# HIGH SENSITIVITY HOMOGENEOUS ENZYME IMMUNOASSAY FOR BENZODIAZEPINES AND METABOLITES

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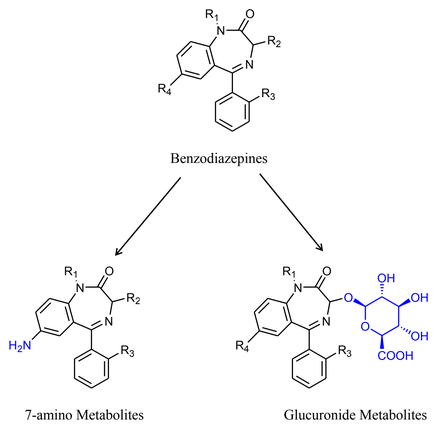
## BACKGROUND

Benzodiazepines are widely prescribed for treating various conditions such as anxiety, insomnia, and seizures due to their sedative and anxiolytic properties. Despite their therapeutic benefits, concerns persist regarding their potential for misuse, dependence, and addiction. Urine drug screening immunoassays serve as an important tool in monitoring benzodiazepine usage, ensuring adherence to prescribed regimens, identifying misuse or abuse, and facilitating necessary medical interventions. However, limitations of benzodiazepine immunoassays are well-documented in literature, with issues surrounding their lack of sensitivity to glucuronidated and 7-amino metabolites, as well as many other common or designer benzodiazepines. ARK Diagnostics has developed a highly sensitive homogeneous enzyme immunoassay capable of detecting both classic and designer benzodiazepines, in addition to their glucuronide and 7-amino metabolites in human urine at a cutoff concentration of 200 ng/mL, without the need for glucuronidase or sample pretreatment.




## METHODS

The ARK™ Benzodiazepine Plus Assay is a liquid-stable homogenous enzyme immunoassay consisting of two reagents. The assay uses temazepam as the 200 ng/mL cutoff calibrator. The performance characteristics of this assay, including precision, spiked recovery, specificity, and method comparison to LC-MS/MS, were evaluated on the Beckman Coulter AU680 automated clinical chemistry analyzer.

## RESULTS

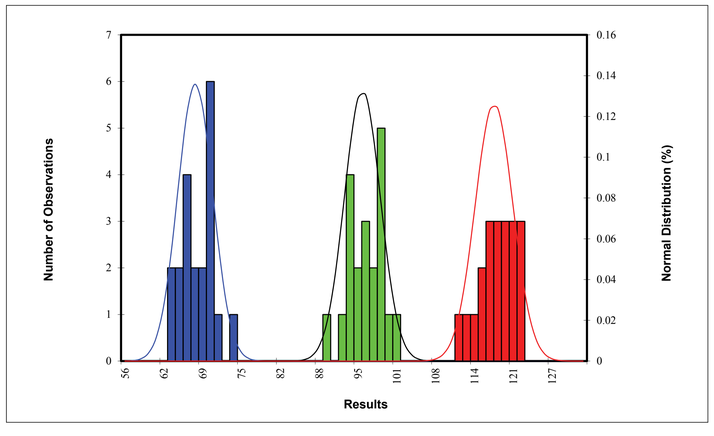
### PRELIMINARY PRECISION

Pooled human urine was spiked with temazepam to achieve concentrations at 25% increments from the cutoff calibrator (200 ng/mL). Twenty (20) replicates of each sample were assayed in semi-quantitative mode.

| Temazepam (ng/mL) | Cutoff (%) | Mean (ng/mL) | SD | CV (%) |
|---|---|---|---|---|
| 150 | -25 | 139.8 | 5.06 | 3.6 |
| 200 | Cutoff | 189.1 | 5.47 | 2.9 |
| 250 | +25 | 230.9 | 6.22 | 2.7 |

### HISTOGRAM OVERLAP ANALYSIS (QUALITATIVE ANALYSIS)

Frequency of distribution of temazepam values for each sample is shown by histogram analysis. Twenty replicates each of Negative Control (150 ng/mL), Cutoff Calibrator (200 ng/mL), and Positive Control (250 ng/mL) were assayed together in a single run. The distributions of measurements did not overlap.




