TECHNICAL NOTE

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Simple and Rapid Color Screening Tests for Flunitrazepam (Rohypnol)

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ABSTRACT: Three new color/screening tests for flunitrazepam (Rohypnol) are reported. The two-step tests are simple, sensitive, highly specific, and effective for both cut and uncut flunitrazepam and standard over-the-counter preparations of flunitrazepam (i.e., Rohypnol tablets.)

KEYWORDS: forensic science, flunitrazepam, Rohypnol, color test, screening test, field test

Flunitrazepam (aka Rohypnol, ''Roofies'') tablets have been increasingly submitted to forensic laboratories as seized evidence (1–7), with over 4500 cases reported to the U.S. Drug Enforcement Administration since the mid 1980's (8). A member of the benzodiazepine class of hypnotics/sedatives, flunitrazepam was first synthesized by Sternback (9), and was patented in both the United States and the United Kingdom (10). At the present time, it is manufactured and marketed by Hoffman LaRoche, Inc. under the trade name ''Rohypnol.'' Although commercially unavailable in the United States, Rohypnol tablets are available ''over the counter'' (OTC) throughout Central and South America, and a thriving black market exists for these ''diverted'' products. In addition, rapidly increasing numbers of counterfeit Rohypnol tablets are also being identified by forensic laboratories (8). All flunitrazepam-containing products are currently listed as a Schedule IV controlled substances under current (May, 1998) U.S. statutes (11).

Over the past few years, this drug has received extensive media attention because of the manner in which it is abused in sex-related crimes. In a typical scenario, a tablet is placed in the alcoholic beverage of an unsuspecting victim; the combination of flunitrazepam and ethanol results in a synergistic and pronounced physiological effect, with symptoms including temporary memory loss, loss of motor control and unconsciousness. The combined effects render the victim both incapable of self-defense and unable to recall details of sexual assault. The U.S. media has therefore coined the term ''the date-rape drug'' for Rohypnol tablets.

This means of flunitrazepam abuse has exploded in the United States over the past three years. In response, legislation has been proposed (and, in some states, passed) to move the drug to higher schedules, and also to increase the penalties associated with its use in sexual assault. Hoffman LaRoche, Inc. has removed their 2 mg tablets from the market (immediately resulting in the above referenced production of counterfeit 2 mg tablets), and has tentatively agreed to add a coloration agent to their commercial 1 mg tablets in order to help potential victims recognize adulteration of clear or lightly colored alcoholic beverages. These new 1 mg tablets are due to be marketed in Central and South America in late 1998; however, no details concerning the type or concentration of the coloration agent are known at this time.

In both forensic and toxicological laboratories, flunitrazepam is routinely detected and identified via standard IR and gas chromatography/mass spectrometry (GC/MS) screening techniques (12–15). Two general color tests have been reported for flunitrazepam: formaldehyde/sulfuric acid and the Janovsky Reagent (16). In both cases, however, the tests gives analogous results for a wide variety of structurally related benzodiazepines, and they are therefore of limited utility. To date, no specific color or ''field test'' has been reported for presumptive identification of this drug. Herein, we report three new color/screening tests for flunitrazepam. The tests are simple, rapid, definitive, and do not require any specialized chemicals, equipment or techniques. All three work on both pure flunitrazepam and any flunitrazepam containing tablet, capsule or powder.

Standards and Reagents

Pure (reference grade) flunitrazepam and all other controlled substances used in this study were obtained from the reference collection of this laboratory; the flunitrazepam standard was originally acquired from Sigma. Standard pharmaceutical-grade tablets of Rohypnol (sealed OTC blister packs prepared by various subsidiaries of Hoffman LaRoche, Inc.) were acquired from seized exhibits, and contained either 1 mg of flunitrazepam in a 100 milligram tablet, or 2 mg in a 200 mg tablet. No counterfeit tablets were analyzed in this specific study; however, all counterfeit tablets analyzed to date in this laboratory imitated the 2 mg Rohypnol tablets, and were otherwise unremarkable. Dimethylformamide (DMF) and dimethylsulfoxide (DMSO) were both reagent grade Matheson, Coleman and Bell or Aldrich products. All other chemicals were acquired from Aldrich, Mallinckrodt or Merck, and were reagent grade or better. The flint glass/soda lime test tubes used in the development of the test were manufactured by Kimble (#fs73000-1075.)

