Use of Fluorescamine ("Fluram") to Detect Amphetamine in Urine by Thin-Layer Chromatography

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A "Fluram" (fluorescamine; 4-phenylspiro[furan-2(3H)-1'-pthalan]-3,3'-dione) spray reagent will detect as little as 250 ng of amphetamine in an extract of amphetamine-containing urine, after it has been separated by thin-layer chromatography. The fluorescence is stable for about 20 h and can be renewed by respraying. Fluram spray does not interfere with reagent sprays used to detect other drugs of abuse. Amphetamine on the thin-layer plate can be measured by extracting the fluorescent area of silica gel and measuring the fluorescence in a fluorimeter. Recovery of 1.0 μg and 2.0 μg of amphetamine averaged 69% and 67%, respectively.

Additional Keyphrases: thin-layer chromatography on silica gel • toxicology • drug abuse

"Fluram" (fluorescamine; 4-phenylspiro[furan-2(3H)-1'-pthalan]-3,3'-dione) has been shown to be a sensitive fluorogenic reagent for primary amines (1). We report here its application to the detection of amphetamine in toxicologic analysis. As little as about 250 ng of amphetamine can be detected after conventional thin-layer chromatographic procedures.

Materials and Methods

Reagents

All chemicals are of reagent-grade quality.

Extracting solvents (by vol): chloroform–isopropanol (96:4) (2) and (3:1) (3); dichloroethane–ethyl acetate (4:6) (4).

Sulfuric acid: 0.1 mol/liter of methanol.


Spray reagents: (a) Triethylamine, 10 ml, is dissolved in and diluted to 100 ml with acetone. (b) Fluram, 10 mg, (Roche Diagnostics, Division of Hoffmann-La Roche Inc., Nutley, N. J. 07110) is dissolved in 100 ml of acetone. These solutions are stable for at least three months.

Amphetamine standard, 100 μg per ml: A stock solution is prepared by dissolving 13.6 mg of amphetamine sulfate (equivalent to 10.0 mg of amphetamine base) in 100 ml of water or methanol. Working amphetamine standards are prepared by diluting the stock standard to contain 50 μg and 25 μg per milliliter.

Negative urine control: Several hundred milliliters of drug-free urine are adjusted to pH 11 with sodium hydroxide (2.5 mol/liter) and extracted twice with 2 volumes of chloroform–isopropanol (3:1 by vol). The organic extracts are discarded. The residual urine is used as the negative urine control and for the preparation of the urine amphetamine control.

Urine amphetamine control, 1 μg amphetamine per milliliter: Ten milliliters of a negative control urine to which 10 μg of amphetamine has been added is submitted to the isolation and detection procedures described below.

Equipment for thin-layer chromatography: Thin-layer chromatography plates, coated with a 250-μm layer of silica gel, without indicator, which have been heat-activated, cooled, and stored in a desiccator. Glass developing tanks of suitable size with ground-glass covers. Disposable capillary pipets, 10-μl and 50-μl capacity. Air blower, hair-drier type. Viewing box equipped with a long-wavelength (360 nm) ultraviolet lamp. Chromatographic spray equipment; in this study, the two-container external propellant type was used. Columns, polypropylene, 10 × 135 mm, filled with about 1.1 g of washed and conditioned XAD-2 resin (20–50 mesh) as described (4–7). Centrifuge tubes, polypropylene, with cap, 50 ml (Falcon No. 2070).

Procedure

Isolation. Amphetamine present in urine specimens (usually, 10–15 ml samples are taken) is isolated by extraction at pH 9.5 with two volumes of chloroform–isopropanol (96:4) (2), or by extraction at pH 11 with two volumes of chloroform–isopropanol (3:1) (3), or by absorption on XAD-2 ion-exchange resin (4–7) and elution with 15–20 ml of either chloroform–isopropanol (3:1) (3) or dichloroethane–ethyl acetate (4:6) (4). The solvent extracts are treated with 0.1 ml of acidified methanol and evaporated.1 The residue is dissolved in 50 μl of methanol, and 10–20 μl aliquots are spotted on a thin-layer plate.