### ANALYTICAL RECOVERY

Spike recovery was evaluated using in-house prepared samples. Ten (10) samples were tested in semi-quantitative mode using the AU680 analyzer. One calibration curve was generated, and five (5) replicates of each sample were assayed. Mean, SD, %Nominal and %CV were calculated for each level. Percent nominal ranged from 95.0 to 103.7%.

| Samples (ng/mL) | Mean (ng/mL) | SD | CV (%) | Nominal (%) | N |
|---|---|---|---|---|---|
| 50 | 50.3 | 2.23 | 4.4 | 100.5 | 5 |
| 75 | 77.5 | 7.68 | 9.9 | 103.3 | 5 |
| 120 | 121.2 | 4.92 | 4.1 | 101.0 | 5 |
| 240 | 228.1 | 14.89 | 6.5 | 95.0 | 5 |
| 400 | 399.3 | 18.04 | 4.5 | 99.8 | 5 |
| 700 | 725.7 | 22.42 | 3.1 | 103.7 | 5 |
| 1000 | 976.3 | 62.74 | 6.4 | 97.6 | 5 |

### SPECIFICITY - BENZODIAZEPINES

The cross-reactivity to benzodiazepine compounds with the ARK Benzodiazepine Plus Assay was tested in semi-quantitative mode to obtain the concentration of each compound equivalent to the 200 ng/mL cutoff.

| Compound | Concentration Approximately Equivalent to the Cutoff (ng/mL) | Cross-reactivity (%) |
|---|---|---|
| 2-OH-ethylflurazepam | 86 | 233 |
| 3-OH Flubromazepam | 143 | 140 |
| 3-OH Phenazepam | 152 | 132 |
| 4-OH-alprazolam | 3472 | 6 |
| 7-aminoclonazepam | 141 | 142 |
| 7-aminoflunitrazepam | 204 | 98 |
| 7-aminonimetazepam | 93 | 215 |
| 7-aminonitrazepam | 115 | 175 |
| Alprazolam | 36 | 548 |
| Bromazepam | 248 | 81 |
| Bromazolam | 30 | 669 |
| Chlordiazepoxide | 583 | 34 |
| Clobazam | 1543 | 13 |
| Clonazepam | 170 | 118 |
| Clonazolam | 646 | 31 |
| Clorazepate | 115 | 174 |
| Delorazepam | 74 | 270 |
| Demoxepam | 329 | 61 |
| Desalkylflurazepam | 78 | 256 |
| Deschloroetizolam | 51 | 392 |
| Diazepam | 90 | 222 |
| Diclazepam | 355 | 56 |
| Estazolam | 40 | 500 |
| Etizolam | 21 | 939 |
| Flualprazolam | 32 | 626 |
| Flubromazepam | 90 | 222 |
| Flubromazolam | 89 | 225 |
| Flunitrazepam | 334 | 60 |
| Flurazepam | 122 | 164 |
| Halazepam | 2165 | 9 |
| Ketazolam | 111 | 180 |
| Loprazolam | 1352 | 15 |
| Lorazepam | 112 | 179 |
| Lorazepam Glucuronide | 164 | 122 |
| Lormetazepam | 301 | 66 |
| Meclonazepam | 42 | 476 |
| Medazepam | 32,492 | 1 |
| Midazolam | 495 | 40 |
| N-Desmethylclobazam | 319 | 63 |
| N-desmethylflunitrazepam | 140 | 143 |
| Nimetazepam | 209 | 96 |
| Nitrazepam | 198 | 101 |
| Norchlordiazepoxide | 264 | 76 |
| Nordiazepam | 80 | 250 |
| Oxazepam | 147 | 136 |
| Oxazepam Glucuronide | 160 | 125 |
| Phenazepam | 300 | 67 |
| Prazepam | 914 | 22 |
| Pyrazolam | 63 | 318 |
| Temazepam | 200 | 100 |
| Temazepam Glucuronide | 170 | 118 |
| Tetrazepam | 3380 | 6 |
| Triazolam | 144 | 139 |
| α-OH bromazolam | 52 | 383 |
| α-OH Flualprazolam | 46 | 433 |
| α-OH Flubromazolam | 49 | 408 |
| α-OH-alprazolam | 65 | 308 |
| α-OH-midazolam | 117 | 171 |
| α-OH-Triazolam | 82 | 244 |

### SPECIFICITY –STRUCTURALLY UNRELATED COMPOUNDS

No interference was observed with the addition of concentrations of 100,000 ng/mL for 112 opiates, opioids, and other structurally unrelated compounds. Acetaminophen and ibuprofen were added at a concentration of 500,000 ng/mL, and norpseudoephedrine was added at a concentration of 50,000 ng/mL, all without any observed interference.

### METHOD COMPARISON

A total of one hundred and three (103) unaltered, un-pretreated with glucuronidase, clinical human urine specimens that are not individually identifiable were analyzed for benzodiazepines with the ARK Benzodiazepine Plus Assay in semi-quantitative mode and the results were compared to LC-MS/MS.

