Identification of Urinary Benzodiazepines and their Metabolites: Comparison of Automated HPLC and GC–MS after Immunoassay Screening of Clinical Specimens

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Abstract

An automated high-performance liquid chromatographic method, benzodiazepines by REMEDI HS, was used to analyze benzodiazepines and their metabolites after β-glucuronidase hydrolysis of 1-mL urine specimens from the following: 924 clinic and hospital patients whose specimens had previously been found to be presumptively positive using either EMIT® or Triage® immunoassay methodologies and 128 individuals whose specimens had screened negative by EMIT d.a.u. REMEDI analyses did not correlate with the immunoassay results in 136 of the positive and three of the negative urine specimens. Gas chromatographic–mass spectrometric (GC–MS) confirmatory analyses were performed on these discordant specimens using 3 mL β-glucuronidase-hydrolyzed urine followed by extraction with chloroform–isopropanol (9:1) and derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide. Two benzodiazepines, flunitrazepam and clonazepam, and their 7-amino metabolites were analyzed without prior derivatization. The analyses established 87% concordance between REMEDI and GC–MS versus 13% concordance with immunoassay for the subset. GC–MS analysis of these 142 specimens demonstrated two reasons for the nonconcurrency between REMEDI and EMIT: EMIT had given either false-negative or false-positive results and EMIT had given a positive result even though the determined metabolites were below the 200-ng/mL cutoff for the immunoassay and the 80-ng/mL cutoff for REMEDI. A total of 23 specimens were found to contain only lorazepam by REMEDI and GC–MS, 15 of which had been screened by Triage. A reevaluation of these 23 specimens by EMIT d.a.u demonstrated that 11 were positive. This finding was in contrast to previous reports that EMIT will not detect lorazepam glucuronide in urine. An unexpected finding was the REMEDI identification and subsequent GC–MS confirmation of 7-aminoflunitrazepam, a urinary metabolite of flunitrazepam that is not available in the United States and that represented illicit use by four patients. A distinct advantage of REMEDI proved to be its capability in identifying demoxepam, a major metabolite of chlordiazepoxide; GC–MS analysis could not detect this metabolite because of its thermal decomposition to nordiazepam. To further evaluate the specificity of REMEDI, we conducted GC–MS analyses in a random fashion on 55 additional nondiscordant urine specimens that were identified as either positive or negative, as well as 22 specimens identified as containing 7-aminoclonazepam by REMEDI. Concurrence was observed between the two methods for all specimens, with the exception of one apparent false positive for α-hydroxylorazepam by REMEDI. The reproducibility of the REMEDI method was found to be excellent; it was assessed by comparing results of 266 specimens that were reprocessed in different batches and for known calibrators and controls also processed with each batch. Study results demonstrated that the automated REMEDI assay for urinary benzodiazepines and their metabolites was comparable with GC–MS but had distinct advantages over GC–MS because of the following reasons: simplicity of the assay, less time required for analyses, and provision of additional information concerning the parent benzodiazepine.

Introduction

Benzodiazepines are widely prescribed drugs in both the United States and other nations, and their availability depends on local regulation and, in some instances, illegal distribution. In the United States, three groups of benzodiazepines are available based on their chemical structures (1) (Figure 1), namely, 1,4-benzodiazepines, diazolobenzodiazepines, and triazolobenzodiazepines (Table 1). Identification of the administered benzodiazepine by analysis of a urine specimen is complicated by the fact that most of these drugs undergo extensive phase I and II metabolism and only minor amounts of the unchanged drug are excreted and by the fact that some of the 1,4-benzodiazepines have common metabolic pathways (Figure 2). Further complicating the analytical identification is the fact that many methodologies cannot distinguish between the various metabolites. Most laboratories use some type of immunoassay screen for detecting the presence of a benzodiazepine; examples are radioimmunoassay, enzyme multiplied immunoassay (EMIT®), fluorescence polarization immunoassay, latex agglutination (ON-Trac®), immobilized monoclonal antibodies (Triage®), or enzyme-linked immunosorbent assay (SINGLESTEP™).
Regardless of the screening technology used, many reports of false-positive and false-negative results for the benzodiazepines have appeared (2-10). Another commonly used screening technique, thin-layer chromatography (TLC), was reported to produce false-negative results for some benzodiazepines (11), and furthermore, the individual metabolites cannot be identified because conversion to aminobenzophenones is required for visualization. Therefore, confirmation of screening techniques is required to assist in developing a definitive clinical diagnosis or to assess forensic implications.

