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Abstract

Benzodiazepines are central nervous system depressant drugs often detected in biological samples from driving under the influence (DUI) offenders. They are associated with marked psychomotor impairment and represent up to 20% of all Miami-Dade County, Florida DUI urine samples analyzed in our laboratory annually. Flunitrazepam emerged in the mid-1990s as an illegal drug in the U.S. that was predominantly abused recreationally and associated with sexual assaults. Immunoassays for benzodiazepines do not discriminate between different benzodiazepines, and certain metabolites, such as 7-aminoflunitrazepam, react poorly with immunoassay reagents. A simple and sensitive method for the detection and quantitation of major benzodiazepines and metabolites by gas chromatography with mass selective detection is presented. This method was used to confirm benzodiazepines in general and flunitrazepam in particular. Data collected over a three-and-a-half-year period are summarized. Whereas flunitrazepam was present in up to 10% of DUI cases in 1995 and 1996 and had fast become the most frequently encountered benzodiazepine in Miami-Dade County DUI-related urine samples, a dramatic drop in case numbers followed the legal reclassification of the drug as a Schedule I substance in Florida in February 1997. Flunitrazepam was often used alone or in combination with cannabis and cocaine. A recent rise in clonazepam cases coincides with the decrease in flunitrazepam confirmation and may indicate a new trend in the abuse of benzodiazepines in South Florida.

Introduction

Since the introduction of chlordiazepoxide (Librium®) in 1960, benzodiazepines remain among the most prescribed pharmaceu-

cials. Used as anxiolytic, sedative/hypnotic, anticonvulsant, and muscle relaxant, they are relatively safe drugs. However, the combination of central nervous system (CNS) depressants such as alcohol and a benzodiazepine results in excessive sedation, mental and motor function impairment, and potential respiratory depression (1,2). The clinical and forensic toxicology laboratory must be able to screen, confirm, and quantitate the main benzodiazepines and metabolites at low concentrations in biological specimens. These compounds are frequently detected in samples related to drug intoxications and traffic accidents and have been implicated in sexual assault cases (3–6).

In recent years, the benzodiazepine sedative hypnotic, flunitrazepam (Rohypnol), has emerged as a significant abused drug in certain areas of the country, particularly in Texas (7), Florida, Louisiana, and Arizona (8). It is not approved for medical use in the United States by the Food and Drug Administration. Elsewhere, flunitrazepam is indicated for the treatment of insomnia and is used as a pre-anesthetic medication because it is primarily a hypnotic drug rather than an anxiolytic, muscle relaxant, or anticonvulsant (9). Flunitrazepam is generally abused with alcohol to produce a prolonged and profound intoxication (10). It is also abused by heroin and cocaine addicts, possibly to reinforce the depressant effect or to blunt the "crash" after a binge of stimulant (11). Occasionally, it is used to incapacitate a victim prior to the commission of rape, hence the coining of the term "date-rape drug" (5). The observed prevalence of DUI cases involving flunitrazepam in Miami-Dade County resulted in a special emphasis being placed on confirming and quantitating 7-aminoflunitrazepam.

Benzodiazepines undergo extensive metabolism, resulting in one or more pharmacologically active or inactive metabolites that may be common to more than one drug. Furthermore, the parent drug may be present in the urine at extremely low concentrations because of high protein binding, low dose, timing of specimen collection, or metabolism (12). 1,4-Benzodiazepines such as

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were formed using N-methyl-N-t-butyl-dimethylsilyl trifluoroacetic acid. The primary urinary metabolites of flu-}

zepam are desalkylflurazepam and hydroxyethylflurazepam (16). The main urinary metabolites of clonazepam and flunitrazepam are the 7-amino compounds with a further reduction of the amine function to the 7-acetamido moiety (17,18). There appears to be some conjugation of the 7-amino metabolites of both clonazepam and flunitrazepam. Lorazepam, oxazepam, and temazepam are conjugated directly to glucuronic acid and excreted. In order to distinguish the many benzodiazepines and their metabolites, the analytical method must provide high specificity and sensitivity. Urinary concentrations typically range from nanograms per milliliter to low micrograms per milliliter during therapeutic use but may reach much higher values in DUI cases in which the person is impaired at the time of the arrest. These metabolic patterns were the basis to monitor diazepam and its main metabolite nordiazepam; alprazolam and hydroxyalprazolam; triazolam and hydroxytriazolam; oxazepam (either as a parent drug or as a common metabolite of various benzodiazepines); 7 aminoflunitrazepam and 7-aminoclonazepam, principal metabolites of flunitrazepam and clonazepam, respectively; desalkylflurazepam and hydroxyethylflurazepam, principal metabolites of flurazepam; and temazepam and lorazepam, as parent drugs. This manuscript presents a sensitive method for the gas chromatographic–mass spectrometric (GC–MS) detection and quantitation of these 13 benzodiazepines and metabolites, with a special emphasis on flunitrazepam and its principal metabolite, 7-aminoflunitrazepam. This method was applied to study the prevalence of flunitrazepam in Miami-Dade County DUI cases from 1995 to mid-1998. Patterns of and trends in the use of Rohypnol with cocaine, cannabis, and other benzodiazepines and the possible emergence of clonazepam abuse are examined.

