Effect of β-Glucuronidase on Urinary Benzodiazepine Concentrations Determined by Fluorescence Polarization Immunoassay

Per Simonsson,1,3 Anders Lidén,2 and Stellan Lindberg1

In samples from patients treated with oxazepam, β-glucuronidase increased the immunoreactivity of urinary benzodiazepines analyzed by fluorescence polarization immunoassay (FPIA). Increasing concentrations of β-glucuronidase added to samples from drug-free controls did not influence the results. In the absence of β-glucuronidase, 22 of 35 samples from patients undergoing detoxification gave positive results at a cutoff concentration of 200 μg/L. Pretreatment with β-glucuronidase increased the number of drug-positive samples to 33. The drug-negative samples were obtained from two patients who had been oxazepam-free for at least 1 week. Thus, β-glucuronidase can be used to increase the sensitivity of the urinary benzodiazepine FPIA without reducing the specificity of the method.

Indexing Terms: oxazepam/drugs of abuse/therapeutic drug monitoring/metabolism

Glucuronidation of benzodiazepines occurs during their metabolism and elimination. The immunoreactivity of glucuronidated benzodiazepines is reduced by glucuronidation, which may lead to false-negative results in immunoassays developed for detecting benzodiazepines in urine. The addition of β-glucuronidase to urine samples containing benzodiazepines can increase the sensitivity of fluorescence polarization immunoassays (FPIA) used in clinical practice (1, 2) as has been discussed in the literature (3, 4); it is therefore of interest to further investigate the effect of β-glucuronidase on commonly used methods for analysis of urinary benzodiazepines.

Oxazepam, one of the most extensively prescribed benzodiazepines in clinical practice, is eliminated in urine after glucuronidation in the liver (5). Therefore, we used oxazepam as a model benzodiazepine for studying the effect of glucuronidase treatment on FPIA of benzodiazepines in urine.

Materials and Methods
Clinical Material

Thirty-five samples from five patients undergoing successive detoxification from long-term benzodiazepine abuse were analyzed. The daily dose of oxazepam for these inpatients varied from 0 to 250 mg. Samples were taken in the morning prior to medication. Alterations in the prescription of oxazepam were done at least 1 week before sampling. No other psychoactive drugs were administered during the period of investigation. In plasma samples taken in parallel with urine samples, no benzodiazepines other than oxazepam were found by HPLC. Urines from 14 benzodiazepine-free subjects were the controls. The study was approved by the Ethical Committee of Lund University.

FPIA Analysis

Urine (200 μL) was incubated for at least 30 min at room temperature with 10 μL of β-glucuronidase (EC 3.2.1.31; 10 kU/g at 25°C, from Escherichia coli K12, dissolved in 500 mL/L glycerol, from Boehringer Mannheim, Mannheim, Germany) (2). Final concentration during incubation was 0.5 kU/L. In studies aimed at optimizing the β-glucuronidase concentration, the enzyme was added in increasing volume to yield final concentrations of 0.25, 0.5, 0.9, and 2.0 kU/L. The sample was then transferred to an ADx sample cup. Benzodiazepines were analyzed in an ADx analyzer (Abbott Labs., Abbott Park, IL) according to the instructions from the manufacturer. If found to contain benzodiazepines above the upper range of the method (>2400 μg/L), the samples were reanalyzed after dilution with H2O.