A number of confirmatory techniques are widely used for the benzodiazepines, such as high-performance liquid chromatography (HPLC) followed by UV detection (12,13), gas chromatography–mass spectrometry (GC–MS) (14-17), or gas chromatography–electron capture detection (GC–ECD) (18,19). The use of GC for the separation of the benzodiazepines is limited by the requirement for an elevated injection port temperature; the high temperature transforms a major metabolite of chlordiazepoxide, demoxepam, into nordiazepam, which is a major metabolite of a number of 1,4-benzodiazepines (20,21) (Figure 2). The decomposition of this N-oxide metabolite during either GC–MS or GC–ECD analysis renders the identification of chlordiazepoxide administration indistinguishable from other 1,4-benzodiazepines that also have nordiazepam as a metabolite (22). An additional complicating factor is that most benzodiazepines taken in therapeutic amounts are found in nanogram-per-milliliter concentrations in urine, which make it necessary to use electron impact (EI) MS with selected ion monitoring (SIM). Because of the wide variety of urinary parent benzodiazepines and their metabolites, it is often necessary to limit the number of analytes detected simultaneously using EI-MS with SIM because of the following reasons: (a) differences in chromatographic and thermal stability properties that require some analytes to be derivatized while others are analyzed without derivatization, (b) requirements for monitoring of multiple ions to ensure selectivity, (c) diverse polar and mass characteristics requiring different preanalytical work-up and chromatographic conditions, and (d) differences in the detection limits as a result of the varying concentrations found in urine because of the diverse dosages used to achieve a therapeutic effect.

Of the potential confirmatory methods, TLC and HPLC do not produce pyrolytic decomposition of the benzodiazepines. However, as already noted, TLC cannot distinguish between different members of the class if conversion to benzophenones is used before chromatography. HPLC was used by a number of workers to determine various benzodiazepines in blood (12,23-26) and urine (13,22,27) and can be used for identification of demoxepam (22); hence, it permits the assignment of chlordiazepoxide as the parent drug (Figure 2).

The REMEDi HS drug profiling system, an automated HPLC-based instrument, is widely used to identify basic and neutral drugs (28-31). A commercial kit (columns, reagents, and software) is now available for the analysis of urinary benzodiazepines and their metabolites. The utility of this technology was evaluated with urine specimens from a control group of specimens that had tested negative for benzodiazepines. GC–MS analyses were performed on all specimens whose results were disparate between the immunoassay and REMEDi methods. A representative number of nondiscordant specimens that had tested either positive or negative for various benzodiazepines and their metabolites were randomly chosen and also tested by GC–MS to further determine the specificity of the REMEDi method.

**Experimental**

*Chemicals and reagents.* Acetone, ethyl acetate, sulfuric acid, potassium dichromate, sodium acetate trihydrate, and sodium bicarbonate were obtained from Mallinckrodt. Nordiazepam, oxazepam, oxazepam-d₅, diazepam-d₆, lorazepam, temazepam, 2-hydroxyethylflurazepam, α-hydroxyalprazolam,
α-hydroxytriazolam, clonazepam, 7-aminoclonazepam, flunitrazepam, and 7-aminoflunitrazepam were obtained from Radian as methanolic solutions containing either 1 mg/mL or 100 μg/mL. The following benzodiazepines were supplied by Roche Pharmaceuticals: midazolam, α-hydroxymidazolam, and 4-hydroxymidazolam. Chloroform, isopropanol, and methanol were obtained from Burdick & Jackson, and N,N-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce. Chloroform–isopropanol (9:1) was prepared as a volume-to-volume ratio solution. β-Glucuronidase, type H2, from *Helix pomatia* (89,400 units/mL) was from Sigma. The 50 mM sodium bicarbonate solution (pH 11) was prepared by dissolving 2.1 g NaHCO3 in 500 mL of reagent-grade water. The 2M sodium acetate solution (pH 4.8) was prepared by dissolving 68.05 g sodium acetate trihydrate in 250 mL of reagent-grade water.

**GC–MS internal standard solutions.** Two ampules of oxazepam-d5 each containing 100 μg/mL in methanol were transferred quantitatively to a 100-mL class A volumetric flask and diluted to volume with methanol to give a final concentration of 2 μg/mL. The solution was transferred to a 100-mL Repipeter® (Labindustries) fitted with a fixed 0.5-mL syringe, and the solution was stored at 5°C when not in use. The syringe delivered 0.5 ± 0.01 mL (1000 ng) of the internal standard solution. The diazepam-d5 internal standard solution was prepared in a similar manner.

