to perform and can be used for analysis of numerous samples within a short time.

References


Automated On-Line Hydrolysis of Benzodiazepines Improves Sensitivity of Urine Screening by a Homogeneous Enzyme Immunoassay, Jeri D. Ropero-Miller, Diana Garside, and Bruce A. Goldberger* (Univ. of Florida College of Med., Dept. of Pathol., Immunol., and Lab. Med., P.O. Box 100275, Gainesville, FL 32610-0275; *author for correspondence: fax 352-846-1586, e-mail goldberg.pathology@mail.health.ufl.edu)

Benzodiazepines are used therapeutically as antidepres-
sant, anxiolytic, anticonvulsant, hypnotic, muscle relax-
ant, and preanesthetic agents. Moreover, benzodiazepines
are often abused for their euphoric effects. Consequently,
benzodiazepines are commonly detected in toxicological
analyses.

Immunosassays of benzodiazepines are plagued by sev-
eral factors that make reliable detection difficult. Complic-
ated metabolic pathways, including N-dealkylation,
C-hydroxylation, and glucuronidation, effectively reduce
the parent compound to more polar, and thus easily
excreted metabolites. Some of these metabolites are phar-
macologically active; in some cases, they are present in
higher concentrations and have a longer elimination half-
life than the parent compound [1, 2]. Many, especially the
glucuronide conjugates, which appear at high concentra-
tions in the urine, do not readily cross-react with com-
cmercial immunoassays. In contrast, other benzodiazepine
metabolites demonstrate high cross-reactivity, depending
on the immunoassay utilized [3].

In the US, 13 benzodiazepines of various potencies are
commonly prescribed. Therapeutic daily doses have sub-
stantially decreased with the newer generation of benzo-
diazepines [2]. Variations in dosage and in ultimate
excretion patterns complicate the ability to accurately
detect benzodiazepine use [4].

Accumulated research findings indicate pretreating urine specimens with β-glucuronidase (EC 3.2.1.31) in-
creases the diagnostic sensitivity of benzodiazepine im-
munosassays. In addition, even untreated specimens with
concentrations of benzodiazepine analytes below the cutoff
concentration may screen positive because of the
presence of highly cross-reactive benzodiazepine meta-
bolites [3, 5]. Also, oxaprozin (Daypro®; G.D. Searle), a
structurally unrelated nonsteroidal antiinflammatory
drug, produces false-positive results in several commer-
cial immunoassays [6–8].

In this study we evaluated whether automated on-line
hydrolysis of benzodiazepine analytes would improve the
sensitivity of the Boehringer Mannheim Corp. (BMC) cloned
drug, produces false-positive results in several commer-
cial immunoassays [3–5].

All CEDIA presumptive positive specimens (n = 50) were
assayed by GC/MS. Before GC/MS analysis, the urine
specimens were hydrolyzed with β-glucuronidase (Sigma Chemical Co.), followed by solid-phase extraction
(Clean Screen® SPE columns, ZSDAU020; United Chem-
ical Technologies, Horsham, PA). The extracts were deria-
thized with methyl-(tert-butyldimethylsilyl)-trifluoroac-
etamide (Pierce Chemical Co.) for quantification of
nordiazepam, oxazepam, temazepam, lorazepam, hydroxytriazolam. All specimens that were negative by
the R1 reagent system (5 μL of β-glucuronidase solution per milliliter of R1) for automated on-line hydrolysis of conjug-
gated benzodiazepine analytes. (The modified CEDIA has
also been evaluated recently by Bellet et al. [9].) All speci-
mens were analyzed with a Hitachi 717 (BMC) automated
chemistry analyzer, with a positive cutoff concentration of
200 μg/L (nitrazepam). All instrument settings were accord-
ing to published manufacturer’s guidelines for the CEDIA
benzodiazepine assay.

All CEDIA presumptive positive specimens (n = 50)
were assayed by GC/MS. Before GC/MS analysis, the urine
specimens were hydrolyzed with β-glucuronidase (Sigma Chemical Co.), followed by solid-phase extraction
(Clean Screen® SPE columns, ZSDAU020; United Chem-
ical Technologies, Horsham, PA). The extracts were deria-
thized with methyl-(tert-butyldimethylsilyl)-trifluoroac-
etamide (Pierce Chemical Co.) for quantification of
nordiazepam, oxazepam, temazepam, lorazepam, hydroxytriazolam. All specimens that were negative by
GC/MS were subsequently reextracted and derivatized with
N,O-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.) for quantification of 7-aminoclonazepam,
7-aminoflunitrazepam, and 7-aminonitrazepam.

