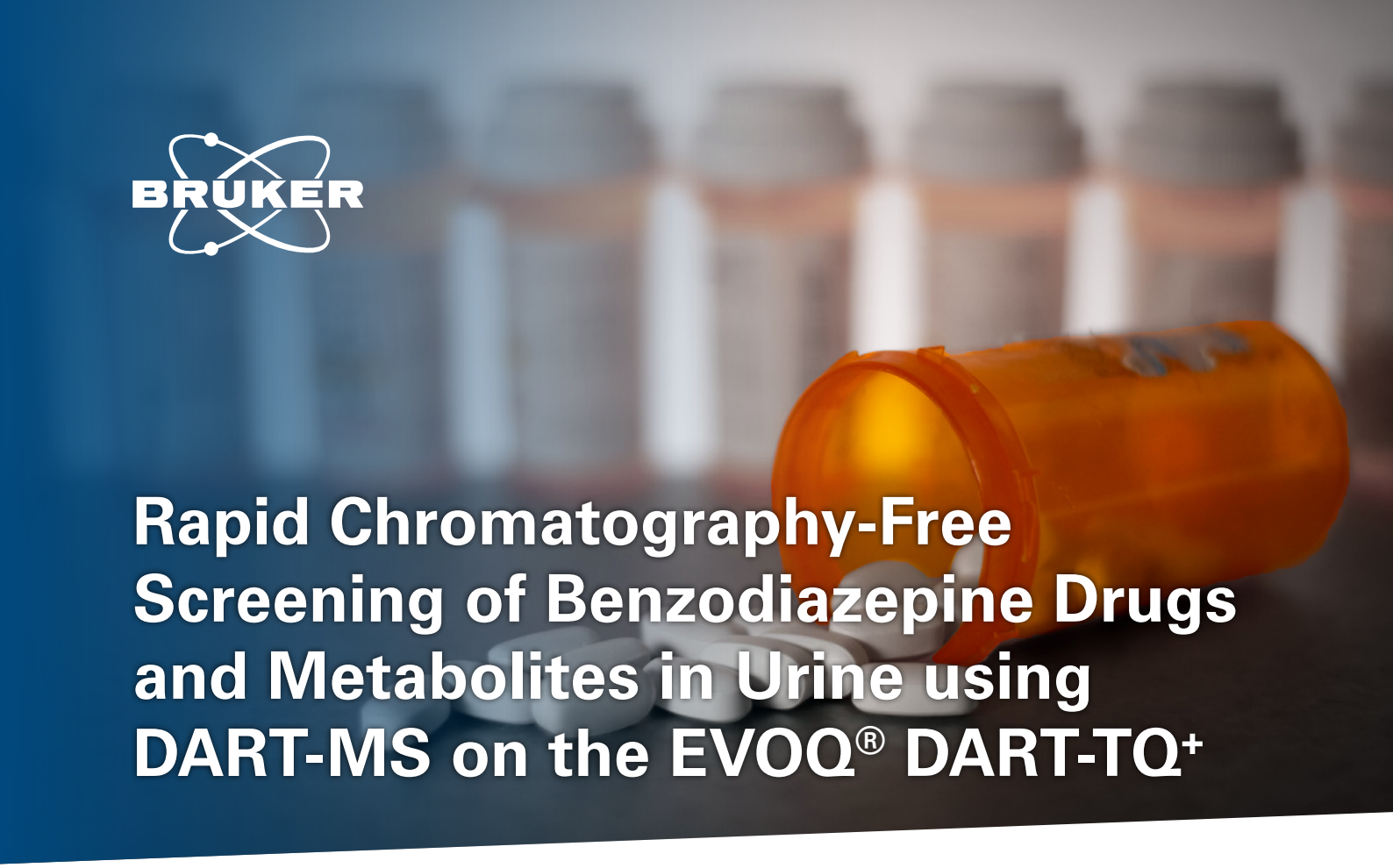

Rapid Chromatography-Free Screening of Benzodiazepine Drugs and Metabolites in Urine using DART-MS on the EVOQ® DART-TQ+

Analysis of urine-based benzodiazepine drugs and associated metabolites using the EVOQ® DART-TQ+ mass spectrometer with a Direct Analysis in Real Time (DART) ion source provides rapid and accurate quantitative confirmatory screening results in a chromatography-free workflow. This combination offers an easy and cost-effective alternative to immunoassay-based screening by improving both throughput and selectivity when compared to immunoassay evaluation of human urine samples.