**Urine specimens and initial immunoassay screening.** Urine specimens were obtained from four different medical centers in the United States and their primary urinary metabolites are listed in Table I.

### Table I. Names of the 1,4-Benzodiazepines, Diazolobenzodiazepines, and Triazolobenzodiazepines Available in the United States and Their Primary Urinary Metabolites

<table>
<thead>
<tr>
<th>Parent</th>
<th>Metabolite(s)</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R6</th>
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<tr>
<td>Chlordiazepoxide*</td>
<td></td>
<td>H</td>
<td>NHCH₃</td>
<td>H</td>
<td>→O*</td>
<td>CI</td>
<td>H</td>
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<tr>
<td>Norchlordiazepoxide</td>
<td>H</td>
<td>NH₂</td>
<td>H</td>
<td>→O*</td>
<td>CI</td>
<td>H</td>
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<tr>
<td>Demoxepam</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>→O*</td>
<td>CI</td>
<td>H</td>
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</tr>
<tr>
<td>Diazepam</td>
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<td>=O</td>
<td>H</td>
<td>-</td>
<td>CI</td>
<td>H</td>
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<tr>
<td>Nordiazepam</td>
<td>H</td>
<td>=O</td>
<td>H</td>
<td>-</td>
<td>CI</td>
<td>H</td>
<td></td>
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<tr>
<td>Oxazepam*</td>
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<td>=O</td>
<td>OH</td>
<td>-</td>
<td>CI</td>
<td>H</td>
<td></td>
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<td>Temazepam*</td>
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<td>=O</td>
<td>OH</td>
<td>-</td>
<td>CI</td>
<td>H</td>
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<tr>
<td>Prazepam</td>
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<td>=O</td>
<td>H</td>
<td>-</td>
<td>CI</td>
<td>H</td>
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<td>OH</td>
<td>-</td>
<td>CI</td>
<td>-</td>
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<td>Nordiazepam, oxazepam</td>
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<tr>
<td>Clorazepate</td>
<td>H</td>
<td>(OH)₂</td>
<td>CO₂H</td>
<td>-</td>
<td>CI</td>
<td>H</td>
<td></td>
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<tr>
<td>Lorazepam</td>
<td>H</td>
<td>=O</td>
<td>OH</td>
<td>-</td>
<td>CI</td>
<td>Cl</td>
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<tr>
<td>2-Hydroxyethylflurazepam</td>
<td>- (CH₂)₂NH(C₂H₅)₂</td>
<td>=O</td>
<td>H</td>
<td>-</td>
<td>CI</td>
<td>F</td>
<td></td>
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<tr>
<td>Desalkyflurazepam</td>
<td>H</td>
<td>=O</td>
<td>H</td>
<td>-</td>
<td>CI</td>
<td>F</td>
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<tr>
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<td>=O</td>
<td>OH</td>
<td>-</td>
<td>CI</td>
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<tr>
<td>Clonazepam</td>
<td>H</td>
<td>=O</td>
<td>H</td>
<td>-</td>
<td>NO₂</td>
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<tr>
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<td>H</td>
<td>=O</td>
<td>H</td>
<td>-</td>
<td>NH₂</td>
<td>Cl</td>
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<tr>
<td>7-Acetamidoclonazepam</td>
<td>H</td>
<td>=O</td>
<td>H</td>
<td>-</td>
<td>NH₂COCH₃</td>
<td>Cl</td>
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<td>* Bold name indicates availability as a prescription drug in the United States.</td>
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<td>α-Hydroxymidazolam</td>
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<td>H</td>
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<td>4-Hydroxymidazolam*</td>
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<td>OH</td>
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<td><strong>Triazolobenzodiazepines</strong></td>
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<td>Alprazolam</td>
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<td>H</td>
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<td>α-Hydroxyalprazolam</td>
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<td>Estazolam</td>
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<td>4-Hydroxyestazolam*</td>
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<td>OH</td>
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<tr>
<td>Triazolam</td>
<td>CH₃</td>
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<tr>
<td>α-Hydroxytriazolam</td>
<td>CH₃OH</td>
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<td>* Bold name indicates availability as a prescription drug in the United States.</td>
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<td>1 N-Oxide.</td>
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<td>2 Present predominantly as the 3-glucuronide.</td>
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<td>3 Also available as the parent drug. Metabolism is mainly as 3-glucuronide.</td>
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<td>4 A minor metabolite.</td>
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the Little Rock area (Arkansas Children's Hospital, Baptist Medical Center, and McClellan and Fort Roots Veterans Administration Hospitals). A total of 877 specimens had been screened by EMIT technology on a variety of clinical chemistry analyzers (Roche Monarch, Olympus REPLY, Beckman Synchron CX, and Syva ETS), whereas 37 had been evaluated with the Triage technology on specimens originating from the emergency department of each facility. The specimens were obtained from a variety of in- and out-patients whose age groups ranged from neonates to older adults. Additionally, 128 specimens submitted as part of our forensic urine drug-testing program and that had been screened negative for benzodiazepines by EMIT d.a.u.™ on the Syva ETS were also evaluated. All medical centers used the manufacturers' recommendations for analyses and a cutoff of 200 ng/mL. This study was approved and judged exempt from the requirement for informed consent by the University of Arkansas for Medical Sciences Human Research Advisory Committee, and specimens were supplied to the laboratory in a coded fashion.