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Experimental Procedures

Three equally valid procedures may be utilized; all three involve two steps, each resulting in specific color formation. Each of the two-step procedures are definitive for flunitrazepam. Samples may be either cut or uncut flunitrazepam or powder scraped from Rohypnol tablets.

General Test Procedure #1—Direct Heating Method—Variant A

A small amount of sample (2 to 3 mg) is added to a flint glass/soda lime test tube (Kimble #fs73000-1075) followed by five to ten drops of DMF or DMSO; the sample is then heated at 100°C. Samples containing flunitrazepam produce a deep purple color within four minutes. Cooling the sample and adding two drops of concentrated hydrochloric acid produces an immediate canary-yellow color. Note that, based on this study, any other type of test tube (i.e., other than flint glass/soda lime) used in the above test will fail. Also note that both DMF and DMSO are flammable solvents, and use of a flame source for heating is therefore not recommended.

General Test Procedure #1—Direct Heating Method—Variant B

A small amount of sample (2 to 3 mg) is added to a test tube (any type), followed by five to ten drops of DMF or DMSO and a small amount of barium oxide, barium hydroxide or finely ground flint glass/soda lime glass (Kimble #fs73000-1075); the sample is then heated at 100°C. Samples containing flunitrazepam produce a visible, deep purple color within four minutes. Cooling the sample and adding two drops of concentrated hydrochloric acid produces an immediate canary-yellow color. Note that if barium oxide or barium hydroxide (both strong bases) are used, due care should be exercised when adding the concentrated hydrochloric acid. Also note that both DMF and DMSO are flammable solvents, and use of a flame source for heating is therefore not recommended.

General Test Procedure #2—Strong Base (Cold) Method

A small amount of sample (2 to 3 mg) is added to a test tube (any type), followed by five to ten drops of DMF or DMSO and a small amount of solid sodium hydroxide. Samples containing flunitrazepam give an immediate red-purple (wine) color. [Note: The immediate red-purple (wine) color observed in this test is different than the slowly developing, deep purple color which is observed in either variant of test #1!] Addition of two drops of concentrated hydrochloric acid (again) produces an immediate canary-yellow color. Note that sodium hydroxide is a strong base, and due care should be exercised when adding the concentrated hydrochloric acid. Also note that due to the difficulty in mixing the reagents, the use of spot plates for this procedure is not recommended.

Results and Discussion

To the author's knowledge, the development of a purple color upon heating flunitrazepam in DMF or DMSO has never been previously reported. In the present work, the observation of color development was a fortuitous result of unrelated research on flunitrazepam in DMF. It was initially thought that the color arose simply from a previously unknown reaction of flunitrazepam with DMF. Upon preliminary exploration of the experimental parameters, however, it became rapidly apparent that color development occurred only in a specific type test tube, that being made from flint glass/soda lime glass. Contamination of the test tubes was quickly eliminated as a possible source of catalysis. Use of finely ground flint glass/soda lime glass as a catalyst for (successful) color tests in other types of test tubes confirmed a surface catalysis effect. Discussions with technical experts from Kimble indicated that barium oxide was a unique component in the flint glass/soda lime test tubes being utilized. Additional testing in other types of test tubes substituting small amounts of barium oxide in place of finely ground flint glass/soda lime glass gave positive color tests. Substitution of barium hydroxide also gave a strong positive test, while barium acetate and barium carbonate gave weakly positive tests. Overall, however, the use of flint glass/soda lime test tubes gave the best results.

Investigation into alternative solvents confirmed that DMSO also gave a positive test, and with somewhat deeper coloration than with DMF. However, use of tetrahydrofuran, acetonitrile, chloroform or toluene did not give any coloration (17). DMF saturated with hydrochloric acid gave a slight yellow color upon heating. Water gave a bright yellow solution upon heating, while methanol gave a yellow color upon evaporation. Other dipolar aprotic solvents (e.g., N-methylpyrrolidone or hexamethylphosphortriamide) were not investigated in this study.