Materials and Methods

Chemicals

Standards of 7-aminoflunitrazepam, 7-aminoclonazepam, oxazepam-d6, α-hydroxylprazolam, α-hydroxytriazolam, desalkylflurazepam, and hydroxyethylflurazepam were obtained as 1 mg/mL methanolic solutions from Radian (Austin, TX). β-Glucuronidase from E. coli-type IXA and additional drug standards diazepam, nordiazepam, oxazepam, temazepam, lorazepam, alprazolam, and triazolam were obtained from Sigma Chemical (St. Louis, MO). Ammonia, methylene chloride, hexane, and potassium phosphate monobasic and dibasic were analytical grade (Fisher Scientific, Pittsburgh, PA). Derivatives were formed using N-methyl-N-t-butyl-dimethylsilyl trifluoroacetic amide (MTBSTFA) and silylation-grade acetonitrile (Pierce Chemical Co., Rockford, IL). Helium-compressed UHP-grade 5.0 and nitrogen-compressed zero-grade 4.0 were used as carrier gas and for evaporation, respectively (Praxair Distribution Southeast, LLC, Orlando, FL).

Screening

Abuscreen OnLine® test kits for benzodiazepines were purchased from Roche Diagnostics Systems (Nutley, NJ). The assays were performed on a Cobas Mira Plus® according to manufacturer’s specifications. The calibrator for this assay was nordiazepam at 100 ng/mL.

Cross-reactivity of 7-aminoflunitrazepam with Roche OnLine reagent

Negative urine samples were fortified with 50 to 1000 ng/mL analyte, and the immunoassay screening value was compared to the arbitrary cutoff value of 1000 given by the commercially available Roche 100-μg/mL nordiazepam calibrator. This value of 1000 represents the factor-corrected normalized absorbance value (ΔA) of the calibrator and is according to the standard operating procedure manual of the instrument. Table I compares the normalized factor-corrected absorbance values of certified negative and positive urine controls. Negative screening values are the result of interfering proteins and nonspecific organic substances as per the manufacturer.

Instrumentation

The instrument used was a Hewlett-Packard 5890 series II GC coupled to an HP 5970 or 5972 mass selective detector (MSD). The GCs were equipped with electronic pressure control and an HP 7663 autosampler.

Enzymatic hydrolysis, extraction, and derivatization procedure

Urine calibrators containing 7-aminoflunitrazepam, 7-aminoclonazepam, alprazolam, hydroxylprazolam, desalkylflurazepam, hydroxyethylflurazepam, diazepam, nordiazepam, oxazepam, lorazepam, temazepam, triazolam, and hydroxytriazolam at 50, 100, 250, 500, and 1000 ng/mL were extracted along with unknown samples from DUI arrests in Miami-Dade County, FL. A commercially available benzodiazepine-positive control containing 200 ng/mL desalkylflurazepam, nordiazepam, hydroxyalprazolam, lorazepam, temazepam, and oxazepam (Quality Assurance Service Corp., Augusta, GA), an inhouse urine control containing 50 ng/mL of each of the analytes and a negative urine control (certified-negative urine) were also extracted with each batch. Each urine aliquot was fortified with 250 ng/mL oxazepam-d6 as an internal standard. The sample volume was 3 mL. Samples were buffered to pH 6.8 with 1 mL of 0.1 M potassium phosphate buffer, and 5000 units of β-glucuronidase from E. coli, type IXA, was added to each tube. Hydrolysis was stopped after a 2-h incubation at 37°C. The samples were alkalinized to pH > 9 by the addition of 750 μL of concentrated ammonia. Benzodiazepine compounds were extracted with 10 mL of a hexane/methylene chloride mixture (4:3, v/v) using a rotator at slow speed for 15 min. The tubes were centrifuged at 3000 × g for 5 min. The aqueous layer was frozen in a dry ice–acetone bath and the organic layer was transferred to 16 × 125 silanized centrifuge
was deactivated by vapor phase silanization prior to and 300~ respectively. The column temperature was held with helium as the carrier gas. The 4-mm single taper borosilicate glass insert contained a 2-cm glass wool plug was deactivated by vapor phase silanization prior to use. The injector and transfer line temperatures were maintained at 295~ for 1.5 min initially and ramped at 30~ to 265~ held for 1.2 min, and then ramped at 4~ to 305~ with a final holding time of 8 min. This resulted in a total chromatography time of 24.53 min. Table II lists the ions that were monitored by SIM and the retention times relative to oxazepam-d<sub>9</sub> of the individual compounds. Diazepam, alprazolam, triazolam, and 7-aminojifenitrizepam were the only four analytes not derivatized by MTBSTFA. The structurally characteristic high mass ions were chosen based on the review of the full scan mass spectra of each analyte and published literature (19,20). They produced stable and consistent ion ratios, indicating a lack of interference from the urine matrix.