Preparation of in-house calibrators and controls. The in-house urine calibrators and controls were prepared with urine collected from laboratory personnel. Each lot of urine collected was shown to be devoid of exogenous benzodiazepines by both EMIT d.a.u. and GC-MS screening and had 30 mg sodium azide added per 4 L urine. Calibrators were prepared by quantitatively transferring the contents of ampules, containing either 1 mg/mL (nordiazepam, oxazepam, lorazepam, temazepam; one ampule per drug) or 100 µg/mL (2-hydroxyethylflurazepam, α-hydroxymidazolam, and α-hydroxytriazolam; 10 ampules per drug), to a 1-L class A volumetric flask and diluting to volume with urine to give a final concentration of 1000 ng/mL. Subsequent dilutions of this stock were made with the urine to give, in addition to the 1000 ng/mL calibrator, other calibrators with concentrations of 500, 250, 125, 62.5, and 31.25 ng/mL. Additionally, another set of calibrators containing 1000, 500, 250, and 125 ng/mL of this same group of benzodiazepines but without 2-hydroxyethylflurazepam was prepared and used in REMEDI analyses. In a similar manner, two different sets of calibrators were made containing the former concentrations. One set contained midazolam, 4-hydroxymidazolam, and α-hydroxymidazolam, and the other contained clonazepam, 7-aminoclonazepam, flunitrazepam, and 7-aminoflunitrazepam. Controls were prepared in a similar manner on a different day and contained only oxazepam at

Figure 2. A schematic of the major metabolism of benzodiazepines. Those drugs contained within a box are available in the United States and are the parent administered compound. (Adapted from S.C. Harvey. Hypnotics and sedatives. In The Pharmacological Basis of Therapeutics, 7th ed. A.G. Gilman, L.S. Goodman, T.W. Rall, and F. Murad, Eds., Macmillan Publishing, New York, NY, 1985, p 347.)
concentrations of 0, 131, and 480 ng/mL. Each urine calibrator and control was pipetted as 3.5-mL portions into individual storage containers and frozen at -30°C until use. Each lot of calibrator and controls was validated by GC–MS with commercially available positive urine control samples containing nordiazepam (Curtin Matheson Scientific) and through interlaboratory comparisons in the College of American Pathologists forensic urine drug and toxicological surveys for nordiazepam, oxazepam, α-hydroxyalprazolam, and α-hydroxytriazolam.

**HPLC analysis of urine specimens.** A REMEDI HS drug profiling system was used for all analyses. The benzodiazepine analytical kit, consisting of three cartridges and five solvents for the liquid chromatographic separation, was supplied by the manufacturer (32). A beta version of the software (version 5.0) was also supplied for data analysis. All reagents were prepared according to the manufacturer's instructions. A 1-mL aliquot of the urine specimen had 100 μL each of buffered internal standard (triazolam and ethyl oxazepam glucuronide) and hydrolysis reagent added, after which it was mixed and incubated in an oven at 37°C for 2 h. The mixture was centrifuged at 5000 rpm for 5 min, and the supernatant was injected directly into the REMEDI system. Each day that specimens were processed, a set of in-house calibrators (125, 250, 500, and 1000 ng/mL) and controls (0, 131, and 480 ng/mL of oxazepam), prepared as already described, were analyzed. Also analyzed were a manufacturer-supplied Check Mix™ containing bromazepam, α-hydroxyalprazolam, oxazepam, lorazepam, and temazepam and a Limit Check™ containing 7-aminoclonazepam, 7-aminofluorazepam, demoxepam, α-hydroxyalprazolam, α-hydroxytriazolam, oxazepam, lorazepam, temazepam, and nordiazepam. Thus, a daily batch consisted of these calibrators and controls plus 41 urine specimens. The manufacturer’s claimed cutoff value for seven benzodiazepine metabolites, namely, oxazepam, temazepam, nordiazepam, lorazepam, α-hydroxyalprazolam, α-hydroxytriazolam, and hydroxyethylflurazepam, was 80 ng/mL; for the other metabolites, namely, demoxepam, 7-aminoclonazepam, and 7-aminofluorazepam, the cutoff ranged from 150 to 600 ng/mL.