Keywords:
Chromtography-free; DART-MS; benzodiazepine drugs; tqControl; quantitation

Abstract

Immunoassays (IA) are typically used as the test method for initial Urine Drug Screenings (UDS) for drugs of abuse in the field of forensic toxicology. This is in part due to the rapid generation of results and ease of adaptability to automation that IA affords. However, IA results are considered presumptive and not confirmatory in their accuracy due to the high frequency of false positives attributed to cross-reactivities with ubiquitous co-analytes. Because of the number of potential interferents in these assays, a positive IA result must therefore be confirmed by another hyphenated analytical approach, typically a chromatography-based method. LC-MS and GC-MS are commonly used as confirmatory assays due to their high degree of sensitivity, specificity, and accuracy. While chromatography-based approaches are well established and achieve sub-ng/mL detection limits, they rely on costly consumables and solvents and are limited in throughput due to time-consuming chromatography steps and requisite sample preparation. In this work, we report the development of an alternative, chromatography-free method using Direct Analysis in Real Time-Mass Spectrometry (DART-MS) that is shown to accurately identify and quantitate eight benzodiazepine drugs and six associated metabolites. IA-based assays currently used for the detection of these drugs suffer from significant false positives and false negative test results. The optimized DART-MS workflow achieves a throughput rate similar to that of IA at 96 samples in 40 min. This chromatography-free workflow also meets the low limits of detection and low %RSD for high-repeatability in urine matrices by avoiding interference from matrix or co-analytes.

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Introduction

Benzodiazepines: Uses, risks, and monitoring

Benzodiazepines (BZD) are a class of synthetic substances that exert agonistic effects on Gamma-aminobutyric acid (GABA)- releasing neurons via modulation of BZD receptors in the brain[1]. These drugs are commonly used in the treatment of psychiatric and neurological conditions including anxiety, insomnia, muscle spasms, and epilepsy. Clinical guidance suggests the use of these drugs in cases of short-term treatment solutions only, as longer-term administration may lead to undesired tolerance and dependence. Supratherapeutic doses of these medications are often self-administered to avoid withdrawal symptoms or to induce opioid-like effects. Due to high levels of abuse of BZD drugs, these analytes and their metabolites are monitored as drugs of abuse via UDS and in routine Prescription Drug Monitoring Programs (PDMP).

Challenges and techniques in Benzodiazepine urine analysis

Detection and measurement of BZD analytes and their metabolites in urine is challenging due to large variations in concentration based on dosage, administration time, and the identity of the specific form of BZD. Traditional Urine Drug Monitoring (UDM) is comprised of two types of tests: presumptive UDS using IA followed by a confirmatory test using a hyphenated chromatographic analytical technique such as LC-MS or GC-MS. IA-based detection is commonly used as a first-step screening tool due to ease of automation, rapid results, and cost efficiency. However, BZD-targeted IAs suffer from significant issues with cross-reactivity and interferences leading to significant numbers of false positive and false negative results[2]. The high negative predictive value of IAs for BZDs excreted as glucuronidated metabolites was vastly improved in the 1990's through the addition of beta-glucuronidase in analytical workflows[3]. Even with the substantial improvements cited, the performance of IAs is still not considered confirmatory.

Advantages of MS-based analysis

Compared to IA-based testing, MS-based techniques like tandem-MS (MS^n) can identify and quantify trace-level analytes with an increased level of selectivity and add important structural information about analytes of interest. Conventionally, MS and MS^n analysis exist as hyphenated techniques preceded by either LC or GC chromatographic separation to further improve selectivity and sensitivity in complex mixtures[4]. While chromatography improves selectivity, measurement using these techniques often requires between 10 and 30 min per sample leading to severe bottlenecks in analytical workflows.

DART-MS: A rapid, chromatography-free technique

For certain analytes, direct ambient ionization techniques such as DART coupled with MS often eliminate the need for chromatographic separation before MS analysis[5]. DART ionization in positive mode begins with the generation of metastable helium atoms that, through a cascade of reactions in the ambient environment, leads to protonated water clusters to ionize analytes with sufficient proton affinity in the DART reaction zone. This gas flow of metastables is heated to desorb analytes from solid scaffolds as ionization occurs, and the ions are then transferred directly into the MS for analysis. Because desorption conditions can be altered to favor lower boiling point and higher boiling point substances, DART is effective in separating compounds simply by changing parameters that control desorption and ionization. DART-MS, therefore, offers a rapid chromatography-free alternative with improved selectivity that significantly reduces false positive and false negative screening results commonly encountered in urine drug screens.