### Calculations

Analyte concentrations were calculated by comparison with matrix-matched standard curves. Least-squares regression analysis of the peak-area ratios (analyte/internal standard) resulted in a standard curve equation that was used to calculate the concentrations of the unknowns.

### Validation

Recovery for the various benzodiazepines and metabolites was assessed in the classical manner: certified-negative urine samples (n = 8) were fortified with internal standard and extracted. The solvent was evaporated; 100 μL of a methanolic solution containing 100 ng of each analyte was then added to the dry residue and evaporated prior to the reconstitution in the derivatizing reagent. In parallel, urine samples (n = 8) were fortified with internal standard and 100 ng of each analyte and carried through the entire procedure. Peak-area ratios were compared to yield recovery values. The limits of detection (LOD) of the various analytes were determined by the analysis of 12 benzodiazepine-negative urine samples and the application of the following method: LOD = mean peak area integration of the quantitation ion + 3 SD. The limit of quantitation (LOQ) was an administrative cutoff at 50 ng/mL, which is above the values obtained with the formula LOQ = mean peak area integration of the quantitation ion + 10 SD. Within-run and between-run variability were calculated for the 100 ng/mL control. Correlation coefficients and y-intercepts approaching zero were used to assess linearity. Flash-derivatization was compared to conventional derivatization as follows: four 100-ng/mL fortified controls reconstituted in derivatizing solution were heated 15 min at 60°C and then injected. In parallel, four 100-ng/mL fortified controls were reconstituted in derivatizing solution and injected with no preheating, that is, "flash-derivatized". Peak-area ratios were averaged and compared.

### Miami-Dade Police Department Drug Influence Evaluation (DRE)

Psychophysical measurements and field sobriety tests were administered by a DRE officer when alcohol and/or other substances impaired the driver's ability to operate a motor vehicle.

### Results

### Validation

The method was fully validated. Recovery was quantitative for diazepam, nordiazepam, desalkylflurazepam, 7-aminoflu

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**Table I. Immunoassay Screening Factor-Corrected Absorbance Values For Certified-Negative and Positive Urine Controls Using the Roche OnLine reagent**

<table>
<thead>
<tr>
<th></th>
<th>Positive Control</th>
<th>Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>1179 ± 83</td>
<td>-62 ± 243</td>
</tr>
<tr>
<td>Median</td>
<td>1158</td>
<td>-5</td>
</tr>
<tr>
<td>Range</td>
<td>[1082-1450]</td>
<td>[-1011-202]</td>
</tr>
<tr>
<td>N</td>
<td>36</td>
<td>36</td>
</tr>
</tbody>
</table>

* Positive Roche benzodiazepine controls containing 150 ng/mL nordiazepam from 36 separate assays averaged a screening value of 1179 while negative controls averaged a value of -62. A 100-ng/mL nordiazepam Roche OnLine calibrator was processed in duplicate in each assay and set to read a cutoff value of 1000 (factor correction).