**Analysis of urine specimens with GC–MS.** To 3 mL of urine were added 0.5 mL of either oxazepam-d₅ or diazepam-d₅ internal standard solution, 100 μL of 2M (pH 4.8) acetate buffer, and 50 μL β-glucuronidase followed by 5 s of vortex mixing and incubation for 2 h in a water bath at 55°C. This solution was then cooled to room temperature, followed by addition of 1 mL 50 mM sodium bicarbonate solution (pH 11) and 3 mL of chloroform–isopropanol (9:1), rotation on a hematologic mixer for 10 min, and centrifugation at 5°C and 2600 rpm for 10 min. The lower organic layer was removed and evaporated to dryness under nitrogen at 50°C. To the resultant residue were added 20 μL ethyl acetate and 30 μL BSTFA, after which the solution was vortex mixed and heated in a sand bath at 75°C for 15 min. For those specimens that were to be analyzed for clonazepam, 7-aminoclonazepam, flunitrazepam, 7-aminofluorazepam, and the diazepam-d₅ internal standard, 50 μL ethyl acetate was added, and the solution was vortex mixed. This solution was transferred to an autosampler vial for GC–MS analysis. An ethyl acetate blank was processed after each specimen analysis. Each batch processed during the study contained a set of six calibrators (31.25, 62.5, 125, 250, 500, and 1000 ng/mL) and three controls (0, 131, 480 ng/mL of oxazepam). The precision and accuracy in recovery of the two positive controls processed on each batch run gave mean (range) oxazepam concentrations of 124.5 (122.1–126.8) and 479.1 (473.5–484.7) ng/mL. A mean correlation coefficient of 0.95 or greater was determined for each calibrator set used in the batch analyses. If the determined concentration was greater than 1000 ng/mL, the specimen was appropriately diluted and reprocessed. The limit of quantitation was 40 ng/mL for oxazepam and lorazepam and 65 ng/mL for the other benzodiazepines. The limit of detection (LOD) was 10 ng/mL for all benzodiazepine metabolites except 2-hydroxyethylflurazepam, α-hydroxyalprazolam, α-hydroxytriazolam, α-hydroxymidazolam, 7-aminoclonazepam, and 7-aminofluorazepam, which had an LOD of 35 ng/mL.

**GC–MS instrumentation and chromatographic conditions.** All urine GC–MS analyses were performed on a Hewlett-Packard model 5972B positive ion electron impact quadrupole mass spectrometer interfaced with a model 5890 gas chromatograph, a model 7673 autosampler, and an HP Vectra 90 MHz Pentium computer using the Drug-Quant software (revision A.00.00) or a Hewlett-Packard model 5971A positive ion electron impact quadrupole mass spectrometer interfaced with a model 5890 gas chromatograph, a model 7673 autosampler, and a model HP Apollo 9000 series 400 Unix data station using Target™ software (revision C.02.02). The gas chromatographs were each equipped with a capillary column (12 m × 0.2-mm i.d., 0.33-μm film thickness) containing an HP-1 cross-linked methyl silicone gum and operated using a program with an initial temperature of 70°C (1 min) that was then increased to a final temperature of 290°C at a rate of 15°C/min; the final hold was 4.33 min (total run time, 20 min). This program was slightly modified for the analysis of midazolam and its metabolites, namely, a final temperature of 275°C was used (total run time, 19 min). The carrier gas was helium; the initial inlet pressure was 8.99 psi/min, followed by a program of 14.5 psi (0.01 min) and a final pressure of 9.0 psi (flow rate, 1.15 mL/min) for 20 min. The injector port was operated at 250°C and fitted with a Merlin septum (HP-5181-8839) and a glass mixing chamber assembly (HP-19251-60504) containing a 10-mm silane-treated glass wool plug (HP-5080-8764) that was 30 mm from the end next to the gold-plated seal (bottom injector plate). The glass mixing chamber assembly was changed daily and cleaned as previously reported by Valentine et al. (33). The gold-plated seal was changed at the time of column installation and ion source cleaning. The mass spectrometers were autotuned daily with perfluorotributylamine, were operated in the SIM mode with a 50 ms dwell time, and were set at 400 V greater than the multiplier value obtained in the daily tune. Specimens were analyzed monitoring the following retention times and ions (where q is the quantitative ion): nordiazepam, 12.6 min, m/z 343 (q), 342, and 343; oxazepam-d₅, 13.3 min, m/z 433 (q), 435, and 436; oxazepam, 13.3 min, m/z 429 (q), 430, and 431; lorazepam, 13.9 min, m/z 429 (q), 430, and 431; temazepam, 14.9 min, m/z 343 (q), 345, and 372; midazolam, 14.3 min, m/z 310 (q), 312, and 325;

