# Ultra-fast Analysis of Benzodiazepines in Human Urine using Dilute and Shoot Methodology and SPE-MS/MS

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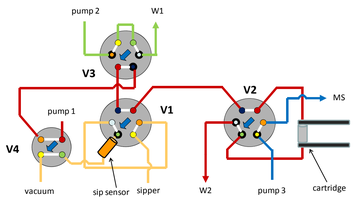
## Introduction

Liquid-liquid extraction and protein precipitation represent time-consuming and cumbersome steps in human urine sample preparation, the elimination of which would greatly speed the rate with which research labs can analyze human samples. Dilute and shoot has been proposed as a preparation-free alternative, though its utility can be limited by matrix effects resulting from the lack of pre-analysis sample cleanup. Here, we apply the dilute and shoot method to the detection of a panel of benzodiazepines in human urine using an ultra-fast online SPE cleanup prior to MS/MS analysis. With a cycle time of ~9 seconds per sample, the system offers a considerable speed advantage over chromatography-based dilute and shoot methodologies while maintaining data quality.

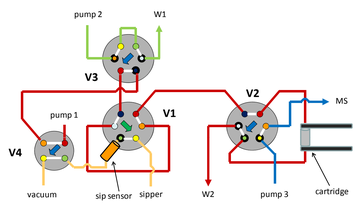
## The RapidFire-MS System

The Agilent RapidFire High-throughput Mass Spectrometry System is an ultra-fast liquid handler that quickly desalts biological samples upstream of mass spectrometric detection. The instrument aspirates a portion of each well of a 96- or 384-well assay plate and applies the sample to an SPE column chosen to retain the analyte(s) of interest. After a washing step, the sample is reverse eluted and sent to a mass spectrometer for detection. The entire cycle time between aspiration of one well and the next is usually between 7 and 10 seconds, as opposed to the several minutes required by most HPLC methods. The RapidFire-MS system can be interfaced with an array of mass spectrometer types, depending on assay requirements.

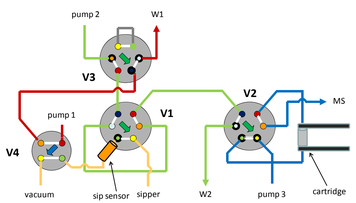
**States 1&4: Aspirate & Re-equilibrate**




**State 2: Wash**




**State 3: Elute**

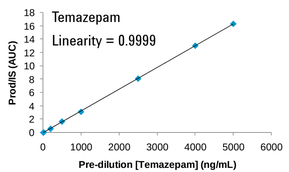



## Experimental

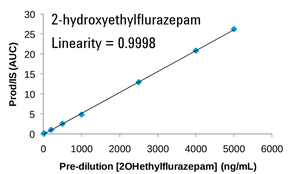
Mass spectral conditions for each compound and internal standard pair were optimized on the Agilent 6460 triple quadrupole (QQQ) mass spectrometer. Calibration standards were prepared by spiking blank human urine with each of a panel of benzodiazepines to final concentrations ranging from 10 ng/mL to 5,000 ng/mL. Calibration standards and QC samples were diluted 1:300 in $ddH_2O$ containing the appropriate internal standard (corresponding deuterated benzodiazepine) at 1 ng/mL. Sample analysis was performed at a rate of ~9 seconds per sample using a RapidFire 300 system coupled to an Agilent 6460 QQQ mass spectrometer operating in positive ESI mode. The SPE method employed a C4 column, a wash buffer of $ddH_2O$ supplemented with 0.01% trifluoroacetic acid and 0.09% formic acid and an elution buffer of 100% acetonitrile containing 0.01% trifluoroacetic acid and 0.09% formic acid. Data analysis was performed using RapidFire Integrator v3.4 software and GraphPad Prism 5. Accuracies were calculated using a $1/x^2$ weighted fit. This methodology is capable of throughputs of ~400 samples per hour.

| Compound | MRM Info Q1 | MRM Info Q3 | Internal Standard | MRM Info Q1 | MRM Info Q3 |
|---|---|---|---|---|---|
| Nordiazepam | 271 | 140 | Nordiazepam - D5 | 276 | 140.1 |
| Oxazepam | 287.1 | 241.2 | Oxazepam - D5 | 292.1 | 246.1 |
| Temazepam | 301.1 | 255.1 | Temazepam - D5 | 306.1 | 260.1 |
| Lorazepam | 321.1 | 275 | Lorazepam - D4 | 325.1 | 279.1 |
| Estazolam | 295.1 | 267.1 | Estazolam - D5 | 300.1 | 272.1 |
| Diazepam | 285 | 193.1 | Diazepam - D5 | 290.1 | 198.1 |
| 2-Hydroxyethylflurazepam | 333.1 | 109.1 | 2-Hydroxyethylflurazepam - D4 | 337.1 | 113.1 |
| a-Hydroxyalprazolam | 325.1 | 297.1 | a-Hydroxyalprazolam - D5 | 330.1 | 302.3 |
| a- Hydroxytriazolam | 359.1 | 331 | a-Hydroxytriazolam - D4 | 363.1 | 335 |
| Chlordiazepoxide | 300.1 | 227.1 | Chlordiazepoxide - D4 | 305.1 | 232.1 |
| Hydroxymidazolam | 342.1 | 203 | Hydroxymidazolam - D4 | 346.1 | 203 |
| 7-aminoclonazepam | 286.1 | 121.1 | 7-aminoclonazepam - D5 | 290.1 | 121.1 |
| 7-aminoflunitrazepam | 284.1 | 135 | 7-aminoflunitrazepam - D7 | 291.1 | 138.1 |

