A Sensitive Immunoassay for Flunitrazepam and Metabolites

K. Walshe¹, A.M. Barrett¹, P.V. Kavanagh², S.M. McNamara², C. Moran³, and A.G. Shattock¹,*

¹Department of Medical Microbiology, University College, Dublin, Ireland; ²Department of Pharmacology and Therapeutics, Trinity College, Dublin, Ireland; and ³Drug Treatment Centre, Pearse Street, Dublin, Ireland

Abstract

The objective of this study was to develop an immunoassay that would be capable of detecting flunitrazepam and/or cross-reacting metabolites in urine and comparing the results with those obtained by gas chromatography–mass spectrometry. Doses of Rohypnol™ varying between 0.5 and 4 mg were given to volunteers, and urine was collected for up to two weeks postingestion. These samples were analyzed by an ELISA that was developed using an antibody raised to flunitrazepam and a drug-enzyme conjugate prepared by attaching 7-aminoflunitrazepam to horseradish peroxidase. Significant levels of flunitrazepam and/or cross-reacting metabolites were detected in urine for up to one week after ingestion. The immunoassay is selective with only diazepam cross-reacting at a level of 1000 pg/L.

Introduction

Flunitrazepam, Rohypnol, is a low-dose benzodiazepine used primarily to relieve insomnia. Although illegal in the United States, it is commercially available in a number of European countries. Once ingested, it is extensively converted to a series of metabolites, 7-aminoflunitrazepam being the major one. With current methods of analysis, it is rarely detected in urine after 72 h, and many immunoassays even fail to detect the drug or its metabolites within this timeframe (1).

Abuse of flunitrazepam is widely accepted within the drug-taking community (2). In particular, it is known to be used in drug co-abuse (3), often in combination with heroin or cocaine; it is also increasingly implicated in date rapes (4). Methadone-maintenance programs require regular screening of participants for a range of drugs, and although members of the medical profession suspect that many participants take flunitrazepam, they are frustrated by the inability of existing commercial tests to detect the drug.

Recently, five such kits were evaluated in the laboratory for their ability to detect both flunitrazepam and its major metabolite, 7-aminoflunitrazepam, in urine at levels between 5 and 1000 µg/L (5). The results clearly demonstrated that the drug could not be detected at the levels claimed by the manufacturer. In addition, analysis of urine samples from volunteers who had taken 1–3-mg oral doses of flunitrazepam displayed mostly negative responses. With low-dose misuse, particularly in cases of date rape, it is clear that the drug would not be detected by existing screening tests.

In order to address this problem, a sensitive ELISA that will detect flunitrazepam and 7-aminoflunitrazepam at levels lower than 5 ng/mL in urine for up to 10 days after ingestion was developed.

Materials and Methods

Materials

All benzodiazepines used in this study were obtained from Sigma Chemical Co. unless otherwise stated. 3-Hydroxyflunitrazepam and des-methylflunitrazepam were donated by Roche. 7-Aminoflunitrazepam (6) and 7-acetimidoflunitrazepam (unpublished) were synthesized.

Biological samples

Oral doses of flunitrazepam (0.5, 1, 3, or 4 mg) were administered in the form of 1 mg Rohypnol tablets to healthy volunteers (50–95 kg) with no history of drug or alcohol abuse. Urine samples were collected prior to ingestion and at regular intervals afterward.

Standards

Stock solutions of 7-aminoflunitrazepam were prepared in methanol (1000 mg/L). Working standards at 5, 25, 50, 100, 200, 400, 600, 800, and 1000 µg/L were made using drug-free urine. A linear correlation was established between optical density and log of the standards concentration, and a corresponding least-squares equation was then used to determine concentrations relative to 7-aminoflunitrazepam. For biological
samples, the amount of drug detected in urine is presented in this paper as apparent concentration relative to a 7-aminoflunitrazepam standard.

Cross-reactivity
The cross-reactivity of 13 benzodiazepines and 5 known metabolites of flunitrazepam, each at 1000 µg/L in drug-free urine, was investigated with the in-house ELISA.

EMIT™ immunoassay
The EMIT d.a.u. assay (Syva Co., San Jose, CA) was performed on a Roche Cobas Mira analyzer according to manufacturer’s specifications using internal oxazepam cutoff calibrators at 200 µg/L. All samples collected from volunteers were analyzed in the same assay to avoid interassay variation. Similarly, standards, potentially cross-reactive compounds, and all volunteer samples were analyzed in the same assay.