Regardless of whether flint glass/soda lime test tubes or barium oxide catalysis are used, test #1 (both variants) is extremely specific (*vide infra*); however, the need for extended heating at 100°C precludes its use for routine field testing. Therefore, additional test variants were investigated that did not require heating. Substitution of either solid sodium hydroxide or sodium methoxide for barium oxide both gave an *immediate* red-purple (wine) color *without* heating; however, as noted in the experimental section, the color is not identical to the deep purple that is obtained in the heated variants. Ammonium hydroxide or sodium carbonate, or the organic bases triethylamine, pyridine or 4-dimethylaminopyridine, all gave colorless solutions, while diethylamine gave a yellow color. Finally, use of two drops of 10% sodium hydroxide gave a burnt-orange color.

General Applicability and Specificity

The utility of any color/screening test is directly related to its general applicability and specificity (i.e., number of false positives.) As previously noted, flunitrazepam is a member of the very broad class of benzodiazepines, which include dozens of currently marketed pharmaceuticals, and therefore represents an unusually stringent target molecule for specific screening via color test. In addition, Rohypnol tablets have the usual excipients and diluents present in most commercial OTC preparations. The recent (and increasing) appearance of counterfeit Rohypnol tablets—a development that will likely dramatically increase with the marketing of colored tablets by Hoffman LaRoche—further complicates the situation. Notably, the substitution (whether intentional or inadvertent) of other benzodiazepines for flunitrazepam in counterfeit tableting operations is widespread, and mandates comprehensive testing of other commonly available controlled and OTC substances.

One hundred and eleven compounds were therefore screened using all three testing variants (Table 1); the compounds included various benzodiazepines and a wide variety of controlled substances, adulterants, diluents and excipients. Of the two general tests, #1 (both variants) proved to be extraordinarily specific, with

