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Identification of Flurazepam (Dalmane®) and a Primary Metabolite in Urine by Thin-Layer Chromatography

Flurazepam hydrochloride (Dalmane®)[5] is a relatively new hypnotic agent gaining widespread usage in adults as a "sleeping pill." Flurazepam is structurally related to the diazebenzapine tranquilizers, diazepam (Valium®) and chlorodiazepoxide hydrochloride (Librium®). It is not classified by Drug Enforcement Administration (DEA) regulations as a drug of abuse; however, any hypnotic, sedative, or tranquilizer which has widespread usage always has the potential for abuse. Until the recent mention by Sturner and Garriott [*1*] in their article on L-DOPA poisoning, few methods have been available for the detection of flurazepam in blood or urine or both with the exception of those by Swartz et al [*2,3*] and de Silva and Strojny [*4*]. A relatively simple, reliable method for the detection of flurazepam in urine could assist in identifying persons abusing or overdosed with the drug. This report describes a simple thin-layer chromatographic (TLC) method for the qualitative identification of flurazepam in urine based upon the presence of flurazepam and a primary urinary metabolite, 7-chloro-1-(2-hydroxyethyl)-5-(2-fluorophenyl)-1,dihydro-2H-1,4-benzodiazepin-2-one (FM).

Materials

All chemicals used should be American Chemical Society (ACS) Reagent Grade.

Standard Solutions

Flurazepam Hydrochloride—Stock standard, 1 mg/ml in ethanol acidified with two drops of 1*N* hydrochloric acid per 25 ml of solution. This solution is stable for three weeks if refrigerated (Hoffman-LaRoche, Inc., Nutley, N.J.).

Flurazepam Hydrochloride—Working standard, 100 μg/ml in ethanol. This solution is stable for one week if refrigerated.

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[5] Dalmane®, Librium®, and Valium® are registered trademarks of Hoffman-LaRoche, Inc., Nutley, N.J.

7-chloro-1-(2-hydroxyethyl)-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one—Stock standard, 1 mg/ml in ethanol acidified with 2 drops of 1*N* hydrochloric acid per 25 ml of solution. This solution is stable for three weeks if refrigerated (Hoffman-LaRoche, Inc., Nutley, N.J.).

7-chloro-1-(2-hydroxethyl)-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one—Working standard, 100 μg/ml in ethanol. This solution must be refrigerated and prepared fresh weekly.

Reagents

Sodium Citrate Buffer—pH 1.0 (±0.1). To 260 gm sodium citrate dissolved in distilled water add 256 ml concentrated hydrochloric acid. Mix well and dilute to 2000 ml with distilled water.

Bicarbonate Buffer—pH 9.5. Add saturated sodium carbonate solution to a saturated sodium bicarbonate solution to pH 9.5. This requires approximately equal parts of the 2 solutions.

sym-*Diphenylcarbazone*—0.01% weight/volume (w/v) in acetone.

Acidic Mercuric Sulfate—2% w/v. Add 100 ml of distilled water to 5 g of mercuric oxide, followed by 20 ml of concentrated sulfuric acid. Cool and dilute to 250 ml with distilled water.

IKI Solution—Dissolve 2 g iodine and 2 g potassium iodide in 50 ml of 95% ethanol. Add 50 ml of 25% hydrochloric acid and mix well.

Neutral Iodoplatinate Solution—Mix equal parts iodoplatinate Solution A (0.79% chloroplatinic acid in water) and Solution B (19.35% potassium iodide in water) (both Solutions A and B from Brinkman Instrument Inc., Westbury, N.Y. 11590, Catalog No. 35041303).