**Table II. Relative Retention Times (rRT) and Mass-to-Charge Ratio of Selected Ions for Quantitation and Identification**

<table>
<thead>
<tr>
<th>Order of elution</th>
<th>rRT</th>
<th>Quantitation ions m/z</th>
<th>Identification ions m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>0.778</td>
<td>283</td>
<td>256</td>
</tr>
<tr>
<td>Desalkylflurazepam-TBDMS*</td>
<td>0.793</td>
<td>345</td>
<td>347</td>
</tr>
<tr>
<td>Nordiazepam-TBDMS</td>
<td>0.801</td>
<td>327</td>
<td>329</td>
</tr>
<tr>
<td>7-Aminojifenitrizepam</td>
<td>0.937</td>
<td>255</td>
<td>254, 282</td>
</tr>
<tr>
<td>Oxazepam-di-TBDMS</td>
<td>1.000</td>
<td>462</td>
<td>464, 518</td>
</tr>
<tr>
<td>Oxazepam-d&lt;sub&gt;3&lt;/sub&gt;-TBDMS</td>
<td>1.002</td>
<td>457</td>
<td>459, 513</td>
</tr>
<tr>
<td>Temazepam-TBDMS</td>
<td>1.075</td>
<td>357</td>
<td>283, 359</td>
</tr>
<tr>
<td>7-Aminonclonazepam-TBDMS</td>
<td>1.106</td>
<td>342</td>
<td>344, 399</td>
</tr>
<tr>
<td>Lorazepam-d&lt;sub&gt;3&lt;/sub&gt;-TBDMS</td>
<td>1.157</td>
<td>491</td>
<td>493, 513</td>
</tr>
<tr>
<td>2-Hydroxyethylflurazepam-TBDMS</td>
<td>1.199</td>
<td>389</td>
<td>391</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>1.271</td>
<td>279</td>
<td>308, 273</td>
</tr>
<tr>
<td>Triazolam</td>
<td>1.395</td>
<td>313</td>
<td>342, 315</td>
</tr>
<tr>
<td>Hydroxyalprazolam-TBDMS</td>
<td>1.639</td>
<td>381</td>
<td>382, 383</td>
</tr>
<tr>
<td>Hydroxytriazolam-TBDMS</td>
<td>1.797</td>
<td>415</td>
<td>417</td>
</tr>
</tbody>
</table>

* TBDMS: tert-butyldimethylsilyl derivatives.
trazepam, oxazepam, alprazolam, and triazolam. Recoveries were 87% for temazepam, 70% for hydroxy metabolites of alprazolam and triazolam, 55% for 2-hydroxyethylflurazepam, and 47% for 7-aminoclonazepam.

An administrative quantitation cutoff value was chosen at 50 ng/mL based on the standard curve range and the high concentrations typical of DUI cases. However, limits of detection were 4 ng/mL for 7-aminoflunitrazepam; 2 ng/mL for alprazolam, triazolam, and their hydroxy metabolites; 1 ng/mL for temazepam, lorazepam, 7-aminoclonazepam, oxazepam, and 2-hydroxyethylflurazepam; and below 1 ng/mL for diazepam, nordiazepam, and desalkylflurazepam.

Coefficients of variation ranged from 2.9% for diazepam to 7.7% for temazepam. Coefficients of variation within run were less than 5% for all analytes. Linearity was observed from 50 to 1000 ng/mL ($r > 0.990$) for all analytes. The standard curve was subsequently chosen to bracket 50 to 500 ng/mL, and samples with concentrations above the curve were adequately diluted with certified-negative urine to fall within this range. Typical chromatograms of controls and cases are shown in Figures 1A and 1B, respectively.

Flash derivatization was accomplished in the insert upon injection. Table III shows that for all benzodiazepines, the difference with conventional derivatization (preheating, 15 min at 60°C) was marginal, with, in most cases, a larger peak-area ratio obtained after flash derivatization, in particular for 7-aminoflunitrazepam and 7-aminoclonazepam, where the peak-area ratios after preheating were only 80% of those obtained following flash derivatization in the insert. Lorazepam, oxazepam, and temazepam gave peak-area ratios that were slightly smaller on average than those obtained with conventional derivatization.

### Immunoassay screening and GC–MS quantitation of 7-aminoflunitrazepam

GC–MS quantitation results were compared with immunoassay screening values on 85 samples submitted in 1995 and