The LC-MS/MS confirmatory method was performed by a licensed reference laboratory. Briefly, the method involves treating specimens with glucuronidase, adding internal standards, and injecting into a column for LC-MS/MS. Detection peaks and their limits of quantitation (LoQ) in ng/mL are: 7-aminoclonazepam (5); alprazolam (1); hydroxyalprazolam (1); lorazepam (10); diazepam (5); nordiazepam (5); oxazepam (5); temazepam (1); midazolam (1); hydroxymidazolam (1).

Results are summarized in the tables below, where the LC-MS/MS result represents the sum across all benzodiazepine peaks identified. All ARK-positive samples in this study were confirmed by LC-MS/MS to have benzodiazepine concentrations of at least 20 ng/mL.

| | LC-MS/MS Results (ng/mL) | | | |
|---|---|---|---|---|
| ARK Result | Low Negative Less than 50% below the Cutoff (< 100 ng/mL) | Near Cutoff Negative Between 50% below the Cutoff and the Cutoff (100 - 199.9 ng/mL) | Near Cutoff Positive Between the Cutoff and 50% above the Cutoff (200 - 299.9 ng/mL) | High Positive Greater than 50% above the Cutoff (> 300 ng/mL) |
| Negative (< 200 ng/mL) | 20 | 1 | 1 | 1* |
| Positive (≥ 200 ng/mL) | 11** | 16 | 12 | 41 |

| Sample ID | ARK Assay (ng/mL) | LC-MS/MS (ng/mL) | Benzodiazepines present by LC-MS/MS |
|---|---|---|---|
| 6** | 227 | 20 | clonazepam |
| 11** | 213 | 27 | clonazepam |
| 15** | 219 | 39 | clonazepam |
| 16** | 203 | 39 | clonazepam |
| 17** | 226 | 43 | alprazolam, hydroxyalprazolam |
| 19** | 225 | 50 | clonazepam |
| 20** | 214 | 52 | alprazolam, hydroxyalprazolam |
| 27** | 321 | 74 | alprazolam, hydroxyalprazolam |
| 29** | 212 | 83 | alprazolam, hydroxyalprazolam |
| 30** | 220 | 86 | alprazolam, hydroxyalprazolam |
| 31** | 259 | 90 | lorazepam |
| 84* | 143 | 725 | alpha hydroxymidazolam, midazolam |

**Strong reactivity with clonazepam, 7-aminoclonazepam, alprazolam, hydroxyalprazolam, lorazepam, and lorazepam glucuronide contributed to the positive results obtained with the ARK Benzodiazepine Plus Assay.

*Un-pretreated sample showed the presence of 1.7 ng/mL alpha hydroxymidazolam and 0 ng/mL midazolam.

## CONCLUSIONS

The ARK™ Benzodiazepine Plus Assay enables the sensitive, rapid, and reliable measurement of benzodiazepines and their metabolites in human urine, without the need for hydrolysis or pretreatment. The assay addresses the present constraints of currently available benzodiazepine immunoassays, including their insufficient sensitivity, limited cross-reactivity to metabolites, and the requirement for glucuronidase pretreatment. The ARK™ Benzodiazepine Plus Assay is applicable to a wide range of clinical chemistry analyzers.

### PROPOSED INTENDED USE

The ARK™ Benzodiazepine Plus Assay is a homogeneous enzyme immunoassay intended for the qualitative and/or semi-quantitative determination of the presence of benzodiazepines and their metabolites in human urine at a cutoff concentration of 200 ng/mL. The assay is intended to be used in laboratories and provides a simple and rapid analytical screening procedure to detect benzodiazepines in human urine. The assay is designed for use with a number of clinical chemistry analyzers. This assay is calibrated against Temazepam. This product is intended to be used by trained professionals only.

The semi-quantitative mode is for the purpose of (1) enabling laboratories to determine an appropriate dilution of the specimen for confirmation by a confirmatory method, such as Gas Chromatography/Mass Spectrometry (GC/MS) or Liquid Chromatography/tandem Mass Spectrometry (LC-MS/MS), or (2) permitting laboratories to establish quality control procedures.

The ARK Benzodiazepine Plus Assay provides only a preliminary analytical test result. A more specific alternative chemical method must be used in order to obtain a confirmed positive analytical result. Gas Chromatography/Mass Spectrometry (GC/MS) or Liquid Chromatography/tandem Mass Spectrometry (LC-MS/MS) is the preferred confirmatory method. Clinical consideration and professional judgment should be exercised with any drug test result, particularly when the preliminary test result is positive.

### REGULATORY STATUS

In Development.
This product is not FDA cleared for sale in the US.