Method

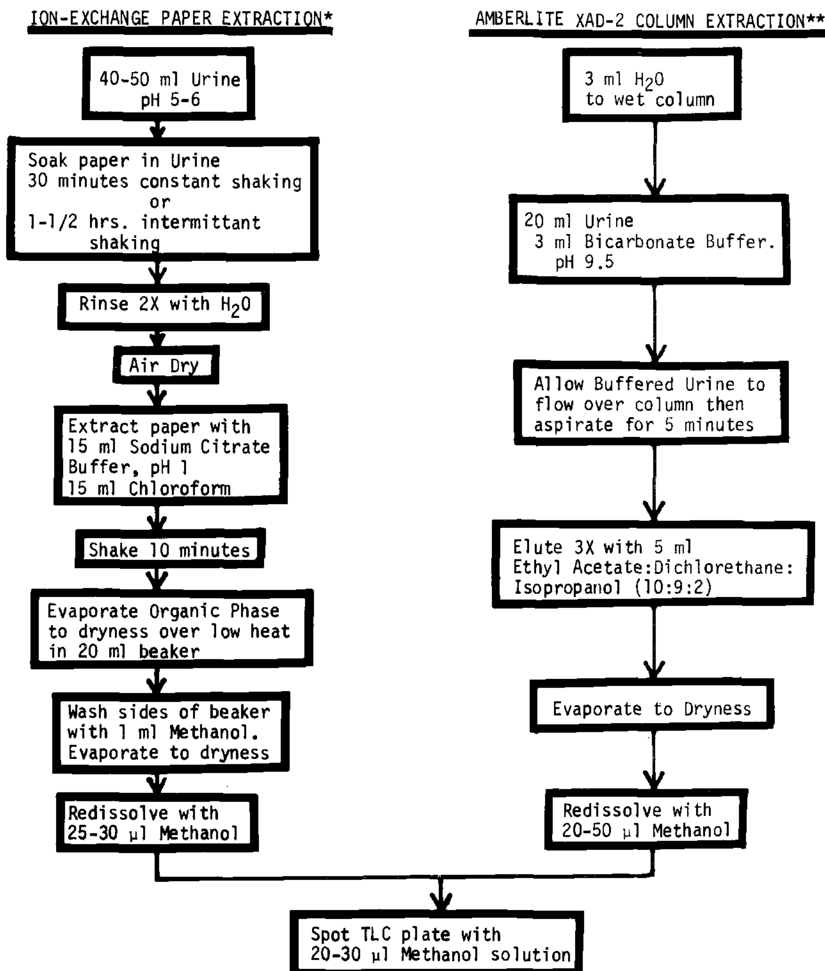
Two techniques are described which provide a satisfactory extraction of flurazepam and a primary metabolite (FM) from urine for their qualitative identification by TLC (Fig. 1). The conditions for TLC identification of the agents are also described (Fig. 2).

Ion Exchange Paper Extraction

Forty to fifty millilitres of urine are acidified with 1*N* hydrochloric acid to pH 5-6 and the flurazepam and FM extracted from the urine with cation-exchange resin paper (Reeves-Angel SA-2 paper), according to the method described by Kaistha and Jaffe [5]. After removal from the urine, the paper is rinsed twice with distilled water and air dried. The paper is then placed in a stoppered bottle containing 15 ml of sodium citrate buffer, pH 1, plus 15 ml of chloroform and agitated on a reciprocating shaking machine for 10 min. The aqueous phase is discarded and the organic phase evaporated to dryness over low heat in a 20-ml beaker. The sides of the beaker are then rinsed with 1.0 ml of methanol and the methanol evaporated to dryness in the same beaker. The resulting residue is redissolved with 25 to 30 μl of methanol. Twenty to thirty microlitres of the final methanolic solution are spotted on a 250-μm silica gel G TLC plate.

Column Extraction

Drug-Skreen® cartridges (Brinkman Instruments, Inc., Westbury, N.Y.), which are filled with an amberlite XAD-2 resin, are used to extract the flurazepam and FM. The columns are moistened with approximately 3 ml of distilled water prior to extraction of



*Reeve Angel SA-2 cation exchange paper.
** Brinkman Drug Skreen® cartridges.

FIG. 1—*Flow chart of extraction procedures for flurazepam and FM.*

the drug and its metabolite from the urine specimen. Twenty millilitres of urine are mixed with 5 ml of bicarbonate buffer, pH 9.5, and allowed to either flow over the XAD-2 column by gravity or be pulled through the filled cartridge by application of a small amount of suction. The column is then aspirated by gentle suction for 5 min after the buffered urine has been removed from the cartridge to remove excess aqueous solutions. Next, the column is eluted three times with 5-ml aliquots of a mixture of ethyl acetate:dichlorethane:isopropanol (10:9:2). The three eluates are pooled and evaporated to dryness in a 20-ml beaker, the beaker rinsed with 1.0 ml of methanol, and the methanol evaporated to dryness. The residue is dissolved in 20 to 50 μl of methanol. Twenty to thirty microlitres of this final methanolic solution are spotted onto a silica gel G TLC plate.