<table>
<thead>
<tr>
<th>Benzodiazepine</th>
<th>% peak-area ratio, heated/flash deriv.</th>
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<tbody>
<tr>
<td>Diazepam</td>
<td>98.8</td>
</tr>
<tr>
<td>Desalkylflurazepam</td>
<td>99.7</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>99.6</td>
</tr>
<tr>
<td>7-Aminoflunitrazepam</td>
<td>83.1</td>
</tr>
<tr>
<td>Oxazepam-d$_3$</td>
<td>101.2</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>101.2</td>
</tr>
<tr>
<td>Temazepam</td>
<td>106.5</td>
</tr>
<tr>
<td>7-Aminoclonazepam</td>
<td>80.3</td>
</tr>
<tr>
<td>Lorazepam</td>
<td>100.2</td>
</tr>
<tr>
<td>2-Hydroxyethylflurazepam</td>
<td>100.0</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>93.6</td>
</tr>
<tr>
<td>Triazolam</td>
<td>93.3</td>
</tr>
<tr>
<td>Hydroxyalprazolam</td>
<td>95.4</td>
</tr>
<tr>
<td>Hydroxytriazolam</td>
<td>94.3</td>
</tr>
</tbody>
</table>

Figure 1. Typical chromatograms. The analytes (peak identification number; retention time) were eluted as follows: diazepam and desalkylflurazepam (1, 2; 8.19, 8.21 min), nordiazepam (3; 8.30 min), 7-aminoflunitrazepam (4; 9.79 min), oxazepam-d$_3$, and oxazepam (5, 6; 10.11, 10.13 min), temazepam (7; 10.89 min), 7-aminoclonazepam (8; 11.22 min), lorazepam (9; 11.50 min), 2-hydroxyethylflurazepam (10; 12.00 min), alprazolam (11; 12.89 min), triazolam (12; 14.02 min), hydroxyalprazolam (13; 16.40 min), and hydroxytriazolam (14; 17.98 min). A, Chromatograms of a control urine fortified with 100 ng/mL of all analytes (top) and of a certified-negative control urine (bottom). B, Chromatograms of two DUI urine samples. The quantitations were as follows: 7-aminoflunitrazepam, 7-aminoclonazepam, alprazolam, and hydroxytriazolam, 204, 179, 74, and 146 ng/mL, respectively (top); nordiazepam, oxazepam, temazepam, alprazolam, and hydroxyalprazolam, 124, 1163, 882, 218, and 295 ng/mL, respectively (bottom).
1996. Immunoassay screening for benzodiazepines revealed that 7-aminoflunitrazepam had poor cross-reactivity with Roche OnLine reagent by kinetic interaction of microparticles in solution (KIMS). Figure 2 shows that 7-aminoflunitrazepam gave less than 40% signal intensity at 200 ng/mL and did not reach the cutoff value at 1000 ng/mL. Each point is the average of four separate measurements. The 85 cases were then classified in three groups (Table IV). POS were samples with an immunoassay value above the cutoff value of 1000 given by the 100-ng/mL nordiazepam calibrator. POS/NEG were samples with immunoassay values above 400 but below the cutoff value of 1000. And NEG were samples with immunoassay values below 400. Thirty-seven out of 85 samples, or 44%, would normally be reported as “negative” (POS/NEG and NEG groups) based on screening alone and would not have been further confirmed by GC–MS. Lowering the cutoff value to 400 resulted in GC–MS confirmation of 7-aminoflunitrazepam in 30 out of 37 “negative” samples (POS/NEG group), leaving seven samples (8%) with immunoassay readings below 400 (NEG group).

Patterns of flunitrazepam use in Miami-Dade County DUI

Analysis of 1407 DUI urines was performed during the period from January 1995 through June 1998. Marijuana was confirmed in 45.2% of these samples (range 40.9% in 1997 to 47.4% in 1996) by the presence of the major urinary metabolite, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THCCOOH). Most THCCOOH positive samples did not contain cocaine (52.1%). Cocaine was confirmed in 35.8%, steadily declining from 38.6% in 1995 to 27.4% in 1998. Most cocaine cases were also confirmed for THCCOOH (59.6%). Opiates were confirmed in 5.6%, steadily rising from 5.1% in 1995 to 8.9% in 1998. Benzodiazepines were confirmed in 14.4% of these samples, rising from 10.9% in 1995 to 18.6% in 1998. Flunitrazepam was present in 86.2% of all benzodiazepine cases in 1995, and was the main benzodiazepine found in urine samples from drivers suspected of DUI in Miami-Dade County for two consecutive years (Figure 3). A sharp decline in flunitrazepam positive samples occurred in 1997 and 1998, with 7.1% and 14.3% of all benzodiazepine cases being confirmed for the presence of the urinary metabolite 7-aminoflunitrazepam, respectively (Figure 3). As flunitrazepam cases dropped, clonazepam cases increased (Figure 4). Clonazepam use was based on the confirmation and quantitation by GC–MS of the major urinary metabolite 7-aminoclonazepam. Clonazepam was seldom confirmed in urine samples from DUI-suspected drivers. Only one urine was positive for 7-aminoclonazepam in 1995 (out of 58 benzodiazepine cases), followed by 5 out of 67 in 1996. Clonazepam was then confirmed with an increasing frequency in 1997, representing 12.5% of all benzodiazepine cases and reached an alarming 23.8% of benzodiazepine cases in the first six months of 1998.

