Development and Comparison of Two Rapid Screening Methods using DART-MS for the Detection of Designer Benzodiazepines in Violative Drug Products

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Abstract

Designer benzodiazepines (DBZDs) comprise a growing class of designer drugs that have similar chemical structures and pharmacological effects as their FDA-approved counterparts. The Forensic Chemistry Center (FCC) of the U.S. FDA routinely provides the FDA Office of Criminal Investigations with laboratory-based services to analyze suspect drug products, such as tablets and powders, potentially containing approved, unapproved, undeclared, and/or illicit drugs that pose a major public health risk. DBZDs have been identified in these products at the FCC with increased frequency. Additionally, such violative products have been encountered at international mail facilities and express courier hubs which can enter the U.S. supply chain if not impeded at these ports of entry. Therefore, the development of screening methods that can rapidly analyze potential violative products is an area of interest to the FDA and the FCC.

Direct analysis in real time ambient ionization mass spectrometry (DART-MS) for rapid screening has become a powerful tool to combat this concerning trend. The DART ionization source has the flexibility to be interfaced with a portable mass spectrometer for use within a mobile laboratory, or with a high resolution instrument within a traditional laboratory. In this research, eight samples (tablets and powders) from adjudicated criminal cases were analyzed, each containing one or more DBZDs (clonazolam, diclazepam, etizolam, flualprazolam, flubromazepam, flubromazolam, meclonazepam, and/or metizolam) that were identified and quantified using liquid chromatography-mass spectrometry (LC-MS) and high performance liquid chromatography coupled with ultra-violet spectroscopy (HPLC-UV), respectively, prior to rapid screening.

For rapid screening, the suspect products were analyzed using an IonSense DART ionization source coupled to either a Waters QDa detector with a thermal desorption unit (portable instrument) or to a Thermo Scientific Q Exactive Orbitrap (high resolution instrument). While the Orbitrap offered greater accuracy and multiple-component detection capabilities (88%) compared to the QDa (56%), the QDa detected at least one DBZD in 97% of the samples examined making it very attractive at ports of entry along with its mobility and ease of use. In this presentation, the advantages and disadvantages of the two rapid screening methods, such as sample preparation, throughput, software, library, etc., will be discussed.

Introduction

- Benzodiazepines (BZDs) are a class of drug that works to sedate or calm a person.[1] Common BZDs include alprazolam (Xanax) and diazepam (Valium). Nonfatal and fatal BZD overdoses increased from 2019 to 2020.[2] Most overdoses occur in combination with other drugs, such as synthetic opioids.

- Illicit designer BZDs (Figure 1) are novel psychoactive substances (NPS) marketed as “legal highs” and are functional analogs with similar chemical structures and clinical effects.[3] Many are taken in the form of tablets that are clandestinely produced to look indistinguishable from an authentic product; however, purity, potency, and active pharmaceutical ingredient (API) can be inconsistent.[3,4]

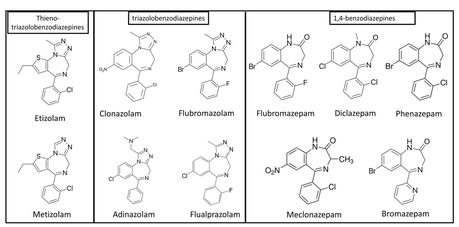

Figure 1. Benzodiazepines currently unapproved by the U.S. FDA[5]

- Therefore, there is increasing need to develop analytical methods to screen violative products at ports of entry, such as satellite laboratories at international mail facilities. Direct analysis in real time mass spectrometry (DART-MS) has become a valuable technique in the current satellite laboratory program at the FCC.

- In this work, the development of rapid methods for the detection of designer BZD in FDA-regulated products was conducted with a high resolution MS in the laboratory (DART-HRMS) and a portable MS (DART-TD-QDa). The advantages and disadvantages will be compared with respect to sample preparation, accuracy/precision, multi-component detection, and data collection and analysis.