DART-MS analysis of BZD drugs

In this work, we perform a liquid-liquid extraction on urine samples containing the eight common BZD drugs and their metabolites. Samples were processed using a ToxBox® custom drug panel (PinPoint® Testing) and analyzed via DART-MS on an EVOQ® DART-TQ$^+$ (Bruker Daltonics) mass spectrometer to successfully measure eight compounds and associated metabolites with good linearity ($R^2 > 0.995$), good repeatability (3-12% RSD), and good recovery (97-107%) across the linear range for each analyte.

Overview of a toxicology workflow

Methods

Sample preparation

100 µL of certified drug-free urine, 100 µL Solution A, and 300 µL deionized (DI) water were added to each well in a 96 deep-well ToxBox® customized Benzodiazepine Validation plate from PinPoint Testing. The ToxBox custom drug panel kit consisted of PinPoint reagent Solutions A-C, a preloaded 96-well plate with selected analytes for an 8-point triplicate calibration curve, triplicate QC samples, and sample and calibration blanks. The entire plate was agitated for 10 min at 500 rpm on a horizontal plate shaker followed by the addition of 650 µL of PinPoint Solution B to each well and aspirated 10X to mix. Samples were timed to allow phase separation for 10 min. Next, the 550 µL aqueous layer was removed from the bottom of each well and discarded. The remaining organic layer was evaporated under nitrogen at 60 psi for 20 min followed by reconstitution in 100 µL of PinPoint Solution C. Reconstituted samples were agitated at 500 rpm on a horizontal plate shaker and a 2 µL aliquot from each well was transferred onto a Bruker DART QuickStrip HTS-96 screen and allowed to dry completely under nitrogen gas at 40°C for 15 min.

DART-MS Analysis

The prepared QuickStrip HTS-96 screen was loaded onto the automated transmission stage of an EVOQ® DART-TQ+ (Bruker Daltonics) triple quadrupole mass spectrometer fitted with a fully integrated DART ion source. DART-MS analysis was done in scanning mode via MRM with each analysis requiring approximately 20 s/sample as outlined in tables 1, 2, and 3. Samples were analyzed and processed using tqControl software (Bruker Daltonics), a single interface for instrument control and data analysis. Each calibration level was analyzed in triplicate and data were fitted to a linear regression model with QCs at three levels using the DART and MS settings as indicated in tables below.

Table 1. DART method parameters

DART Parameter	Value
Gas flow temperature	300 °C
Grid Voltage	50 V
Scanning Speed	0.5 mm/sec
Ionization gas	He
Polarity	Positive
Array	96-well plate

Table 2. EVOQ® DART-TQ+ MS method parameters

MS Parameter	Value
Cone temperature	300 °C
Cone gas pressure	23 psi
CID cell pressure	1.25 mTorr
Collision gas	Ar
Detector voltage	Dynamic
Polarity	Positive

Compound Transitions

For all analytes and metabolites, the MRM transitions, the optimized collision energies, and scan times are shown in the table below.

Table 3. EVOQ® DART-TQ+ MS method compound transitions

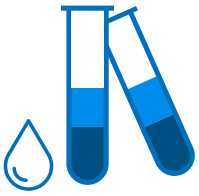
Analyte	MRM Transition (m/z)	CE (V)	Scan Time (msec)	Q1 Resolution	Q3 Resolution
Alprazolam	309-->281	22	25	0.7	0.7
Alprazolam-d5	314-->286	22	25	0.7	0.7
Clonazepam	316-->270	22	25	0.7	0.7
Clonazepam-d4	320-->274	20	25	0.7	0.7
Lorazepam	321-->275	17	25	0.7	0.7
Lorezepam-d4	325-->279	18	25	0.7	0.7
Diazepam	285-->193	22	25	0.7	0.7
Diazepam-d5	290-->198	22	25	0.7	0.7
Temazepam	301-->255	19	25	0.7	0.7
Temazepam-d5	396-->260	20	25	0.7	0.7
Oxazepam	287-->241	21	25	0.7	0.7
Oxazepam-d5	292-->246	22	25	0.7	0.7
Flurazepam	388-->315	21	25	0.7	0.7
Flurazepam-d10	398-->315	25	25	0.7	0.7
Triazolam	343-->308	22	25	0.7	0.7
Triazolam-d4	347-->243	39	25	0.7	0.7
2-Hydroxyethylflurazepam	333-->211	35	25	0.7	0.7
2-Hydroxyethylflurazepam d4	337-->211	35	25	0.7	0.7
7-Aminoclonazepam	286-->222	22	25	0.7	0.7
7-Aminoclonazepam d4	290-->121	33	25	0.7	0.7
7-Aminoflunitrazepam	284-->135	30	25	0.7	0.7
7-Aminoflunitrazepam d7	291-->138	30	25	0.7	0.7
alpha-Hydroxyalprazolam	325-->297	25	25	0.7	0.7
alpha-Hydroxyalprazolam d5	330-->221	35	25	0.7	0.7
alpha-Hydroxymidazolam	342-->324	21	25	0.7	0.7
alpha-Hydroxymidazolam d4	346-->328	21	25	0.7	0.7
Desalkylflurazepam	289-->140	35	25	0.7	0.7
Desalkylflurazepam d4	293-->230	29	25	0.7	0.7