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2-hydroxyethylflurazepam, 14.7 min, m/z 288 (q), 287, and 388; 4-hydroxymidazolam, 15.0 min, m/z 398 (q), 399, and 400; α-hydroxymidazolam, 15.2 min, m/z 310 (q), 398, and 413; α-hydroxyalprazolam, 16.6 min, m/z 381 (q), 383, and 396; α-hydroxytriazolam, 17.4 min, m/z 415 (q), 417, and 430; diazepam-d₅, 13.5 min, m/z 261 (q), 287, and 289; clonazepam, 15.7 min, m/z 314 (q), 315, and 280; 7-aminoclonazepam, 15.5 min, m/z 285 (q), 256, and 257; flunitrazepam, 14.8 min, m/z 312 (q), 285, and 286; and 7-aminoflunitrazepam, 14.6 min, m/z 283 (q), 255, and 254. A ratio of qualifier ions to quantitative ion of ±20% was used for all analyses. The autosampler was set to perform five purges of the syringe before drawing 2 μL for injection. A viscosity delay of 1 was used along with ten washes of ethyl acetate. Samples were injected in a splitless mode with a purge-on time of 2 min followed by a 20:1 split.

Results and Discussion

A total of 924 urine specimens that were presumptively positive for benzodiazepines using either the EMIT or Triage methodologies were each evaluated by REMEDi. Of this number, 788 (85.3%) specimens were found by REMEDi to contain one or more benzodiazepines or their metabolites, and 136 (14.7%) were found to be negative. This latter discordant group of specimens was submitted to GC–MS analysis. Fifty-six were found to be negative; 55 contained one or more benzodiazepines and their metabolites, each of which were below the 80-ng/mL cutoff specified by the manufacturer; eight specimens contained oxazepam above the 80-ng/mL cutoff (mean, 142.1 ng/mL; range 93.5–213.7 ng/mL); two specimens contained temazepam at levels of 138.6 and 183.7 ng/mL; and five specimens contained temazepam at levels greater than the 80-ng/mL cutoff but with values that appeared to be artifact because the qualifier ions were greater than the ±20% permitted by the procedure. Reanalysis of these latter specimens gave identical results and hinted that an interfering component not identified in the study was responsible for the result. Additionally, there was insufficient specimen to perform GC–MS confirmation on five specimens, and five specimens could not be satisfactorily analyzed by GC–MS because of failure to extract the internal standard. Reanalysis of these latter specimens, up to three additional times, gave identical results, which suggested that some endogenous constituent in the urine matrix was preventing the internal standard from being extracted or, alternately, was preventing satisfactory derivatization.

An additional 128 urine specimens that were negative for benzodiazepines using the EMIT d.a.u. methodology were also evaluated by REMEDi; only three specimens were found to be positive, containing 7-aminoclonazepam, α-hydroxyalprazolam, and demoxepam, respectively. These three specimens were also evaluated by GC–MS, and the REMEDi findings were validated by the identification of nordiazepam.

To further evaluate the findings of REMEDi, we performed three additional studies. First, 55 of the specimens that had been either positive or negative by immunoassay and con-
specimens containing α-hydroxymidazolam, as identified by both REMEDI and GC–MS, were negative by EMIT d.a.u., and this finding suggested that the benzodiazepine glucuronide-derived antibodies in the Triage device may be superior for detecting the presence of midazolam metabolites. Additional studies will be required to validate this initial finding.