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Introduction References
[1]National Institute of Drug Abuse
[2]S. Liu et al. Morbidity and Mortality Weekly Report 70 (2021) 1136
[3]S. Jones, E. Sisco, I. Marginean. Analytical Methods 12 (2020) 5433
[4]W.C. Samms et al. Journal of Forensic Science 56 (2011) 993
[5]M.M.Kimani et al. Forensic Science International 338 (2022) 111390

Materials and Methods

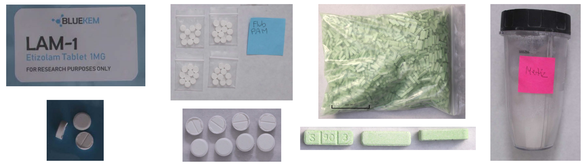

Figure 2. Tablets and powders from adjudicated criminal cases.

- Eight samples (tablets and powders) were acquired from adjudicated criminal cases and were analyzed by the two instruments. Four of the eight samples can be observed in Figure 2.

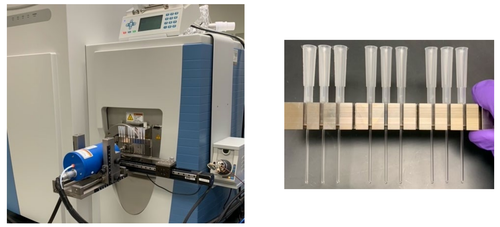

Figure 3. DART-HRMS instrument (left) and holder with DIP-it® tips (right) .

- Instrument was operated with positive polarity with an MS full scan of *m/z* 200-600 with an inclusion list, a resolution of 17,500, and an acquisition time of 0.35 minutes.
- MS method: Dynamic exclusion of 1 second and parent list with the monoisotopic protonated ions for BZDs studied with a mass tolerance of 10 ppm
- Sample preparation: DIP-it® tips were rolled across the surface of the tablets or powders were extracted in acetonitrile
- Using this system, 12 samples can be analyzed in ~6 minutes using an autosampler
- Software: Xcalibur was used for acquisition, mzVault for the library, and TraceFinder for processing

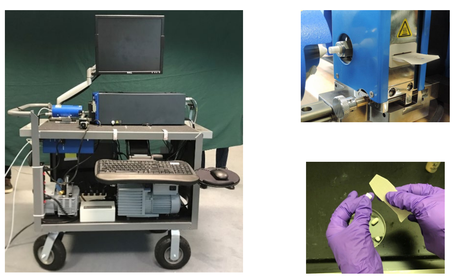

Figure 4. DART-TD-QDa instrument (left), thermal desorption unit (top right), and Teflon®-coated (PTFE) fiberglass sample traps (bottom right).

- Instrument was operated with positive polarity with an MS full scan of *m/z* 50-1250 with an acquisition time of 20 seconds.
- CID voltages 1 through 4 were 15, 30, 45, and 70 V, respectively
- Sample preparation: Tablets or powders were rubbed or pressed against the sample trap and excess powder was wiped off with a Kim Wipe. Additionally, highly concentrated powders could be extracted in acetonitrile and pipetted on traps.
- MS method: Software: MassLynx was used for acquisition, NextGenPIMISA for the library and processing

Results and Discussion

Figure 5. TraceFinder layout for data analysis using DART-HRMS

- Research Sample #1 can be observed as an example in Figure 5. Three tablets were analyzed with three replicates each. Clonazolam and etizolam were observed in all replicates.
- mzVault library can be built for APIs/drugs of interest with reference standards.
- Criteria for match during processing is based on a combination of isotopic pattern of $[M+H]^+$ and MS/MS fragmentation ions.
- Clonazolam highlighted in top right of Figure 5 and sample files collected in top left will be green or yellow if presence is detected in the sample and red if not.
- System is ideal for acquisition and processing large sets of data.