## Representative Data




| | Accuracy (%) | | Accuracy (%) | | Accuracy (%) |
|---|---|---|---|---|---|
| LQC (30 ng/mL) | 99.3 | MQC (2500 ng/mL) | 101.8 | HQC (4000 ng/mL) | 103.1 |
| | 95.7 | | 102.8 | | 104.4 |
| | 97.8 | | 103.1 | | 103.7 |
| | 99.1 | | 103.0 | | 102.3 |
| | 94.8 | | 102.7 | | 101.6 |
| | 99.4 | | 103.4 | | 103.7 |




| | Accuracy (%) | | Accuracy (%) | | Accuracy (%) |
|---|---|---|---|---|---|
| LQC (30 ng/mL) | 99.0 | MQC (2500 ng/mL) | 99.3 | HQC (4000 ng/mL) | 104.4 |
| | 102.2 | | 104.5 | | 97.2 |
| | 93.1 | | 98.4 | | 98.8 |
| | 100.9 | | 98.3 | | 103.5 |
| | 100.8 | | 101.5 | | 103.6 |
| | 94.2 | | 98.4 | | 102.3 |

## Data Summary

| Compound | Linearity | LOD ng/mL | s/n (peak to peak) | LQC (n=5) Accuracy | LQC (n=5) Precision | MQC (n=5) Accuracy | MQC (n=5) Precision | HQC (n=5) Accuracy | HQC (n=5) Precision |
|---|---|---|---|---|---|---|---|---|---|
| Nordiazepam | 0.9998 | <1 | 70.0 | 94.8 | 4.7 | 103.3 | 2.1 | 108.3 | 2.0 |
| Oxazepam | 0.9989 | 13 | 14.8 | 97.3 | 6.1 | 98.8 | 2.4 | 103.0 | 2.1 |
| Temazepam | 0.9999 | < 1 | 45.5 | 97.3 | 2.1 | 103.1 | 0.8 | 103.5 | 1.1 |
| Lorazepam | 0.9999 | 10 | 15.2 | 105.5 | 5.3 | 97.7 | 2.0 | 101.1 | 2.5 |
| Estazolam | 0.9985 | < 1 | 30.5 | 97.8 | 6.7 | 101.6 | 1.7 | 103.4 | 2.0 |
| Diazepam | 0.9987 | < 1 | 20.1 | 89.4 | 3.5 | 103.1 | 2.2 | 105.5 | 1.6 |
| 2-Hydroxyethylflurazepam | 0.9998 | 7 | 27.4 | 97.0 | 4.5 | 101.4 | 3.6 | 102.4 | 2.8 |
| a-Hydroxyalprazolam | 0.9972 | < 1 | 11.9 | 104.4 | 6.1 | 99.7 | 1.7 | 101.0 | 2.8 |
| a- Hydroxytriazolam | 0.9977 | < 1 | 22.1 | 104.0 | 4.6 | 101.3 | 2.3 | 101.1 | 2.7 |
| Chlordiazepoxide | 0.9985 | < 1 | 26.9 | 105.4 | 3.4 | 105.1 | 0.9 | 104.5 | 1.6 |
| Hydroxymidazolam | 0.9973 | 6 | 99.9 | 98.8 | 5.1 | 97.3 | 2.2 | 89.2 | 0.7 |
| 7-aminoclonazepam | 0.9993 | < 1 | 31.7 | 110.3 | 2.1 | 103.3 | 1.5 | 101.7 | 1.7 |
| 7-aminoflunitrazepam | 0.9999 | < 1 | 78.0 | 102.2 | 2.5 | 102.9 | 1.1 | 102.2 | 1.1 |

## Conclusions

- Dilute and shoot feasibility was assessed using a panel of commercially available benzodiazepines.
- The MS response for the tested benzodiazepines was found to be linear over the 10 ng/mL to 5,000 ng/mL range with an $R^2$ of at least 0.995 in each case.
- The limit of detection of the system was approximately 1 ng/mL for the majority of compounds tested.
- The accuracies for each of the compounds were well within ± 15%, and usually less than 10%, at all three QC concentrations (30, 2500, or 4000 ng/mL), suggesting that the system has a robust response over the entire range of concentrations tested.
- The precision on the QC samples was <10% for the LQC and <5% for the MQC and HQC samples, demonstrating the reproducibility of the analysis.
- These experiments establish the RapidFire-MS system as a viable and robust clinical research method for the detection of benzodiazepines in diluted human urine.
- The system further offers the advantage of vastly improved cycle time, as it achieves an analysis rate of ~9 seconds per sample (~400 samples per hour) while producing data that is comparable to LC-MS/MS systems.