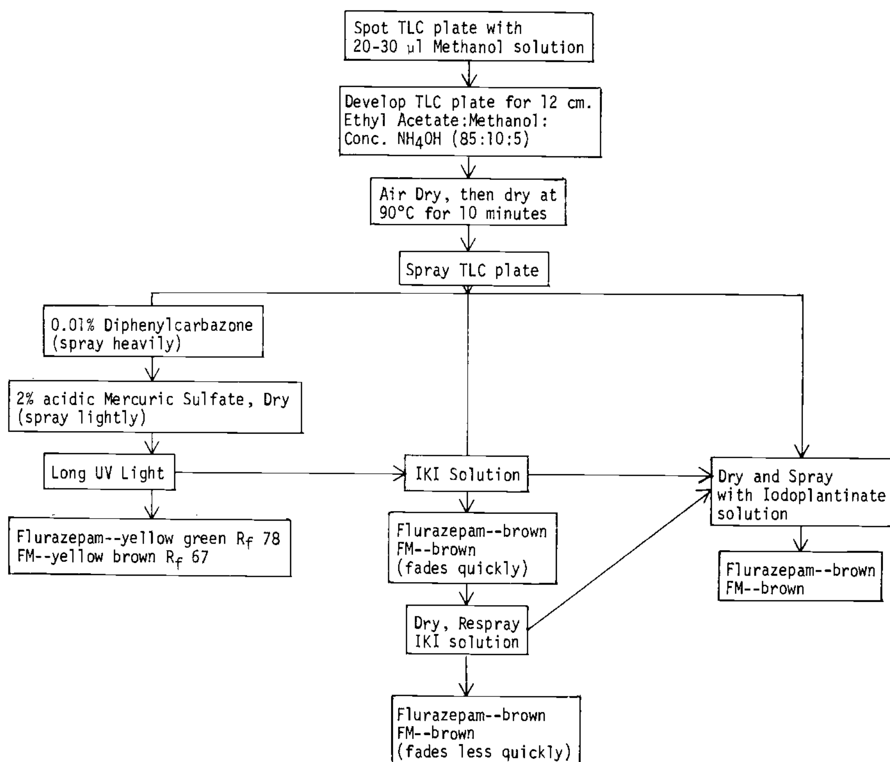


FIG. 2—*Flow chart for the TLC separation and identification of flurazepam and FM.*

Identification of Drug and Metabolite

After spotting the methanolic drug solutions on a silica gel G plate, the plate is placed in a glass developing tank containing ethyl acetate:methanol:ammonium hydroxide (85:10:5) and the solvent front permitted to advance for 12 cm. Five-microlitre aliquots of the working standard solutions of flurazepam and FM are also spotted for reference purposes. After development is complete, the plate is first air dried and then dried in a hot air oven at 90°C for 10 min. Qualitative identification of flurazepam and FM is accomplished by one or a combination of the following procedures (Fig. 2) after the TLC plate has been removed from the oven and cooled.

1. The plate is sprayed heavily with 0.01% diphenylcarbazone followed immediately by a slight spray application of 2% acidic mercuric sulfate. After the plate has dried it is exposed to longwave ultraviolet light. Flurazepam fluoresces with a yellow green color (R_f 78) and FM appears as a yellow-brown fluorescent spot (R_f 67).
2. The IKI solution can be used as a single spray identification for flurazepam and FM or it can be used after the diphenylcarbazone, mercuric sulfate, and ultraviolet light identification procedure. The first visualization of flurazepam and FM with IKI solution produces two distinct brown bands. This color fades rapidly but can be reproduced for a longer period of time if the plate is resprayed a second time with IKI solution. The plate should be permitted to dry between IKI applications.

3. The dried plate can be sprayed with neutral iodoplatinate solution, which also produces a brown color with the flurazepam and FM bands. The iodoplatinate solution can either be applied to a fresh chromatogram or be used following the drying of the IKI solution. The chromogen produced by the reaction with IKI disappears quickly and there is no confusion in this visualization step with the iodoplatinate chromogen.

4. If additional confirmation is deemed necessary or if the bands are very faint or weakly reactive from Treatments 1 through 3, a final light spraying with Dragendorff solution can be used.

Results and Discussion

This work was begun while comparing methods prior to establishing certain types of analytical drug procedures in our laboratory. During the course of this work we used urine from patients known to have received medications as a matter of their therapy to monitor the effectiveness of our procedures. In the urine from some patients we encountered two TLC bands which were not readily identifiable. In reviewing the patients' charts, it was discovered that common to all persons whose urine had these two "unknown" TLC bands present was the peroral administration of 30 mg of flurazepam at bedtime on the night prior to collection of the urine specimen for our laboratory use. Subsequent testing by the method described identified the bands as flurazepam and FM when compared with the pure compounds.