In 1995 and 1996, 7-aminoflunitrazepam was found as the only benzodiazepine confirmed in 81 out of 90 flunitrazepam cases (90%) and represented 72% of all benzodiazepine cases (90 of 125) (Figure 5A). Only 9 of 90 (10%) flunitrazepam cases were positive and confirmed for other benzodiazepines. Patterns of use of flunitrazepam with respect to other drugs of abuse during the same two-year period are presented in Figure 5B. 7-Aminoflunitrazepam was most often found in combination with cocaine and marijuana (36.7%). It was also found with THCCOOH alone (33.3%). In 22.2% of the cases, 7-aminoflunitrazepam was the only drug detected, and few cases showed combination with cocaine alone (7.8%).

In 1997 and 1998, 7-aminoflunitrazepam was the only benzodiazepine confirmed in 5 out of 77 benzodiazepine cases (6.1%) and only represented 9% of all benzodiazepine cases (7 of 77) (Figure 6A). Of flunitrazepam cases, 29% (2 of 7) were confirmed for other prescription benzodiazepines. Patterns of use of flunitrazepam with cocaine and cannabis during the same 18-month period are shown in Figure 6B. 7-Aminoflunitrazepam was most often found
in combination with cocaine and marijuana (42.9%). It was also found with THCCOOH alone (42.9%) and was never the only drug detected. Only one case was combined with cocaine alone.

### Miami-Dade Police Department Drug Influence Evaluation (DRE)

A DRE examination is authorized by departmental policy when blood-alcohol concentrations (BAC) are below the legal cutoff of 0.08 g/dL, as determined by breath-alcohol testing, and drug use is suspected as a cause of impaired driving. BAC data collected in 1998 from 87 DUI suspects are shown in Figure 7: 32 cases (37%) were negative for alcohol, 55 had BAC averaging 0.05 ± 0.02 g/dL, with a median of 0.05 g/dL. DRE examination of drivers found to be under the influence of flunitrazepam revealed classical CNS-depressant impairment. Coordination was poor in all cases as evidenced by the failure to perform standardized roadside tests such as the Romberg balance test and finger-to-nose, one-leg-stand, and walk-and-turn tests. Speech was generally slurred, and eye examination typically revealed bloodshot, watery eyes, most often mydriasis, and the presence of a horizontal gaze nystagmus.

### Discussion

The need for a GC–MS method that allows confirmation and quantitation of the most common benzodiazepines on the

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**Figure 3.** Percentage of DUI urine samples confirmed for 7-aminoflunitrazepam out of all benzodiazepine-positive cases 1995–1998.

**Figure 4.** Percentage of DUI urine samples confirmed for 7-aminoclonazepam out of all benzodiazepine-positive cases 1995–1998.

**Figure 5.** Flunitrazepam patterns of use in 1995 and 1996: relationship with other benzodiazepines (A) and with other drugs of abuse (B). Numbers in the diagrams represent the actual number of cases. Abbreviations: 7-AF, 7-aminoflunitrazepam; BZD, benzodiazepines; COC, cocaine; THC, cannabis.
market, their major metabolites, in particular 7-aminoflunitrazepam, the major urinary metabolite of the illegal drug flunitrazepam, was the impetus for this work. A large body of literature on analytical methods is available for the determination of benzodiazepines in biological samples. Many of the earlier methods lacked sensitivity and failed to identify which drug was ingested or were directed at the confirmation/quantitation of one or two benzodiazepines and would not be useful for forensic purposes when multiple benzodiazepines may be present in a specimen. Benzodiazepines have been analyzed using polarography (21), immunoassays (22,23), thin-layer chromatography (TLC) (24), high-performance liquid chromatography coupled with UV detection (25,26) or fluorimetry (27), electron capture gas–liquid chromatography (28), and GC–MS (29–32). Recently, the use of flunitrazepam has been confirmed indirectly by conversion of the drug and its main metabolites to corresponding benzophenones, increasing dramatically the sensitivity for detection of the illegal drug (33,34). Because multiple benzodiazepines are often found in DUI-related cases, and a number of drugs can be associated with sexual battery, a GC–MS confirmatory method that screens the main available benzodiazepines and metabolites was adopted. DUI or sexual-battery case urine samples analyzed often contained several benzodiazepines.