Concurrently, identical volumes of the amphetamine-positive and amphetamine-negative urine control specimens were similarly extracted, the ex-

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1 When a buffer that contains ammonia is used to adjust the pH, the methanolic acid should be added after the ammonia has evaporated, i.e., when about half the solvent has been removed.
tracts concentrated, and aliquots of the concentrates similarly applied to thin-layer plates.

After the plate is spotted with aliquots of the specimen extracts and the amphetamine-positive and amphetamine-negative urine control extracts, 10 \mu l of each of the amphetamine reference solutions (25, 50, and 100 \mu g/ml; equivalent to 250, 500, and 1000 ng) is spotted.

Development. Freshly prepared developing solvent is added to an unlined developing tank, and the tank is sealed and allowed to equilibrate for 10–15 min. The plate is placed in the developing tank and the solvent is allowed to migrate about 15 cm. The plate is removed, dried preliminarily with warm air from the air blower and then for 10 min in an oven at 75 °C to remove residual ammonia.

Visualization and identification. The dried plate is examined under the long wavelength (360 nm) ultraviolet lamp. The appearance of a bright bluish fluorescence, \( R_f \) about 0.6–0.8, prior to spraying usually indicates the presence of quinine. The plate is sprayed first with the triethylamine solution, followed by the Fluram solution, and then oversprayed with the triethylamine solution. When the plate is re-examined under ultraviolet (360-nm) light, the presence of amphetamine in the extracts is revealed as a bright greenish fluorescence at the same \( R_f \) (usually between 0.70–0.80) (6) as that of the fluorescent spots observed in both the amphetamine-positive control urine and the amphetamine reference areas (but not in the negative urine controls). Fluorescence intensity gradually increases and is greatest after 20–30 min.

Quantitative aspects. To evaluate the chromatographic separation of amphetamine from other urinary constituents as a basis for quantitative determination, we spotted amphetamine solutions containing 0.25, 0.50, and 1.0 \mu g/10 \mu l in duplicate, and developed, dried, and sprayed them with Fluram. The three fluorescent areas were marked, and the corresponding silica gel in these areas was scraped off the plate individually onto small squares of glazed weighing paper and transferred to 10 × 75 mm test tubes. The gel was extracted with 1.0 ml of acetone by vortex-type mixing for about 30 s. The tubes were centrifuged for 10 min to pack the solid, and the supernate was poured into another 10 × 75 mm test tube. A portion of the silica gel above the solvent front, equal in area to the sample and similarly treated, served as a reagent blank. To the three extracts, the reagent blank, and to duplicate 1.0-ml standard solutions (0.25, 0.50, and 1.0 \mu g/ml), we added 2.0 ml of borate buffer (0.2 mol/liter, pH 9.5) (1), followed by 1.0 ml of Fluram solution (10 mg/100 ml of acetone). After vortex-type mixing, we measured the fluorescence of the standard solutions and the silica gel extracts vs. the reagent blank in an Amino filter microfluorimeter. The fluorimeter was standardized daily with a quinine standard [1 \mu g of quinine sulfate per milliliter of \( H_2SO_4 \) (0.1 mol/liter)].

![Photograph of a thin-layer chromatographic plate illuminated at 360 nm](image)

Fig. 1. Photograph of a thin-layer chromatographic plate illuminated at 360 nm
Detection with Fluram of amphetamine in 10 ml urine (1 \mu g/ml) isolated (left to right) by ion-exchange, lane 2; by extraction at pH 11, lane 4; by extraction at pH 9.5, lane 7. Lanes 1, 3, and 6 are extracts of three different drug-free urines. Lane 5 is the amphetamine reference, 1 \mu g

Fig. 2. Amphetamine–Fluram reaction: Calibration curve
Fluorescence was measured in a 10 × 75 mm cuvet in an Aminco microfluorimeter with high-intensity mercury-lamp light source; primary filter, Wratten 7-51 (390 nm); secondary filter, Wratten 4 (>465 nm). The points shown represent mean ± 1 SD

Results

Figure 1 is a photograph of some representative results by this method. The negative control urine shows no fluorescence in the area where the amphetamine-containing urines and the amphetamine reference fluoresce.