A Hewlett-Packard (HP) 5890 Series II gas chromatograph
and HP 7673 automated liquid sampler interfaced to an HP
5972A mass-selective detector (MSD) were utilized. The gas
chromatograph was equipped with a cross-linked 95% di-
methyl/5% diphenyl polysiloxane capillary column [HP-
5MS; 30 m × 0.25 mm (i.d.) × 0.10-μm-thick film]. Automa-
ted injections were made in the splitless mode. The MSD
was operated in the selected-ion monitoring mode with
mass ions monitored at a dwell time of 20 ms. A multipoint calibration curve (25–500 μg/L) and pentadeterated internal standards (Radian Corp., Austin, TX) were used for quantification; i.e., quantification was based on the ion peak area ratio of the analyte to its corresponding pentadeterated internal standard analog. The limit of quantification (LOQ) was established as 25 μg/L. Analytes were identified on the basis of retention time (±1%) and ion ratios (±20%) by comparison with the corresponding values for the internal standards.

Manual pretreatment with enzyme before immunoassay analysis can be very time consuming. By using \( \beta \)-glucuronidase added to the R1 reagent of the CEDIA benzodiazepine assay and subsequent on-line incubation with the urine specimen, conjugated benzodiazepines were cleaved to yield more immunoreactive analytes. Compared with the current CEDIA, the modified CEDIA demonstrated a 26% increase in the number of presumptive positive results, from 37 to 50 specimens. Furthermore, all benzodiazepine-positive specimens demonstrated an increase in \( \Delta \) absorbance rates (mA/min) with the modified assay, the increase ranging from 10 to 269 (mean, 72.56; SD, 70.19).

Results of the GC/MS analyses for the specimens that were negative by the current CEDIA, but positive by the modified CEDIA, are listed in Table 1. The data demonstrate that \( \beta \)-glucuronidase pretreatment improves immunoassay detection of several benzodiazepine analytes, even at concentrations less than the cutoff calibrator. Benzodiazepine analytes for which detection was improved include lorazepam, nordiazepam, oxazepam, and temazepam. Of the 50 total specimens, 3 could not be confirmed by the chosen GC/MS assays and were subsequently submitted to BMC for analysis by alternative GC/MS methods. Two of these specimens were found to be positive for oxaprozin, and one was positive for alprazolam. Combining these GC/MS results with the CEDIA screening results gives a specificity of 99.8% for the current and modified CEDIA methods.

Our study shows that addition of \( \beta \)-glucuronidase to the R1 reagent of the CEDIA DAU benzodiazepine assay increases the detection of benzodiazepine analytes in urine specimens. To confirm the presence of benzodiazepine analytes in urine, a technique should be able to detect concentrations less than the immunoassay cutoff value. This sensitive on-line hydrolysis method provides a rapid and efficient urine screen for the presence of benzodiazepines.

This study was funded in part by Boehringer Mannheim Corp.

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>Current CEDIA</th>
<th>Modified CEDIA</th>
<th>GC/MS result, μg/L</th>
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<tbody>
<tr>
<td>25</td>
<td>9</td>
<td>10</td>
<td>Nordiazepam &lt;25; oxazepam 51; temazepam &lt;25</td>
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<tr>
<td>25</td>
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<tr>
<td>236</td>
<td>-81</td>
<td>53</td>
<td>Lorazepam 334; ( \alpha )-hydroxyalprazolam &lt;25; ( \alpha )-hydroxytriazolam &lt;25</td>
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<tr>
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<td>7</td>
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<tr>
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<td>Lorazepam 334</td>
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<td>( \alpha )-Hydroxyalprazolam 84</td>
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<td>32</td>
<td>Nordiazepam &lt;25; oxazepam 103</td>
</tr>
</tbody>
</table>

\( \Delta \) absorbance rate, mA/min

This study was funded in part by Boehringer Mannheim Corp.

Analysis of Cyanine Dye-Labeled PCR Product and Restriction Fragments by Capillary Electrophoresis and Laser-Induced Fluorescence, \textit{Wen-Shen Wu and Jin-Lian Tsai}\* (Poison Control and Analysis Center and Graduate Institute of Occupational Safety and Health, Kaohsiung Medical College, Kaohsiung 80708, Taiwan R.O.C.; \*address for correspondence: Graduate Institute of Occupational Safety and Health, Kaohsiung Medical College, No.100 Shih-Chuan 1st Rd., Kaohsiung City 80708, Taiwan, Republic of China; fax 07–3162632, e-mail jilits@cc.kmc.edu.tw)

Capillary gel electrophoresis (CGE) has been used for analysis of double-stranded DNA, including products of