The enzymatic hydrolysis of the glucuronides was chosen over an acid hydrolysis for various reasons, including cleanliness of the extracts, generation of the parent compounds (lorazepam, oxazepam, and temazepam), and possibility of measuring the hydroxy metabolites of the prevalent drugs alprazolam and triazolam at the same time. Acid hydrolysis leads to the formation of benzophenones. Several benzodiazepines of interest, such as nordiazepam, chlordiazepoxide, and oxazepam share common benzophenones (35). In addition, because one of the goals was also to quantitate the metabolite 7-aminoflunitrazepam in order to have a database of levels found in DUI and also to compare with Roche OnLine immunoassay values, formation of benzophenones was inadequate. Other metabolites of the drug give the same benzophenone, making it impossible to track the single metabolite 7-aminoflunitrazepam. Other authors have used enzymatic hydrolysis of the samples using \textit{H. pomatia} or \textit{P. vulgaris} 13-glucuronidase treatment (36,37). These enzymes require an acid medium to work (pH 4–5). Glass et al. (37) also showed that incubation at 60°C degraded oxazepam and temazepam during hydrolysis. \textit{E. coli}–IA 13-glucuronidase was therefore chosen so that hydrolysis would be achieved under mild conditions (37°C and pH 6.8).

The solvent used for the extraction of the 13 benzodiazepines and metabolites was based on the method published by Lillsunde and Seppala (38). The authors had used a mix of n-hexane/methylene chloride (70:30) to extract more than 20 benzodiazepines. Modification to a ratio of 40:30 allowed better extraction recoveries for hydroxyalprazolam and hydroxytriazolam.

Derivatization of benzodiazepines allows for better chromatography and improved sensitivity because of the large ion fragment masses obtained, reducing matrix interferences. \textit{t}-Butyldimethylsilyl derivatives are stable, do not require cleanup procedures, and can be directly injected into the capillary column. The detection of 7-amino metabolites of flunitrazepam and clonazepam was substantially improved by taking advantage of the high injector temperature and causing the analytes to flash-derivatize as they were injected onto the column in the splitless mode. Another advantage is a good stability of the extracts in the derivatized form. Standard curves were reproducible after one week of standing at room temperature (data not shown), allowing...
for re-injection of samples the next day or within three days of extraction (weekends) with no risk of loss or degradation of the analyte. Additionally, flash derivatization decreases sample preparation time.