HPLC Analysis

HPLC analysis was a modification of the method published by Brodie et al. (7). Urine or serum (1.0 mL) was supplemented with internal standard [30 μL of 20 μmol/L methylclonazepam (kindly provided by Roche, Basel, Switzerland)]. Saturated sodium tetraborate solution (1.0 mL) was added and the mixture extracted with 5.0 mL of dichloromethane by rotating the tubes containing the samples for 15 min. After centrifugation, the organic phase was transferred to new tubes and evaporated to dryness with nitrogen. The residue was redissolved in 100 μL of the mobile phase used for HPLC [acetonitrile:methanol:20 mmol/L potassium dihydrogen phosphate buffer (pH 3.8), 280:50:670 by vol]. All reagents were analytical grade. Water of analytical grade was prepared with a MilliQ-Ultra ion-exchange device (Millipore, Bedford, MA). The chromatographic system consisted of a Waters (Milford, MA) 510 B pump and 710B autosampler, and a UV-VIS detector from Linear (Reno, NV) operated at 313 nm. The 125 × 4 mm column was packed with RP-selectB (Merck, Darmstadt, Germany). The flow rate was 2.0 mL/min. The retention times of oxazepam and methylclonazepam were 4.2 and 8.7 min, respectively. Oxazepam...
was kindly provided by Pharmacia AB, Stockholm, Sweden. In addition, chlordiazepoxide, nitrazepam, flunitrazepam, clonazepam, nordiazepam, and diazepam were analyzed separately in the same chromatographic system. Each assay was calibrated against a calibration curve (0.5, 1.0, and 2.5 μmol/L). The detection limit was 0.1 μmol/L. The within-batch imprecision (CV) was 6%, and between batches, 10%.

Results

β-Glucuronidase treatment influenced neither the concentrations of the calibrator (nordiazepam) used for FPIA (Fig. 1) nor the immunoreactivity of added unconjugated oxazepam (Fig. 2). β-Glucuronidase treatment had no significant effect on samples from drug-free control subjects. In controls, mean urinary benzodiazepine was 18 (range: 0–94) μg/L without β-glucuronidase and 21 (8–35) μg/L with β-glucuronidase, values well below the cutoff recommended by the manufacturer (200 μg/L).

The addition of β-glucuronidase to samples from oxazepam-treated patients induced a concentration-dependent increase in the measured concentrations of urinary benzodiazepines (Fig. 3). Maximum benzodiazepine concentrations were obtained at 0.5 kU/L β-glucuronidase. Increasing concentrations of β-glucuronidase in samples from drug-free controls did not influence the results.

In the absence of β-glucuronidase, 22 of the 35 samples from patients undergoing detoxification were found to be positive at a cutoff concentration of 200 μg/L (Fig. 4). Pretreatment with β-glucuronidase increased the number of positive samples to 33. Only two samples were negative after β-glucuronidase treatment, and these samples were obtained from two patients who had been oxazepam-free for at least 1 week. A positive correlation (r = 0.89) was found between concentrations in β-glucuronidase-treated and untreated samples. However, the correlation did not appear to be linear: The increase in immunoreactivity was greater in samples in the upper range of benzodiazepine concentrations than in samples containing benzodiazepines below or at the cutoff concentration.

In addition, the benzodiazepine immunoreactivity correlated to the dose of oxazepam administered to the patients. The correlation between urinary benzodiazepine concentrations and daily dose of oxazepam was
0.71 for \(\beta\)-glucuronidase-treated samples and \(r = 0.71\) for untreated samples.

Samples were also analyzed by HPLC. Of the samples not treated with \(\beta\)-glucuronidase, only three samples were found to contain oxazepam at concentrations exceeding the established cutoff limit of 200 \(\mu \text{g/L}\) (Fig. 5). As expected, pretreatment with \(\beta\)-glucuronidase increased the concentrations of oxazepam in urine. In most cases, the concentration of oxazepam was higher than the concentration of benzodiazepine as determined by FPIA, indicating a reduced cross-reactivity of the antibodies used in the immunoassay (Fig. 6). HPLC analysis of \(\beta\)-glucuronidase-treated samples detected oxazepam concentrations above the 200 \(\mu \text{g/L}\) cutoff concentration in all samples except the two from drug-free patients. HPLC analysis thus confirmed the qualitative results of the FPIA method.

**Discussion**

Screening for urinary benzodiazepines has become routine practice in the diagnosis of chemical dependency. The aim of the methods currently in use is to detect the presence of these tranquilizing drugs with high sensitivity and specificity. The market is dominated by a few manufacturers producing well-established analytical systems based on immunochemistry. However, because of the introduction of high-potency benzodiazepines and the complex biological nature of metabolite elimination, the sensitivity of these methods has been questioned (1, 2).