TABLE 1—*Presumptive tests results.*

Chemical Sample	Color in Test #1 Heat Only (100°C)	Color in Test #2 Sodium Hydroxide	Chemical Sample	Color in Test #1 Heat Only (100°C)	Color in Test #2 Sodium Hydroxide
dl-Amphetamine sulfate	N/C	N/C	Mannitol	N/C	N/C
Antipyrine	N/C	N/C	Magnesium sulfate	N/C	N/C
Aspirin	N/C	N/C	Mangenese sulfate	N/C	N/C
Acetylprocaine hydrochloride	N/C	N/C	O3-MAM sulfamate	N/C	N/C
			O6-MAM hydrochloride	N/C	N/C
Acetylcodeine base	N/C	yellow	O6-MAM base	N/C	N/C
Alprazolam	N/C	light green	2-Methylacetanilide	N/C	N/C
Acetaminophen	N/C	slow blue; turns colorless upon addition of concentrated HCl	dL-methamphetamine hydrochloride	N/C	N/C
			Midazolam	N/C	green-yellow
			Methaqualone base	N/C	N/C
Benzocaine	N/C	N/C	Methaqualone hydrochloride	N/C	N/C
Bromodiphenhydramine	N/C	N/C			
Boric acid	N/C	N/C	Morphine base	slight yellow	slight yellow
Benzophenone	N/C	N/C	3,4-MDA sulfate	slight yellow	N/C
Benzoyl tropeine hydrochloride	N/C	N/C	3,4-MDMA	N/C	N/C
			3,4-MDPNS	strong yellow	yellow
Benzphetamine hydrochloride	N/C	N/C	3,4-MDEA	N/C	N/C
			Meperidine	N/C	N/C
Benzctyzine	N/C	N/C	Methyl paraben	N/C	N/C
Corn Starch	N/C	N/C	Nicotinamide	N/C	slight orange
Chlordiazepoxide	slight yellow	bright yellow	Nitrazepam	yellow	yellow
Codeine base	N/C	yellow	Noscapine	yellow-green	N/C
Codeine hydrochloride	N/C	N/C	Norephedrine	N/C	N/C
Cocaine base	N/C	N/C	Nortriptyline hydrochloride	N/C	N/C
Cocaine hydrochloride	N/C	N/C			
Chloroquine base	N/C	yellow	Ophenadrine	N/C	N/C
Clonazepam	N/C	yellow	Oxazolam	N/C	N/C
Camphor	N/C	N/C	Oxazepam	N/C	yellow-green
Chlorphenrime mealate	N/C	N/C	Phenyl-2-propanone	N/C	orange
Clorazepate	N/C	N/C	Phenylpropanolamine	N/C	N/C
Dimethyl terephthalate	N/C	N/C	alpha-dL-Propoxyphene	N/C	N/C
Diphenhydramine hydrochloride	N/C	N/C	Phenolphthalein	N/C	bright purple color that turns colorless after addition of concentrated HCl
2,5-DMPNS	strong yellow	yellow			
Dextrose	N/C	N/C			
Dimethylamphetamine hydrochloride	N/C	N/C			
Diazepam	N/C	dark orange	Pemoline	N/C	N/C
Dipyrone	N/C	N/C	Papaverine hydrochloride	yellow	N/C
Estazolam	N/C	light green			
Ethylmorphine hydrochloride	slight yellow	slight yellow	1-Phenyl-2-nitropropene	N/C	N/C
			Pyrrocaine	N/C	N/C
Ephedrine hydrochloride	N/C	N/C	Prazepam	N/C	orange-yellow
Ephedrine sulfate	N/C	N/C	d-Pseudoephedrine base	N/C	N/C
Fenbendazole	yellow	slight yellow	Potassium guaiacol sulfonate	N/C	N/C
Flurazepam	N/C	N/C			
Flunitrazepam standard	deep purple	deep red-purple (wine color); canary yellow when acidified with HCl	Procaine base	N/C	N/C
			Phentermine hydrochloride	N/C	N/C
			N-phenyl-2-naphthylamine	slight yellow	orange
Mexican flunitrazepam tablet	deep purple	deep red-purple (wine color); canary yellow when acidifed with HCl	Phenolbarbital	N/C	N/C
			Quinine base	N/C	N/C
			Quinine hydrochloride	N/C	N/C
			Quinine sulfate	N/C	N/C
			Stearic acid	N/C	N/C
Brazilian flunitrazepam tablet	deep purple	deep red-purple (wine color); canary yellow when acidified with HCl	Strychnine	slight yellow	N/C
			Scopolamine	N/C	light green
			Salicylic acid	N/C	N/C
			Salicylamide	N/C	N/C
			Sodium bicarbonate	N/C	N/C
Glutethimide	N/C	N/C	Sucrose	N/C	N/C
Griseofulvin	N/C	N/C	Tetracaine hydrochloride	N/C	N/C
Heroin hydrochloride	N/C	N/C	Thiamine hydrochloride	N/C	N/C
Hydromorphone hydrochloride	N/C	light yellow	Triazolam	N/C	dark orange
			Thebaine base	N/C	light yellow
Isonicotinic acid hydrazide	N/C	yellow	Temazepam	N/C	light green
			Theophylline	yellow	N/C
Ibogaine hydrochloride	N/C	yellow	Tripelennamine	N/C	N/C
Inositol	N/C	N/C	Vitamin E acetate	N/C	N/C
Ibuprophen	N/C	N/C	Yohimbine hydrochloride	N/C	N/C
Lorazepam	N/C	light green			
Lactose	N/C	N/C			
Microcrystalline cellulose	N/C	N/C			

N/C = no color, N/A = no applicable, rt = room temperature, MDA = 3,4-methylenedioxyamphetamine, NS = nitrostyrene, MAM = monoacetylmorphine.

no false positives (purple colors) even using just the first (heating) step. Most compounds gave no coloration at all, while a few gave yellow or yellow-green colors. Notably, even closely structurally related benzodiazepines (including nitrazepam) did not give false positives; a few gave yellow colors, but most were colorless. In addition, all Rohypnol tablets tested (regardless of source) gave positive tests that were indistinguishable from the flunitrazepam standard.

As noted in the previous paragraph, test #1 (both variants) is so specific that just the heating stage alone is sufficient to screen for flunitrazepam; however, this is accurate only for the compounds listed in Table 1. The dramatic color change associated with the second step (deep purple to canary-yellow upon addition of concentrated hydrochloric acid) adds an extra measure of specificity, and completion of both steps is therefore strongly recommended for confirmational purposes.