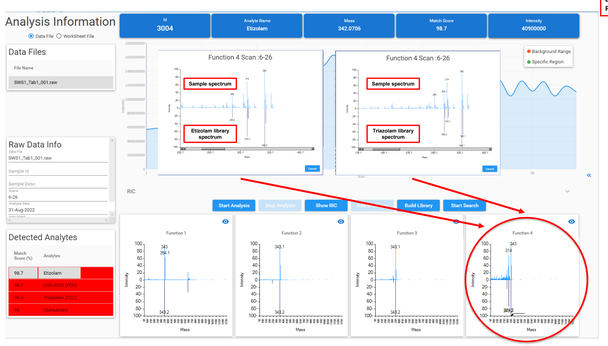

Figure 6. NextGenPIMISA layout for data analysis using DART-TD-QDa (top) and enlarged MS spectra for etizolam (left) and triazolam (right) library matches

- Research Sample #1 can be observed as an example in Figure 6. Three tablets were analyzed with two replicates each. Clonazolam and etizolam were observed in all replicates.
- Library can be built for APIs/drugs of interest with reference standards.
- Criteria for match during processing is based on $[M+H]^+$ and MS/MS fragmentation ions collected using four different collision energies.
- Library matches with name and match score can be observed in bottom left of Figure 6 for the processing software layout. Once highlighted, in each of the four function voltages boxes at the bottom, the ions observed above the x-axis are for the sample and below are the library spectra for the matched compound.
- For most of the BZDs studies, the $[M+H]^+$ dominates Functions 1 through 3, and Function 4 offers much more fragmentation that is valuable to distinguish compounds with similar monoisotopic masses.
- A great match in Function 4 was observed for etizolam (enlarged above; left) . Since the QDa cannot select, individually isolate, and fragment each monoisotopic ion and there is no chromatographic separation, library matches, such as triazolam (enlarged above; right), can result from the combination of fragment ions from each API present in the sample.

*Value in parenthesis was the number of tablets analyzed. For each tablet, N=3. Powders N=3.

Research Samples*	Labeling	DART Screening: HRMS	DART Screening: Accuracy	Other Instrumentation: FTIR	Other Instrumentation: LC-MS	Other Instrumentation: HPLC-UV
#1 tablets (3)	Etizolam (1 mg)	Clonazolam Etizolam	✚ ✚	Clonazolam	Clonazolam Etizolam	2.31 ± 0.09 1.06 ± 0.06
#2 tablets (3)	Flubromazolam (0.5 mg)	Flualprazolam Flubromazepam Etizolam Metizolam	✚ ✚ ✚ 1/9	Flualprazolam Flubromazepam	Flualprazolam Flubromazepam Etizolam Metizolam	0.45 ± 0.03 0.27 ± 0.01 0.14 ± 0.01 N/A
#3 tablets (3)	Nitraz	Flualprazolam Flubromazepam Etizolam	✚ ✚ 7/9	Flualprazolam Flubromazepam	Flualprazolam Flubromazepam Etizolam	2.78 ± 0.09 0.49 ± 0.02 0.11 ± 0.01
#4 tablet (1)	Flubromazolam (0.5 mg)	Flubromazolam Etizolam	✚ ✚	Flubromazolam	Flubromazolam Etizolam	Not analyzed by HPLC-UV
#5 tablets (3)	Flub PAM	Flubromazepam Flualprazolam Etizolam	✚ ✚ ✚	Flubromazepam Flualprazolam	Flubromazepam Flualprazolam Etizolam	Not analyzed by HPLC-UV
#6 tablets (2)	Alprazolam (2 mg)	Meclonazepam Flualprazolam	✚ ✚	Meclonazepam	Meclonazepam Flualprazolam	Not analyzed by HPLC-UV
#7 powder	Metiz	Metizolam	8/9 ✚	Metizolam	Metizolam	Not analyzed by HPLC-UV
#8 powder	N/A	Diclazepam	✚	Diclazepam	Diclazepam Etizolam	Not analyzed by HPLC-UV

