and the Test Reagent B used in this study is the point at which the solid sodium hydroxide is added. In the McKibben test, the sample is first dissolved in the DMSO, and then the base is added. In this study, the base is first dissolved in the DMSO, and then the sample is added. It is unclear how such a simple variation can have such a profound impact; however, the (fortuitous) difference allows for a specific test for diazepam.

Attempted Identification of the Colored Specie: In order to attempt identification of the colored specie, the reddish test solutions resulting from diazepam were extracted with a variety of organic solvents (acetic anhydride, acetone, acetone and chloroform, chloroform, ethyl acetate, formaldehyde, and petroleum ether). The color not only failed to extract into any of the organic solvents, in every case it vanished altogether. In addition, and as was noted in the Experimental section, addition of water to the reddish colored solutions also caused the color to instantly vanish (and the rapid fading of the reddish color when Test Reagent A was used is very likely due to the small percentage of water in that reagent). The results suggest that the reddish color results from formation of a transient charge-transfer complex that is sensitive to virtually any change in polarity or concentration. This sensitivity may explain why the test is so specific to diazepam, why the test fails with powder from tablets, why the LOD is so much higher for diazepam extracted from tablets, and finally why it fails if DMF is substituted for DMSO.

Screening of Lemmon 714 (Quaalude) Tablets: Actual Lemmon 714 tablets contain only methaqualone, whereas nearly all Lemmon 714 mimic tablets seen over the past 25 years have contained diazepam, sometimes adulterated with diphenhydramine [3]. Diazepam is also (uncommonly) substituted for methaqualone in Mandrax mimic tablets (widely abused in South Africa [16]). In a 1982 patent, Fischer and Morris reported a screening test to differentiate tablets containing methaqualone or mecloqualone from mimic tablets containing diazepam or diphenhydramine. Addition of about 7 drops of 85% formic acid to a sample containing methaqualone or mecloqualone, followed by 5 drops of 5% sodium nitrite, then 10 drops of chloroform, results in a yellow color that extracts into the chloroform layer; if diazepam is present, however, it will give a yellow color that is *not* extracted into the chloroform layer [17]. In the present test, methaqualone did not display any color. Thus, the two tests perfectly complement each other for rapid, facile screening of Lemmon 714 (Quaalude) or Mandrax tablets.

Photo 2 - Reddish Color from Diazepam

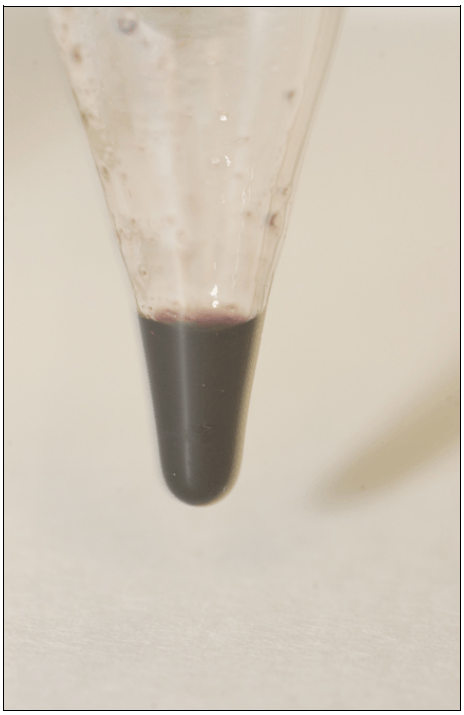

Photo 3 - Purple Color from Flunitrazepam

Photo 4 - Faint Yellow Color from Addition of Conc. HCl to the Reddish Solution (i.e., from Diazepam)

Photo 5 - Distinct Orange Color from Addition of Conc. HCl to the Purple Solution (i.e., from Flunitrazepam)

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Table 1. Tested Compounds, Sources, Extraction Solvents, and Manufacturers.

	Compound	Source	Extraction Solvent	Manufacturer
1	Acetaminophen	Cold and Sinus Tablet	Acetone	Equal USA
2	*alpha*-Lipoic Acid	-	A/G	Standard
3	Alprazolam	Xanax	Ethanol	Pharmacia Pakistan Ltd.
4	Aspirin Sodium Salt	Disprin	Water	Reckitt Benckiser Pakistan
5	Benzophenone	-	A/G	BDH
6	Bromazepam	Lexotanil	Chloroform	Roche Pakistan Ltd.
7	Calcium Carbonate	-	A/G	Sigma
8	Chlorazepate Dipotassium	Tranxene	Water	Searle Pakistan (pvt.) Ltd.
9	Chlordiazepoxide	Librium	Ethanol	Ethical Pharmaceutical Pakistan
10	Chlorpheniramine Maleate	Pritone	Water	Glaxo Smith Kline Ltd. Pakistan
11	Clonazepam	Rivotril	Acetone	Roche Pakistan Ltd.
12	Cocaine	-	Chloroform	Sigma
13	Codeine	Cocodamol	Water	Alpharma Barnstaple UK
14	Dextrose	Splenda	Water	McNeil Nutritionals LLC F. Washington
15	Diazepam	Valium	Chloroform	Roche Pakistan Ltd.
16	Diclofenac Na	Dicloran	Acetone	Sami Pharmaceutical Pakistan
17	Diphenhydramine HCl	Cold and Sinus Tablet	Water	Equal USA
18	Flunitrazepam	Rohypnol	Chloroform	Sigma
19	Flurazepam	Dalmane	Chloroform	Sigma
20	Folic Acid	-	A/G	Sigma
21	Glucose	-	A/G	Merck
22	Guanidine	-	A/G	Sigma
23	Heroin	-	Chloroform	Sigma
24	Ibuprofen	Bruphen	Chloroform	Equal USA
25	Indomethacin	Indomethacin cap.	Chloroform	Chongqing Medicine Pakistan
26	Kanamycin Monosulfate	-	A/G	MP Biomedicals, Inc. Ohio, France.
27	Ketamine	Ketalar	Chloroform	Sigma
28	Lactose	-	A/G	Merck
29	L-Cysteine	-	A/G	Acros
30	L-Glutamine	-	A/G	Sigma
31	Lorazepam	Aitivan	Acetone	Spencer Pharma (pvt) Ltd. Pakistan
32	Mefenamic Acid	Ponstan	Chloroform	Reckitt Benckiser Pakistan
33	Magnesium Sulfate	-	A/G	Merck
34	Maltodextrin	Splenda	Water	McNeil Nutritionals LLC F. Washington
35	Mannitol	-	A/G	Difco Laboratory
36	Methaqualone	-	Chloroform	Sigma
37	Myo-Inositol	-	A/G	Sigma
38	Nalbuphine HCl	Loricin injection	Water	Medicina Pharma Pakistan
39	Naproxen	Synflex	Chloroform	ICI Karachi Pakistan
40	Nicotinic Acid	-	A/G	Sigma