It has been necessary to make minor modifications of the extraction procedure of Kaistha and Jaffe [5] for the convenience of our laboratory. We have replaced the wide mouth jars with screw-capped (teflon-lined) 50-ml centrifuge tubes and have found that intermittent shaking with a reciprocal shaking machine is as successful for extraction of the drug and metabolite by the SA-2 paper as soaking overnight in the urine, as recommended by Kaistha [5]. The SA-2 paper extraction procedure produced a final product for the TLC spotting that when chromatographed resulted in many bands which tailed or obliterated our drug bands. When we began using the Drug-Skreen® cartridge and the recommended filter, which is a phase-separating paper, many of these interfering products were eliminated.

The original evaporation procedure of Kaistha and Jaffe [5], was found to be very time-consuming since it recommended evaporation in tubes with a stream of air flowing into the tube. We have obtained excellent, more rapid results using a heating block (70 to 90°C) with either air blowing over the organic fraction or gentle suction applied to a semiclosed cover on the heating block.

Another advantage of the resin cartridge extraction over the SA-2 paper is the amount of specimen required for processing. The SA-2 paper method requires 40 to 50 ml of urine while the cartridge requires 20 ml of urine. If only a very small quantity of flurazepam or FM is suspected to be present, up to 40 ml of urine can be used with the Drug-Skreen® cartridge extraction procedure. With the larger sample one will encounter more extraneous materials on the final chromatogram, which are apparently extracted by and eluted from the columns.

In vitro studies have shown that extraction of urine specimens by either method described with subsequent separation by TLC can detect flurazepam and FM in amounts as low as 1 μg/ml of original urine sample. Swartz et al [2,3] and de Silva [4,6] state that flurazepam is largely excreted as a glucuronide or sulfate conjugate of FM in the first 24 hours post drug administration. Existing chemical methods by these investigators have included a step for enzymatic treatment of the specimen to break this conjugate

bonding. We do not find hydrolysis necessary to detect the presence of flurazepam or FM using the method described.

Equal success has been experienced in visualizing flurazepam and FM by either single-spray application of the various chromogen-producing chemicals or by sequential application of these same compounds. No quantitative recovery studies were performed for the purposes of this particular method, since we were interested in a simple qualitative identification method for flurazepam and its primary metabolite. When diazepam is also present in the urine, difficulty may be encountered by the inexperienced technologist in distinguishing between it and flurazepam because of the close similarity in color under ultraviolet light and in R_f values. However, none of the following drugs or their metabolites tested by the method described produce any interference in the identification of flurazepam or FM: barbiturates, glutethimide, diphenylhydantoin, methadone, phenothiazines, methaqualone, amphetamines, propoxyphene, quinine, cocaine, morphine, codeine, chlorodiazepoxide, pentazocine, meperidine, and phenylpropanolamine. This lack of interference in the method by these compounds was examined both by addition of the pure drugs to known negative urines and testing urine from persons known to have received the various drugs.

Standards of flurazepam and FM were prepared in both ethanol and chloroform in concentrations of 1 mg/ml. Both were readily soluble in ethanol and FM was equally soluble in chloroform; however, flurazepam was not easily soluble in chloroform. After the compounds were dissolved in the two solvents, it was found that no difference existed in their chromatographic behavior. Shelf life of the standard solutions was extended from a few days to three weeks by addition of two drops of 1*N* hydrochloric acid per 25 ml of each stock standard. Dilute solutions of flurazepam and FM (100 μg/ml) prepared from the acidified ethanolic stock solutions are generally stable for up to three weeks; however, we routinely prepare fresh working standards weekly. Flurazepam and FM working standards are best prepared in a negative urine. Otherwise the R_f value of the standard may be slightly higher than observed for the unknown urine specimen. Flurazepam and FM in terms of color reactions and reaction to long ultraviolet light are the same, regardless of whether the ethanolic standard solution or the drug in urine is chromatographed.

Summary

A relatively simple, routine method has been described for the qualitative identification of flurazepam and its primary human metabolite 7-chloro-1-(2-hydroxyethyl)-5-(2-fluorophenyl)-1,3-dihydro-2H-1,4-benzodiazepin-2-one in urine. We have described two extractions and several identification procedures by which flurazepam and its primary urinary metabolite can be identified by TLC.

Acknowledgments

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