Gas chromatography–mass spectrometry (GC–MS)
The presence of 7-aminoflunitrazepam in the urine samples was confirmed by GC–MS (7). Briefly, this involved the addition of an internal standard, 7-amino-1-methylclonazepam, to urine, solvent extraction, and derivatization to give a trifluoroacetyl derivative. Quantitation was performed using m/z 379 for 7-aminoflunitrazepam and 395 for the internal standard.

Enzyme drug conjugate for in-house ELISA
7-Aminoflunitrazepam (2 mg) and succinic anhydride (2 mg) were dissolved in 200 µL of methanol, mixed, and allowed to stand at room temperature for 2 h. The mixture was evaporated to dryness with air. After the addition of 1 mL of 0.1M sodium hydroxide, the pH was adjusted to 7.5 with 0.1M hydrochloric acid. The conjugation was started by the addition of an aqueous solution of horseradish peroxidase (0.5 mL at 10 g/L) and EDC hydrochloride solution (0.5 mL at 10 g/L). The reagents were gently mixed and allowed to react overnight at room temperature. The conjugate was purified by Sephadex G25 chromatography and eluted with phosphate-buffered saline (PBS) pH 7.5.

In-house ELISA
Ninety-six-well microtitre plates (Nunc Maxisorb, Roskilde, Denmark) were coated with sheep antiflunitrazepam (Serotec Ltd., Oxford, U.K.). One hundred microliters of a 2-mg/L solution in 10mM carbonate buffer (pH 9.6) was added to each well, and the plate was left overnight at 4°C. Following washing with PBS containing 0.5% Tween-20 (PBST), wells were blocked with 200 µL of 1% bovine serum albumin (BSA) in PBST. All plates were freshly prepared before use. Optimum conjugate concentration was obtained by checkerboard titration. Conjugate was diluted in 1% BSA in PBST.

Fifty microliters of the test sample was added in duplicate to the wells, followed by 50 µL of optimally diluted conjugate and incubated for 1 h at 37°C. After washing, 100 µL of tetramethylbenzidine substrate was added to each well for 20 min before stopping the reaction with 50 µL of 2M sulfuric acid. The absorbance was read on a dual wavelength (450/620 nm) spectrophotometer (SLT, Spectra).

Results
A typical standard curve is presented (Figure 1). Using 1-25 (the concentration of drug that reduces the OD of the zero control by 25%) as an indicator of analytical sensitivity, the lowest level of 7-aminoflunitrazepam that could be reliably detected was shown to be 5 µg/L.

All the samples collected from volunteers who had taken 0.5 to 4 mg of flunitrazepam orally were screened by the EMIT d.a.u., using 200-µg/L cutoff calibrator and found to be negative. In contrast, significant levels of flunitrazepam and/or

![Figure 1. Calibration curve for 7-aminoflunitrazepam.](https://academic.oup.com/jat/article-abstract/24/4/296/885337)

![Figure 2. Urinary excretion profile of flunitrazepam taken by volunteers. Results are expressed as B/Bo relative to the time the sample was collected. (●) 0.5-mg dose; (■) 1.0-mg dose; (○) 3.0-mg dose; and (□) 4.0-mg dose.](https://academic.oup.com/jat/article-abstract/24/4/296/885337)

| Table 1. Cross-Reactivity Data for Metabolites by In-House ELISA |
|-------------------|-----------------|
| Metabolite       | Percentage cross-reactivity* |
| 7-Amino flunitrazepam | 100             |
| 7-Acetimido-flunitrazepam | 116.8          |
| Desmethyl-flunitrazepam | 98             |
| 3-Hydroxy-flunitrazepam | 0.6            |
| Flunitrazepam     | 56.8            |

* Percentage cross-reactivity was calculated as the concentration recovered by in-house ELISA divided by the initial concentration (1000 µg/L) multiplied by 100.
cross-reacting metabolites were detected by the ELISA described here (Figure 2). At the 0.5-mg dose, flunitrazepam and/or cross-reacting metabolites were detected for up to 70 h after ingestion. With the 1-mg dose, low levels of flunitrazepam and/or cross-reacting metabolites were still seen one week after sample collection (data not shown). The 3- and 4-mg doses were clearly detectable up to 72 h and one week, respectively.