REMEDi analysis identified the presence of demoxepam in 360 specimens and suggested that the patients had used chlordiazepoxide because demoxepam can only be found as a result of chlordiazepoxide metabolism (Figure 2). This finding was not unexpected because a number of our specimens were from patients in drug rehabilitation programs that routinely use chlordiazepoxide in withdrawal therapy. Clearly, REMEDI analysis was advantageous in permitting the determination of chlordiazepoxide use in these patients because GC–MS analysis could not determine the presence of demoxepam because of its pyrolysis to nordiazepam, a common intermediate in the metabolic pathway of many different 1,4-benzodiazepines (Figure 2). Other benzodiazepines and their metabolites were also found in combination with demoxepam and illustrate the commonality of pathway through nordiazepam as well as the prescribing practice of using combinations of benzodiazepines (e.g., chlordiazepoxide and lorazepam or chlordiazepoxide and alprazolam). Nordiazepam was found in less than 5% of the specimens that also contained demoxepam, which suggests that demoxepam is a principal urinary metabolite of chlordiazepoxide and therefore important in urine analyses of benzodiazepines. The presence of oxazepam along with demoxepam in the majority of these specimens also illustrates the additional metabolic pathways for chlordiazepoxide, namely, those passing through demoxepam, nordiazepam, and oxazepam. Figure 3 illustrates a typical HPLC chromatogram from a patient’s specimen in which chlordiazepoxide and its metabolites were identified.

Making an assignment of whether the patient has been administered diazepam, clorazepate, or prazepam by analysis of urine would be difficult because all three benzodiazepines pass through the common intermediate, nordiazepam. However, only diazepam can give rise to temazepam (Figure 2); therefore, identification of temazepam along with nordiazepam and oxazepam would suggest diazepam administration. Clorazepate is rapidly decarboxylated in the gastric juice of the gut to nordiazepam (35); therefore, no unchanged drug would be expected in the urine. Furthermore, if clorazepate is analyzed by GC–MS, it is quantitatively converted to nordiazepam in the injector port environment (data not shown). Thus, the finding of nordiazepam and oxazepam without temazepam suggests that either clorazepate or prazepam was the administered drug. Only one patient’s specimen was found by REMEDI to contain nordiazepam and oxazepam, as also confirmed by GC–MS, and suggested that, in our population of patients, prazepam and clorazepate were not widely used. Both diazepam and prazepam are excreted to a limited extent in urine, but REMEDI analysis did not detect either of these compounds in the urine specimens. Prazepam is known to be metabolized to 3-hydroxyprazepam, but this metabolite was not part of the REMEDI library evaluated in our study. Likewise, the GC–MS analyses used did not detect this metabolite or its parent drug or diazepam. Further studies will be required to demonstrate whether these additional metabolites and parent drugs can be identified in urine.

Based on our findings that less than 2% of all specimens analyzed by REMEDI or GC–MS contained either α-hydroxytriazolam or hydroxyethylflurazepam, the prescribing of triazolam and flurazepam appeared to be minor in our study population. However, the use of either of these drugs, as well as alprazolam or clonazepam, was easy to differentiate because each had unique and specific metabolites detectable by both REMEDI and GC–MS analyses.

Temazepam can be prescribed as a single drug entity, and, if this is the case, one would expect to identify both temazepam and oxazepam in the urine but not nordiazepam (Figure 2) because oxazepam is a metabolite of temazepam. Evaluations of the urine specimens in this study by REMEDI demonstrated that six specimens met this criteria; two of these specimens were also submitted to GC–MS and confirmed. Only one specimen was found that contained temazepam but not oxazepam and that was observed with lorazepam also present. Thus, these findings suggested that when temazepam is administered as the parent drug, both temazepam and oxazepam will be found in the urine. Additional

![Figure 3. A high-performance liquid chromatographic chromatogram from a patient illustrating the identification of chlordiazepoxide and its major metabolites in a urine specimen. In the chromatogram, peak 9 (2.0 min) is demoxepam, peak 11 (3.0 min) is oxazepam, peak 12 (3.6 min) is chlordiazepoxide, peak 13 (3.9 min) is the internal standard triazolam, and peak 16 (7.0 min) is the internal standard ethyl oxazepam (added as the glucuronide to the specimen).](https://academic.oup.com/jat/article-abstract/20/6/416/777609)
was the first substantiation of these reports. This benzodi-
we had reports that illicit flunitrazepam was in our area, this
was true in the case of demoxepam, which can arise only from
studies with patients receiving only temazepam would be
needed to verify this preliminary finding.

A surprising finding in our study was the presence of 7-
aminoflunitrazepam detected by REMEDI and confirmed by
GC–MS in four specimens. Although at the time of the study
we had reports that illicit flunitrazepam was in our area, this
was the first substantiation of these reports. This benzodi-
azepe is not approved for use in the United States but has re-
portedly come into this country via South America and Mexico,
where it is an approved drug.