#7 required a more concentrated extract

Table 1. Summary of sample analysis using DART-HRMS

- DART-HRMS had the ability to match more components in each research sample giving an accuracy (relative to LC-MS) of ~88%. This setup struggled in detecting the least concentrated component in research samples #2, 3, and 8.
- Some false positives were observed due to overlapping isotopic patterns as many of these BZDs had similar structures and monoisotopic masses. For samples #3 and 5, meclonazepam was observed as library match with both the isotopic pattern and MS/MS fragmentation ions which requires future work to understand this particular false positive.

*Value in parenthesis was the number of tablets analyzed. For each tablet, N=2. Powders N=3.

Research Samples*	Labeling	DART Screening: QDa	DART Screening: Accuracy	Other Instrumentation: FTIR	Other Instrumentation: LC-MS	Other Instrumentation: HPLC-UV
#1 tablets (3)	Etizolam (1 mg)	Clonazolam Etizolam	✚ 4/6	Clonazolam	Clonazolam Etizolam	2.31 ± 0.09 1.06 ± 0.06
#2 tablets (3)	Flubromazolam (0.5 mg)	Flualprazolam Etizolam	4/6 3/6 1/6 with no match ✖	Flualprazolam Flubromazepam	Flualprazolam Flubromazepam Etizolam Metizolam ✖	0.45 ± 0.03 0.27 ± 0.01 0.14 ± 0.01 N/A
#3 tablets (3)	Nitraz	Flualprazolam Etizolam	✚ 5/6	Flualprazolam Flubromazepam	Flualprazolam Flubromazepam Etizolam	2.78 ± 0.09 0.49 ± 0.02 0.11 ± 0.01
#4 tablet (1)	Flubromazolam (0.5 mg)	Flubromazolam	✚	Flubromazolam	Flubromazolam Etizolam	Not analyzed by HPLC-UV
#5 tablets (3)	Flub PAM	Flualprazolam Etizolam	✚ ✚	Flubromazepam Flualprazolam	Flubromazepam Flualprazolam Etizolam	Not analyzed by HPLC-UV
#6 tablets (2)	Alpazolam (2 mg)	Meclonazepam	✚	Meclonazepam	Meclonazepam Flualprazolam ✖	Not analyzed by HPLC-UV
#7 powder	Metiz	Metizolam	✚	Metizolam	Metizolam	Not analyzed by HPLC-UV
#8 powder	N/A	Diclazepam	✚	Diclazepam	Diclazepam Etizolam ✖	Not analyzed by HPLC-UV

Carryover issues

Table 2. Summary of sample analysis using DART-TD-QDa

- DART-TD-QDa was more limited in its ability to analyze multi-component research samples (56% accuracy relative to LC-MS). While this was unsurprising, this configuration did identify at least one BZDs in 97% of the analyses conducted.
- The combination of the DART-TD-QDa and FT-IR, both currently used in FCC satellite laboratories, were able to observe all the BZDs identified using LC-MS with the exception of those with an ✖ .

Conclusion

- Both configurations offered rapid analysis with minimal sample preparation for the detection of BZDs in forensic drug samples. Each had the ability to build a library of compounds to suit the needs of the user.
- DART-HRMS offered greater resolution and a superior ability to achieve multi-component detection more consistently and accurately. It also offered higher throughput if an analyst is preparing and analyzing a larger number of samples due to the autosampler and TraceFinder software. The sample preparation for tablets was also minimally destructive.
- DART-TD-QDa offered a less expensive, mobile device with less space, power, and vacuum requirements. It was ideal for working with one sample at a time and a limited number of analytes which is very attractive for mobile detection outside of a full service laboratory. The combination of mobile orthogonal techniques, such as DART-TD-QDa and FT-IR, in this study was almost equivalent to that of a traditional laboratory.