Cross-reactivity

Two groups of compounds were analyzed for cross-reactivity. The first group included five known metabolites of flunitrazepam in addition to 7-aminoflunitrazepam (Table I). The second group contained a range of benzodiazepines and metabolites (Table II). Solutions at 1000 ng/mL were assayed by in-house ELISA in parallel with the parent compound, 7-aminoflunitrazepam, 7-acetamidoflunitrazepam, and desmethyllumitrazepam, which were all detected at approximately equal sensitivity. In contrast, 3-hydroxyflunitrazepam was less than 1% cross-reactive.

Of the 13 other benzodiazepines tested, diazepam was the only one that demonstrated any major degree of cross-reactivity; the remainder had negligible reaction (Table II).

**Table II. Cross-Reactivity Data by In-House ELISA for a Range of Commercially Available Benzodiazepines**

<table>
<thead>
<tr>
<th>Benzodiazepine</th>
<th>Percentage cross-reactivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lorazepam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Flurazepam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Desalkylflurazepam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Temazepam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Diazepam</td>
<td>27.45</td>
</tr>
<tr>
<td>Demoxepam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Nitrazepam</td>
<td>3.22</td>
</tr>
<tr>
<td>7-Aminonitrazepam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Oxazepam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Alprazolam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Triazolam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>&lt; 0.1%</td>
</tr>
<tr>
<td>Nordiazepam</td>
<td>&lt; 0.1%</td>
</tr>
</tbody>
</table>

* Percentage cross-reactivity was calculated as the concentration recovered by in-house ELISA divided by the initial concentration (1000 µg/L) multiplied by 100.

**GC–MS analysis**

7-Aminoflunitrazepam was confirmed by GC–MS. The results reflected the trend observed with the in-house ELISA (Figures 3A–3D).

**Discussion**

It has long been recognized that existing immunoassay tests designed to detect a broad range of benzodiazepines in many cases fail to identify samples that are genuinely positive (1,2,8). This is particularly relevant in the case of drug abusers on methadone-maintenance programs who are required to conform with strict criteria in order to continue with treatment. Many addicts are aware that detection of flunitrazepam in urine is difficult and continue to take the drug with the knowledge that, in all probability, it will not be detected during the routine screening process. Detection of flunitrazepam is also essential in alleged cases of date rape.

The purpose of this investigation was to develop a sensitive screening assay for the detection of flunitrazepam in urine. This was achieved by using an antibody that recognized predominantly 7-aminoflunitrazepam, the major metabolite of flunitrazepam. Significant quantities of flunitrazepam and/or cross-reacting metabolites were detected in urine samples for up to one week after ingestion of normal therapeutic doses. In contrast, the EMIT d.a.u. assay failed to detect the drug.

Results indicate that this laboratory's ELISA shows a very high selectivity for flunitrazepam and/or cross-reacting metabolites with only diazepam showing any degree of cross-reactivity. However, diazepam is extensively metabolized to nordiazepam, temazepam, and oxazepam with only traces of parent drug found in urine. All of these metabolites were evaluated and found to be non-cross-reactive. Although there may be some cases (e.g., overdose) where diazepam may cause a problem, samples are normally confirmed by another method with integrated laboratory protocols. Certainly, in cases of date rape, diazepam is highly unlikely to cause any problem.
One study suggested that the level of drug detected by immunoassay is generally higher than that found by GC–MS, as the latter is targeted to detected 7-aminoflunitrazepam, whereas the screening method can potentially detect numerous cross-reacting metabolites (9). Similarly, we have found that the apparent level of drug detected by this laboratory's ELISA was significantly higher than that indicated by GC–MS.

The new ELISA will be particularly useful as a screening assay to detect flunitrazepam misuse in date-rape situations. Even if presented within three days of exposure, most, if not all, commercial screening tests would fail to detect the drug.

Acknowledgments

The authors wish to thank B.M. Browne Ltd. for the use of the Cobas Mira clinical chemistry analyzer and Roche Diagnostics U.K. for their gift of the benzodiazepines. This work was funded by the Irish Science and Innovation Agency (Enterprise Ireland) under the Science and Technology Against Drugs Research Programme.

References


Manuscript received November 2, 1998; revision received October 15, 1999.