Chromatography-Free Screening Workflow

of Benzodiazepine Drugs in Human Urine Using DART-MS Analysis

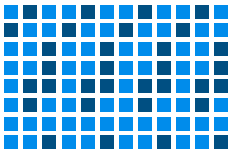
1. SAMPLE PREPARATION

1. Load sample
2. Enzyme hydrolysis
3. Dilute
4. Mix/Agitate
5. Quench enzyme



2. LIQUID LIQUID EXTRACTION

1. Add extraction solution
2. Mix/Agitate
3. Rest
4. Remove bottom aqueous layer
5. Dry down
6. Reconstitute



3. LOAD QUICKSTRIP HTS PLATE

1. Spot 8x12 format
2. Dry down

4. DART-MS ACQUISITION

1. Analysis of 96 samples in under 45 minutes
2. 27 seconds sample to sample

5. DATA REVIEW

1. Review calibration curve linearity and residual plot regression analysis
2. Review precision and accuracy
3. Identitiy screen hits

6. REPORT

1. Export to LIMS
2. Or printout

Results

Validation of DART-MS for quantitative screening of benzodiazepines in urine

DART-MS analysis for the panel of compounds resulted in good linear correlation with respect to the QC samples that were run showing that the results are adequate for use in screening with $R^2 > 0.995$ for all compounds measured. Additionally, the Lower Limit of Quantitation (LLOQ) was calculated for each analyte and demonstrated that this simple chromatography-free workflow is sufficient in detecting the compounds at levels at or below the common cutoffs within urine matrix (Table 4). Performance of this quantitative screening workflow is as good as or better than commonly used IA-based UBS assays, without the high rate of false positives associated with this assay type.

Table 4. Chromatography-free Benzodiazepine workflow quantitative data

Analyte	Range (ng/mL)	R^2	Slope	LOD (ng/mL)	LLOQ (ng/mL)	Recovery QC1 (150 ng/mL)	Recovery QC2 (3000 ng/mL)	Recovery QC3 (8000 ng/mL)	Repeatability (%RSD)
Alprazolam	50-10,000	0.999	0.010	2.2	7.5	101	105	99	9%
Clonazepam	50-10,000	0.999	0.006	16.0	53.4	104	99	101	12%
Lorazepam	50-1,000	0.998	0.005	15.5	51.6	102	105	--	9%
Diazepam	50-10,000	0.998	0.007	10.0	33.2	101	105	106	10%
Oxazepam	50-10,000	0.999	0.007	3.0	10.1	101	105	97	4%
Flurazepam	50-10,000	0.999	0.004	2.5	8.4	97	101	104	7%
Triazolam	50-10,000	0.998	0.005	0.1	0.5	99	110	107	9%
Desalkylflura-zepam	50-10,000	0.997	0.012	3.6	11.9	101	104	101	8%
7-Aminoclona-zepam	50-10,000	0.999	0.003	6.9	22.9	99	98	101	3%
7-Aminofluni-trazepam	50-10,000	0.999	0.006	4.9	16.3	101	102	110	6%
alpha-Hydroxyal-prazolam	50-10,000	0.998	0.012	0.0	0.1	98	100	110	12%
alpha-Hydroxy-midazolam	50-10,000	0.998	0.005	0.2	0.7	102	101	99	10%

Example for Flurazepam

An example of the DART-MS signal collected for Flurazepam is shown in Figure 1 below. This figure illustrates the characteristic raw 'unsmoothed' response that is generated by DART ionization that is used to quantify the sample. Although the nature of the DART-MS signal is not identical to that produced by chromatography-based MS measurements, similar levels of quantitative accuracy can be generated from this baseline-integrated signal from the entire DART sample analysis.

