ENZYMATIC HYDROLYSIS IMPROVES THE SENSITIVITY OF EMIT SCREENING FOR URINARY BENZODIAZEPINES.


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Summary
Screening for benzodiazepines is routinely performed by immunochemical analysis such as enzyme immunoassay. However, the wide range of therapeutic and toxic concentrations of the different benzodiazepines and their extensive metabolism and conjugation hamper the design of an immunoassay protocol that can detect all benzodiazepines and their corresponding urinary metabolites. Only a few investigators have tried to improve the detection of a number of benzodiazepines either by incorporating an enzymatic hydrolysis step before screening or by adding β-glucuronidase to the immunoassay reagents. These studies were performed with spiked samples or with samples collected after controlled intake of therapeutic doses of one single benzodiazepine by healthy volunteers. The purpose of this study was to evaluate whether enzymatic hydrolysis improves the sensitivity of the EMIT d.a.u. Benzodiazepine Assay by comparing the screening results of a large number (n=530) of authentic patient urine samples before (EMIT) and after enzymatic hydrolysis (EMIT-H) reflecting actual practice.

Materials and methods
Urine samples
Urine samples were collected from volunteers with a high expected prevalence of benzodiazepine use, in accordance to the ethical standards of the ethical review committee of the Ghent University Hospital.

EMIT d.a.u. Benzodiazepine Assay
Urine specimens were analyzed by the EMIT d.a.u.© Benzodiazepine Assay. The assay was calibrated daily with the EMIT low (0.2 mg/L oxazepam) and high (1.0 mg/L oxazepam) calibrators and the calibration was validated by assaying positive and negative controls. Unknowns were determined from the calibration curve by linear interpolation. A sample that gave a change in absorbance (ΔA) equal to or higher than the lower calibrator was interpreted as positive.

Enzymatic hydrolysis and GC-MS screening of benzodiazepines
From urine samples, 2 mL of 0.2 M sodium acetate buffer (pH 4.5) and 5500 U of H. pomatia β-glucuronidase were added. The tubes were mixed vigorously and incubated at 56°C for 4.5 h. After centrifugation at 2500 rpm for 10 min, 100 μL of the supernatant was isolated for EMIT-H analysis. The internal standard (N-Me-clonazepam) was added to the remainder of the supernatant that was brought to pH 6.8 by the addition of sodium hydroxide and phosphate buffer, prior to solid-phase extraction on phenyl-type cartridges. The dried extracts were derivatized by acetylation and reconstituted in 20 μL of ethyl acetate. On-column injections of 1-μL aliquots were performed with a HP 7890 autosampler (Avondale, PA) on a Restek (Bellefonte, PA) hydroguard guard column (5 μm 32 mm I.D.) coupled to a SGE (Achrom, Zulte, Belgium) BP1 capillary column (30 m 0.25 mm I.D., 0.25 μm film thickness; nonpolar polydimethylsiloxane phase) via a Universal angled press-to-connect connector (Restek). The GC-MS instrument consisted of a HP 6890 Series Gas Chromatograph coupled to a HP 5973 MSD, used in the electron impact scan mode (1).

Results and discussion
Fig. 1 correlates the GC-MS results with the traditional EMIT immunoassay and the EMIT-H results. Of the 530 samples analyzed, 174 samples were found positive for benzodiazepines by GC-MS. The following compounds were identified (with the corresponding number of samples between brackets): alprazolam (24), bromazepam (48), diazepam (24), flunitrazepam (2), flurazepam (8), lormetazepam (44), oxazepam (74), prazepam (2) and temazepam (34). Classification of the samples as true positives and true negatives was based on these GC-MS results.

To study the effect of changing the cutoff value on sensitivity and specificity, the receiver-operating characteristic (ROC) curves were derived and compared (Fig. 3). By comparing both ROC curves, it is clear that at a fixed specificity, the corresponding sensitivity is systematically higher for the EMIT-H test. For the urine samples screened in this study, the recommended cutoff value of 0.2 mg/L for EMIT is optimal with respect to specificity (100 %) but corresponds to a sensitivity of only 67 %. In the case of the EMIT-H test, the corresponding value for specificity is 96 % and for sensitivity is 87 %. However, the sensitivity of a screening method is considered to be more important than specificity as it is more important to avoid FN than FP results. In routine practice all positive samples are retested for identification and quantification purposes, and after the confirmation assay FP samples will correctly appear as negatives. For our set of samples, the recommended cutoff value of 0.2 mg/L for the EMIT test is far from optimal with respect to sensitivity and it would be advantageous to decrease the cutoff value. On the other hand, the recommended cutoff value of 0.2 mg/L results in high sensitivity for the EMIT-H test and the gain in sensitivity is therefore only marginal when decreasing the cutoff value.

The specificity and sensitivity of the two EMIT screening tests were first evaluated at the cutoff value of 0.2 mg/L oxazepam. The 95 % confidence intervals (CI) were calculated based on the binomial distribution and the specificity and sensitivity of the two screening tests were compared by the Fisher exact test (2). Of the 174 GC-MS positive samples, 117 versus 152 yielded a positive screening result at the cutoff value of 0.2 mg/L oxazepam in the EMIT and EMIT-H test, respectively. This implies that the sensitivity increased from 67% (95% CI: 60-74%) for the EMIT test to 87 % (95% CI: 81-92%) for the EMIT-H test, which is a highly significant gain of 20% (P<0.0001). The increase in EMIT absorbance upon hydrolysis was found to be dependent on the identity of the ingested drug and the largest increase was observed for samples containing flurazepam (Fig. 2).

EMIT-H - EMIT (mg/L)
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0
EMIT
EMIT-H
Fig. 1: Correlation between EMIT and EMIT-H. Of the 530 urine samples analyzed, 356 samples were negative (?) and 174 samples were tested positive (?) by a sensitive GC-MS procedure, used as a reference method. The cutoff value for both immunoassays was 0.2 mg/L oxazepam. EMIT yielded 117 positive and 413 negative results, including 57 false negatives. After enzymatic hydrolysis the number of positives increased to 167, including 15 false positive samples, thus the number of false negatives decreased to 22. The square in the left-hand figure is enlarged to the right-hand figure.

Conclusion
Enzymatic pretreatment of urine samples before EMIT screening for benzodiazepines yields a highly significant gain in sensitivity of 20 % at the cutoff value 0.2 mg/L oxazepam. From the ROC curves it is clear that EMIT-H outperforms EMIT as for a fixed specificity, the corresponding sensitivity is systematically higher for the EMIT-H test. By analyzing this large number of authentic patient samples and by the confirmation analysis not only of the presumptive positives but of all samples with the optimized GC-MS procedure, combined with a detailed statistical evaluation, we produced convincing evidence that EMIT-H is the recommended screening procedure for urinary benzodiazepines in routine practice.

References