Test #2 is nearly as specific as test #1, with only two compounds (acetaminophen and phenolphthalein) giving similar colors (Table 1); the former gives a slowly developing blue color, while the latter gives an immediate and intense purple color. With practice, an experienced analyst can easily distinguish between the three compounds based on the color alone. However, the colored solutions from both acetaminophen and phenolphthalein immediately turn colorless when treated with two drops of concentrated hydrochloric acid, thus rigorously differentiating them from flunitrazepam (which again turns canary-yellow upon addition of concentrated hydrochloric acid.) As with test #1, the dramatic color changes associated with the second step adds an extra measure of specificity, and completion of both steps is again strongly recommended for confirmational purposes.

Sensitivity

Stock solutions of reference grade flunitrazepam were prepared in both DMF and DMSO at 0.2, 0.02, 0.002, and 0.0002 mg/mL, respectively, and tested using General Test Procedure #1—Variant A (i.e., heating in flint glass/soda lime test tubes at 100°C.) Colors were recorded at 1, 2, 3, 5 and 10 min intervals. Purple colors developed for both 0.2 mg/mL stock solutions within 2 min, and were very strong within 3 min. The 0.02 mg/mL stock solutions also developed strong purple colors within 3 min. However, the 0.002 and 0.0002 mg/mL stock solutions failed to produce a recognizable color changes even after 10 min of heating. The results indicate a detection limit of *less than* 20 μg of flunitrazepam. Similar sensitivity levels were observed with General Test Procedure #2. Note that 20 μg of flunitrazepam is equivalent to 2 mg of either type (i.e., 1 or 2 mg) Rohypnol tablets.

Effects of Water

The burnt-orange color obtained when 10% sodium hydroxide solution was substituted for solid sodium hydroxide (*vide supra*) suggests that water has a pronounced and detrimental effect on the color formation. The addition of 10% distilled water by volume to the sample prior to heating temporarily inhibited color development; the purple color eventually developed after more than 15 min of heating. Addition of 20% or more distilled water by volume permanently inhibited color development. The addition of water to a positive test solution quenched the purple color and gave an orange colored solution; this latter solution turned bright yellow after 24 h.

Identification of the Purple Pigment

As previously noted, there are no literature reports of color development from flunitrazepam and DMF or DMSO solutions. Attempted isolation of a suspected pigment responsible for the purple color was problematic, suggesting either miniscule amounts of a pigment with an extremely high extinction coefficient or a reversible mechanism for color development.

Blue or purple colors can be suggestive of a solvated electron or radical; however, addition of a molar excess of benzophenone (a radical scavenger) to the reaction mixture prior to heating did not inhibit color formation (and no color was noted when benzophenone itself was tested) (18).

Most benzodiazepines are synthesized via benzophenone type intermediates, and many benzophenones are themselves deeply colored compounds. However, hydrolysis of flunitrazepam under basic conditions did not result in any color formation, while hydrolysis under acidic conditions gave a yellow color. Similarly, submission of the flunitrazepam precursor benzophenone to the color test procedure did not result in any color formation (suggesting that the color did not result from reaction of a small quantity of the precursor benzophenone). Finally, addition of powdered zinc to the reaction mixture did not affect color formation, suggesting that a redox mechanism is not taking place.

Conclusion

Three simple, sensitive and highly specific color screening tests for flunitrazepam were presented.

The two-step tests are effective on both uncut and cut flunitrazepam and standard OTC preparations (i.e., Rohypnol tablets.) General Test Procedure #1 showed the best specificity, but requires a 100°C heat source for either variant (and is therefore best suited for laboratory screening work). General Test Procedure #2 does not require heating and may therefore be easily adapted for field testing. All three tests performed best in the flint glass/soda lime test tubes; however, test #2 may be performed in any type test tube, including those typically utilized in field test kits.

The worldwide marketing of colored 1 mg Rohypnol tablets in late 1998 *may* render this test invalid for commercial OTC preparations, depending on the type and concentration of the added coloration agent. However, even in this event, the test will remain valid for remaining stockpiles of diverted (uncolored) OTC products, and will also probably remain permanently valid for counterfeit Rohypnol tablets—as it is highly unlikely that any illicit tableting operation will deliberately adulterate their product with a distinct marker compound.

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