A forensic toxicology laboratory will typically submit urine samples to a screening assay, such as an immunoassay, and/or a basic drug screen by GC–NPD or GC–MS. Immunoassays are designed to detect drugs or their metabolites at or above a given threshold determined by the cutoff calibrator. Any presumptively positive sample must then undergo confirmation, and preferably quantitation, using highly sensitive and specific techniques such as GC–MS. In a recent publication, Verstraete et al. (39) clearly showed that nonchromatographic techniques, including radioreceptor assay, can discriminate between positive and negative samples, but cannot reliably determine therapeutic versus toxic concentrations of benzodiazepines. The results from benzodiazepine screening and confirmation assays must be interpreted carefully. Other drugs may interfere with the results and yield false-negative results, such as the nonsteroidal anti-inflammatory drug oxaprozin (40). The parent benzodiazepines and their metabolites produce a unique cross-reactivity profile with the immunoassay antibody. OnLine reagent for the screening of benzodiazepines is unreliable for the detection of 7-aminoflunitrazepam. The assay failed to give a positive screening value in 44% of the cases (n = 85). This agrees with Beck et al. (41), who showed that Emet and FPIA gave false negatives for flunitrazepam in particular, but also for chlordiazepoxide, lorazepam, nitrazepam, and triazolam. The lack of correlation between the immunoassay and GC–MS confirmation of 7-aminoflunitrazepam most likely results from different cross-reactivities of the various compounds with the antibody. Flunitrazepam and 7-aminoflunitrazepam have 25 and 33% cross-reactivity to this antibody, respectively, according to the manufacturer's package insert. A screening value below the cutoff calibrator is not an indication of the absence of this metabolite in the sample. Lowering the cutoff calibrator concentration helps improve performance (42). However this may result in an increase in false positives. Another approach has been to use two separate immunoassay screening techniques (43). Although not suggested for routine use, this would double the cost of the screening, and some automated clinical chemistry analyzers may not allow for the use of different reagents. The decision was not to lower the concentration of the cutoff calibrator, but rather to consider GC–MS analysis of samples giving immunoassay values below that of the cutoff calibrator but well above that of a certified negative. Lowering the value from 1000 to above 399, allowed GC–MS confirmation of 92% more flunitrazepam cases, but 8% of these cases were still missed. Currently, GC–MS, although laborious and costly, remains the best method to detect and quantify 7-aminoflunitrazepam. Benzodiazepines are widely used as anxiolytic or sedative preparations and are present in close to 20% of all positive DUI urine samples processed in Miami-Dade County, FL over the past two years. Interestingly, this figure is much higher than the annual prevalence of use of benzodiazepines in the general U.S. population: below 10% and declining (44). This discrepancy is an indication of the degree of impairment induced by these drugs in a driver, as illustrated with the DRE data. The importance of low concentrations of alcohol is not to be underestimated because of additive pharmacological effects with other drugs. The presence of other drugs and alcohol in most flunitrazepam cases confounds conclusions pertaining to the use of flunitrazepam alone. In particular, the measurement of vital signs yielded variable results, although blood pressure was most often found to be lower than normal, and was generally compensated by an increase in heart rate (baroreceptor reflex). However, in situations where a DRE officer was available, the battery of tests performed showed a clear picture of CNS depression. Benzodiazepines induce both psychomotor and behavioral impairment at therapeutic dosage (45,46) and are associated with a higher risk for road-traffic accidents (3,4). Notably, flunitrazepam was the only benzodiazepine present in the urine sample in most of the cases. In contrast, more than one commercially available benzodiazepine is often confirmed in one urine sample. A possible explanation is the illegal nature of flunitrazepam abuse versus the possible therapeutic use of more than one benzodiazepine preparation for sedative or anxiolytic purposes. Benzodiazepines are often misused by drug addicts (47). The abuse liability of flunitrazepam seems higher than that of other benzodiazepines in South Florida. Personal communications with Miami Beach police users reveal that the feelings of euphoria occur without the overwhelming sedative effect commonly experienced after ingestion of other benzodiazepines. In agreement with this statement, a recent study in healthy male volunteers looked at subjective rating scales as well as psychophysical performance tests under the influence of flunitrazepam or triazolam (48). Flunitrazepam caused significant increase in "liking" scores and pleasurable subjective feelings, whereas triazolam did not. Flunitrazepam is classically associated with heroin addiction in European countries (49). In a study in morphine-dependent mice, withdrawal was attenuated by administration of flunitrazepam (50), suggesting neuropharmacological bases for the combination of flunitrazepam and opiates. This may explain the abuse liability of flunitrazepam reported in opiate addicts (51,52). In all flunitrazepam cases analyzed, no combination of the drug with heroin was found. THC metabolites or THC metabolites and cocaine are most often detected with flunitrazepam. Flunitrazepam was the single most commonly detected benzodiazepine, far surpassing diazepam and alprazolam (22.0 and 21.5%, respectively) in DUI urine samples for two consecutive years. A sharp decline (90.2%) in flunitrazepam samples was observed in 1997 compared to the previous two-year period, while a gradual increase in clonazepam-positive samples was observed during the same period of time. This increase is nearly exponential, having doubled each year (1.7%, 7.5%, 12.5%, and 23.8% in 1995, 1996, 1997, and 1998, respectively). Clonazepam might be sold as a flunitrazepam substitute on the street and could emerge as a new "Rochnal" for South Florida. The change in patterns of drug use coincides with the reclassification of flunitrazepam from Schedule IV to Schedule I. However, the use of benzodiazepines remains very prevalent in Miami-Dade County.

Conclusions

A GC–MS procedure to confirm presumptive positive benzodi-
azepine DUI urine samples screened by Roche OnLine immunoassay was developed. This method is simple, rapid and robust, produces forensically acceptable results, and requires a minimal sample volume. Domestic seizures of benzodiazepines are on the increase and their popularity is growing. Further studies are required to confirm the decrease in flunitrazepam use. DUI urine samples give biased demographic data about the abuse of flunitrazepam because a certain degree of impairment and driving are two reasons for intercepting the sample. Heightened awareness and law enforcement might contribute to the decrease in flunitrazepam use in drivers. Specimens from toxicology screens at the site of employment or in schools and from hospital admission would address more generally the trends in flunitrazepam abuse and identify the segments of population targeted. The authors recommend routine monitoring of the clonazepam metabolite 7-aminoclonazepam. This would identify if the recent increase in confirmation of its use is limited to South Florida and to DUI cases.

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References