41	Nitrazepam	Mogadon	Chloroform	Sigma
42	Oxazepam	Murelax	Chloroform	Sigma
43	Pentazocine	-	A/G	Standard
44	Phenolphthalein	-	A/G	BDH
45	Phenylephrine HCl	Cold and Sinus Tablet	Water	Equal USA
46	Prednisolone	Prednisolone cap.	Ethanol	Ethical Pharmaceutical Pakistan
47	Propoxyphene	Algaphan	Water	Efroze Chemical Industries Ltd. Pakistan
48	Pyridoxine	-	A/G	Sigma
49	Salicylic Acid	-	A/G	Sigma
50	Sorbitol	-	A/G	Sigma
51	Sodium Bicarbonate	-	A/G	Merck
52	Sodium Gluconate	-	A/G	GPR*
53	Sodium Glutamate	-	A/G	Merck
54	Sorbic Acid	-	A/G	Merck
55	Starch	-	A/G	Merck
56	Succinic Acid	-	A/G	Kodak
57	Sucralose	Splenda	Water	McNeil Nutritionals LLC F. Washington
58	Sucrose	-	A/G	Merck
59	Temazepam	Restoril	Acetone	Novartis Pharmaceutical Pakistan
60	Triazolam	Halcion	Chloroform	Sigma
61	Tyrothricin	-	A/G	Sigma
62	Vitamin D	Calcium D Tablet	Chloroform	Spring Valley by Schiff Nutrition Group Inc., USA

* GPR = General Purpose Reagents; A/G = Analytical Grade (Not Extracted).

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[Table 2 Follows.]

Table 2. Test Results.

	Compound	Color		Compound	Color
1	Acetaminophen	N/C	32	Mefenamic Acid	N/C
2	*alpha*-Lipoic Acid	N/C	33	Magnesium Sulfate	N/C
3	Alprazolam	N/C	34	Maltodextrin	N/C
4	Aspirin	N/C	35	Mannitol	N/C
5	Benzophenone	N/C	36	Methaqualone	N/C
6	Bromazepam	N/C	37	Myo-Inositol	N/C
7	Calcium Carbonate	N/C	38	Nalbuphine HCl	N/C
8	Chlorazepate Dipotassium	N/C	39	Naproxen	N/C
9	Chlordiazepoxide	N/C	40	Nicotinic Acid	N/C
10	Chlorpheniramine Maleate	N/C	41	Nitrazepam	Yellow
11	Clonazepam	N/C	42	Oxazepam	N/C
12	Cocaine	N/C	43	Pentazocine	N/C
13	Codeine	N/C	44	Phenolphthalein	N/C
14	Dextrose	N/C	45	Phenylephrine HCl	N/C
15	Diazepam	Red	46	Prednisolone	N/C
16	Diclofenac Na	N/C	47	Propoxyphene	N/C
17	Diphenhydramine HCl	N/C	48	Pyridoxine	N/C
18	Flunitrazepam	Purple	49	Salicylic acid	N/C
19	Flurazepam	Yellow	50	Sorbitol	N/C
20	Folic acid	N/C	51	Sodium Bicarbonate	N/C
21	Glucose	N/C	52	Sodium Gluconate	N/C
22	Guanidine	N/C	53	Sodium Glutamate	N/C
23	Heroin	N/C	54	Sorbic Acid	N/C
24	Ibuprofen	N/C	55	Starch	N/C
25	Indomethacin	N/C	56	Succinic Acid	N/C
26	Kanamycin Monosulfate	N/C	57	Sucralose	N/C
27	Ketamine	N/C	58	Sucrose	N/C
28	Lactose	N/C	59	Temazepam	Green
29	L-Cysteine	N/C	60	Triazolam	N/C
30	L-Glutamine	N/C	61	Tyrothricin	N/C
31	Lorazepam	N/C	62	Vitamin D	N/C

N/C = No Color.

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