It has become increasingly clear that conjugation of drugs not only influences their biological activity but also influences the analysis of these substances (5, 6). Beck et al. have proposed increasing the sensitivity of FPIA by pretreating the samples with \(\beta\)-glucuronidase (1, 2, 4)—an approach that has been questioned by representatives of the manufacturers (3). The arguments raised are related to the possibility that alterations in the standard procedure of an established method may generate analytical problems previously not clarified. Boeckx also expressed concern that \(\beta\)-glucuronidase could generate new products in the sample that could cross-react in the assay and thus lower the specificity of the analysis (3).

The aim of our study was to investigate the effects of \(\beta\)-glucuronidase on a set of samples from patients receiving oxazepam treatment and on urine from drug-free subjects to determine the effect of deconjugation on the sensitivity and the specificity of the FPIA for benzodiazepines. The classification of samples as true positive and true negative was based on HPLC results for the presence or absence of benzodiazepines in urine. The sensitivity of FPIA for detection of oxazepam was increased after pretreatment with \(\beta\)-glucuronidase, in general, by at least 10-fold. In this cohort of inpatients,
where the administration of the drug was well controlled, all samples from patients taking benzodiazepines at doses as low as 5 mg daily were positive 12 h after drug administration. The presence of oxazepam was confirmed by HPLC. The two negative samples came from two patients who had been benzodiazepine-free for >1 week. Serum and urine oxazepam concentrations analyzed by HPLC in these patients were also negative, confirming the cessation of benzodiazepine intake. In contrast, only 22 of the 35 samples were negative by the established protocol from the manufacturer, indicating an unsatisfactory performance of the assay. Thus, β-glucuronidase treatment of urine samples yields an increased sensitivity for benzodiazepines.

A trend towards a nonlinear relation was noted between values from β-glucuronidase-treated and untreated samples. The increase in the immunoreactivity after β-glucuronidase was more pronounced in high-concentration samples than in samples close to the cutoff concentration of the assay. This would not influence the performance of the method in a clinical setting, because the urinary benzodiazepine method is only used as a qualitative assay for screening.

To determine whether β-glucuronidase reduces the specificity of the assay, we used several tests. As mentioned above, negative results were obtained in the two samples from patients who had been benzodiazepine-free for >1 week, and HPLC analysis of serum and urine confirmed the absence of benzodiazepines in these samples. Several other experiments were performed to study possible artifacts that could generate false-positive results. First, β-glucuronidase did not influence the calibrator used. Second, the immunoreactivity and the recovery of added, unconjugated oxazepam were not altered by β-glucuronidase treatment. Finally, β-glucuronidase did not increase the immunoreactivity of 14 urine samples obtained from drug-free subjects. Therefore, we found no indications for a negative effect of β-glucuronidase on the specificity of the FPIA.

In our study, we included patients who were taking oxazepam. However, a number of different benzodiazepines are currently in clinical use, and we must point out that the conclusions, particularly with regard to studies on the sensitivity, are restricted to oxazepam. Glucuronidated oxazepam is also an end product in the elimination of other benzodiazepines such as diazepam and temazepam; it is therefore possible that β-glucuronidase may increase the sensitivity of FPIA for these other benzodiazepines. This aspect is illustrated in the publication of Beck et al. (2).

In conclusion, the results of our investigation highlight the role of conjugation with glucuronide in the urinary elimination of benzodiazepines and points towards an approach that may improve the performance of benzodiazepine analysis in clinical practice.

We thank Lennart Meyer, Department of Psychiatry, Simrishamn, Sweden, for providing the clinical samples, and Lillemor Magnusson for expert technical assistance. Financial support was obtained from the county council of Kristianstad, Sweden, and Goldi and Ludvig Berglund's Foundation.

References