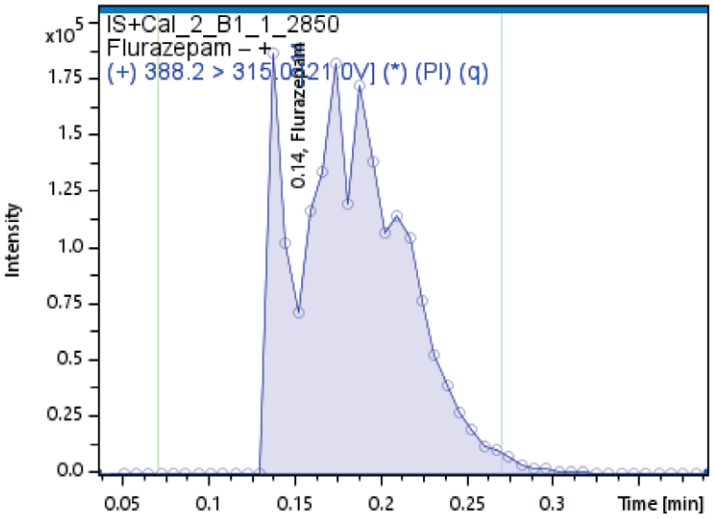


Figure 1. Flurazepam DART signal data at calibration level 2

Figure 2 shows an example of the calibration curve that was generated for Flurazepam with a linear R^2 correlation value > 0.999. Again, this shows the potential of DART-MS as a quantitative, chromatography-free tool and its ability to detect Flurazepam accurately and sensitively at confirmatory levels with high confidence.

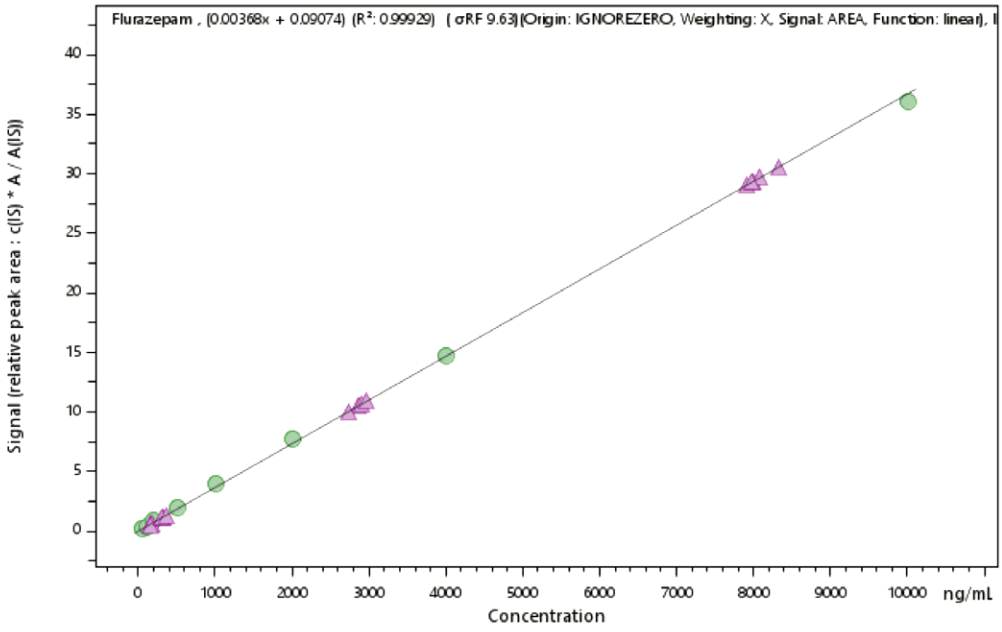


Figure 2. 8-Point calibration for Flurazepam

Conclusions

The work presented herein demonstrates the utility of DART-MS as a viable alternative to current UDS assays in rapid quantitative drug screening for urine. The chromatography-free workflow is faster, more accurate, and quantitative. In addition, the chromatography-free workflow has the benefit of improving predictive values for screening as compared to traditional immunoassay-based screening by preventing non-value-added work to yield higher productivity. This high-performance, quantitative workflow also eliminates the need for expensive and time-consuming chromatography-based confirmatory tests for common false positive results leading to considerable reduction in costly consumables and unnecessary sample preparation and data analysis times.

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