Our evaluation of this new REMEDI methodology has sug-
gested that it could have utility in augmenting the identifi-
cation of the parent benzodiazepine administered to a patient
based on the urinary benzodiazepine metabolites found. This
was true in the case of demoxepam, which can arise only from
chlordiazepoxide administration and cannot be identified by
GC–MS analysis. The method also proved to be less complic-
cated when compared with the arduous procedures required to
perform GC–MS analyses. Even though both techniques
require an initial 2-h hydrolysis of the glucuronides, the
remaining instrumental part of the analysis is weighed heavily
in favor of REMEDI because the hydrolyzed urine, after a brief
centrifugation step, is placed directly on the instrument, and
its analysis requires only about 12 min before a result can be
obtained. This assumes that the instrument has been properly
calibrated for the day, which requires three additional sample
analyses, namely, Check Mix, Limit Check, and a negative con-
control. In contrast, GC–MS analysis requires approximately an
additional 2 h of preanalytical preparation, including liquid–
liquid extraction, centrifugation, evaporation of the organic
solvent extract, derivatization of most analytes, and an instru-
mental analysis time of 19–20 min as well as solvent blanks
between each specimen to prevent carryover. Again, as with
REMDI analysis, GC–MS analysis requires prior injection of
calibrators and controls. Nonautomated HPLC analysis would,
like GC–MS, require a preanalytical treatment of the spec-
imen and would also not offer the additional REMEDI advan-
tages of UV spectral matching with a library and computer
algorithm for compound identification based on two UV wave-
lengths and relative retention times using two internal stan-
dards, one of which is a glucuronide to control hydrolysis.

GC–MS analysis had the advantage in sensitivity for detec-
tion of the urine benzodiazepines and their metabolites. How-
ever, this could be gained only by analyzing 3 mL of urine
instead of 1 mL and by performing the analyses on relatively
new columns, with a new gold-plated bottom injector seal,
and with a freshly cleaned ion source. Without these adjust-
ments in the GC–MS methodology, we could not obtain re-
quisite sensitivity for most analytes, with the exceptions of
oxazepam and lorazepam, with the 62.5-ng/mL in-house cali-
brator. Thus, the sensitivity of the GC–MS method proved to be
highly dependent on the condition of the instrument, and
analyses reported here were performed only under these
optimal parameters. This means that typical working condi-
tions on a GC–MS instrument would preclude much better
sensitivity than the 80-ng/mL cutoff level claimed for REMEDI
by the manufacturer. The question that was unanswered by this
study was whether a cutoff level of 80 ng/mL was adequate to
detect the diazolo- and triazolobenzodiazepines in urine after
a therapeutic dose, which is typically only 5–10% of the dose
given for the 1,4-benzodiazepines. Because the majority of our
study was designed to analyze by REMEDI only those spec-
imens that were positive by immunoassay, some false-negative
specimens may have resulted from patients on low therapeutic
doses of the diazolo- and triazolobenzodiazepines. This was
reinforced by our finding of α-hydroxyalprazolam in one sup-
posedly immunoassay-negative urine specimen by REMEDI
and this same metabolite by GC–MS in a few specimens that
were negative by both REMEDI and immunoassay. Other
authors (6–8,36) reported that therapeutic doses of alprazolam
may produce urine levels below 80 ng/mL even though one
report claimed that EMIT was adequate to detect alprazolam
use in patients receiving the drug (8).

In our opinion, the REMEDI methodology should prove to be
useful in laboratories that routinely perform high volumes of
benzodiazepine confirmations of immunoassay screening results. The savings in time and labor cost as well as the addi-
tional information obtained concerning the identity of the
administered drug will lead laboratory directors to use their
GC–MS instruments for other assignments. Because of the
large numbers of false-positive and false-negative results
typically seen with the benzodiazepine immunoassay tests,
REMDI or nonautomated HPLC or GC–MS analysis should be
considered in critical specimens in which the screening results
are not consistent with clinical or other investigatory find-
ings. Further, if it is necessary to know whether chlo-
diazepoxide was the administered drug, then REMEDI analysis
or an equivalent HPLC analysis should be considered.

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References

2. O. Beck, P. Lafolie, G. Odelius, and L.O. Boreus. Immunological
   screening of benzodiazepines in urine: two techniques evaluated
3. O. Beck, P. Lafolie, P. Hjemdahl, S. Borg, G. Odelius, and
   P. Wirbing. Detection of benzodiazepine intake in therapeutic
   doses by immunoanalysis of urine: two techniques evaluated
4. O. Beck, P. Lafolie, and P. Hjemdahl. Modification of commercial


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