Figure 2 shows a representative amphetamine calibration curve. Fluorescence, in arbitrary units, is linearly related to amphetamine concentration over a range of 0.25 to 2.0 \mu g/ml. In repeated studies, by the procedure described, recovery of 1.0 \mu g amphetamine averaged 69%; 2.0 \mu g amphetamine, 67%.

Discussion

The principal purpose of this study was to determine the ability of a Fluram reagent spray to detect amphetamine in extracts of urine containing less than 1 \mu g/ml of urine, a concentration is generally accepted as the minimal detection limit of the ninhy-
drin reagent spray used for the purpose (8). With Fluram, by comparison, one can readily detect 250 ng of amphetamine on the plate. We made no detailed examination of the extraction efficiency or isolation and thin-layer separation of amphetamine in urine with various solvent systems, because there are numerous reports on these features of the analysis and detection.

Reagent system. Initially, the spray reagent combination that we used duplicated that in the published procedure for determination of primary amines (1); i.e., the developed and dried plate was sprayed first with borate buffer (0.2 mol/liter) pH 8.5, then with the Fluram reagent in acetone. Good fluorescent visualization in the ultraviolet resulted, but the fluorescence was transient (half-life, about 2–3 h). When the described sequence was used (9), the stability improved: the fluorescence is still visible (although less intense) 20 h later, and may be restored by respraying, even as long as 10 days later.

A Fluram concentration of 10 mg/100 ml acetone is sufficient to make amphetamine visible on a thin-layer plate after solvent development, in drug concentrations of 25 ng to 1000 ng/10 μl applied. Other workers have used 30 mg of Fluram per 100 ml of acetone to make amphetamine visible, particularly when using SA-2 ion-exchange paper to extract amphetamine from urine.2 Here, we found no marked differences in the visual fluorescence response whether 10, 20, or 30 mg of Fluram per 100 ml solutions were used as sprays.

Interferences. Obviously, other physiologic substances bearing primary amino groups usually present in drug-free urine extracts will also react with Fluram, as is evidenced in the drug-free urines in Figure 1. Frequently, as many as eight or more well-separated fluorescent areas were observed. However, all have mobilities considerably less than that of amphetamine in the developing solvent system chosen, and so cannot be considered true interferences.

Two possible interferences that may be more frequently encountered in drug-abuse analyses are quinine, Rf: 0.65 (3), and methamphetamine, Rf: 0.72 (6), which have mobilities close to that for amphetamine (Rf, 0.7–0.8) when the developing solvent described in this report is used. Quinine (sometimes used as a diluent for “street” drugs) can be detected before spraying because it fluoresces when exposed to 360-nm light and will continue to fluoresce. Methamphetamine, a secondary amine, will not react with Fluram, but can be detected subsequently with ninhydrin, if its presence is suspected (7).

Quinine can be readily separated from amphetamine by developing the plate with ethanol–dioxane–benzene–ammonium hydroxide (5:40:50:5 by vol) (10). However, our main focus was to see how Fluram reagent performed in the most frequently used screening technique.

We evaluated the effect of the spray sequence on frequently used detection reagents for other drugs: to 10 ml negative control urine was added 0.5 and 1.0 ml of a solution3 containing amphetamine, 80 μg/ml; “Doriden,” 50 μg/ml; pentobarbital, 50 μg/ml; phenobarbital, 80 μg/ml; phenothiazine, 50 μg/ml; quinine, 5 μg/ml; methadone, 100 μg/ml; and morphine; 10 μg/ml. The mixed solution was extracted at pH 9.5 and the procedure completed. After amphetamine was made visible by Fluram spray, the plates were sprayed with other identification reagents in common use (3). We saw no interference with any of these.

Our consistent recovery of 67–69% of 1–2 μg of amphetamine from plates was obtained by a single brief extraction of the fluorescent silica gel area. Undoubtedly this can be improved.

Except for the Amberlite XAD-2 resin-column isolation method, all operations were carried out in polypropylene centrifuge tubes. This decreased losses of amphetamine reported to occur by irreversible adsorption on active sites of uncoated glass surfaces and, because polypropylene is both solvent resistant and does not wet, the separation of organic and aqueous phases was enhanced (11).

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References

2 Dr. Arthur deT. Valk, Jr., personal communication.

3 The reference control solution used in the drug-abuse analyses program of the New York City Department of Health.