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Fast polarity switching and MRM triggered automatic MS/MS applied to benzodiazepines and their metabolites in clinical and forensic analysis

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Introduction

Benzodiazepines are now among the most commonly-prescribed drugs, which increases their potential for addiction and abuse, and they are often found in combination with other drugs in drug-related fatalities or drug facilitated sexual assault cases. Using fast polarity switching and MRM triggered automatic MS/MS, a new method package was developed for the simultaneous screening and quantification of benzodiazepines and benzodiazepine-like substances, which are available in Japan and that are relevant in clinical and forensic cases. This method, and associated MS/MS data base, will be applied to both low dose and high dose benzodiazepines abuse in forensic analysis.

Methods

A standard sample solution was prepared by mixing 35 benzodiazepines, Zopiclone, Zolpidem and their metabolites in water (Table 1). Human urine samples (1mL) were initially adjusted to pH 5 with acetic acid and extracted into chloroform / isopropanol (3:1) by vortex mixing. After centrifugation, the organic phase was transferred to a clean tube and evaporated to dryness by a gentle stream of N2. The residue was dissolved in 500uL of water/methanol (9:1).

Table 1. List of Compounds

Hypnotic Drugs	Metabolites	
Alprazolam	a-Hydroxyalprazolam	
Bromazepam	3-OH ABBP	
Brotizolam	a-Hydroxybrotizolam	4-Hhydroxybrotizolam
Chlordiazepoxide	Desmethyldiazepam	Oxazepam
Clobazam	N-desmethylclobazam	
Clonazepam	7-Aminoclonazepam	7-Acetamidoclonazepam
Clorazepic acid	Desmethyldiazepam	Oxazepam
Clotiazepam	Desmethylclotiazepam	Clotiazepam Y-10247
Cloxazolam	Delorazepam	
Delorazepam	-	
Diazepam	Desmethyldiazepam	Oxazepam
Estazolam	Estazolam M-II	Estazolam M-IV
Ethyl loflazepate	Desalkylflurazepam	
Etizolam	8-Hydroxyetizolam	a-Hydroxyetizolam
Fludiazepam	Desalkylflurazepam	
Flunitrazepam	3-Hydroxyflunitrazepam	7-Aminoflunitrazepam
	7-Acetamidoflunitrazepam	
Flurazepam	1-Ethanolflurazepam	Desalkylflurazepam
Flutazolam	1-Ethanolflurazepam	
Flutoprazepam	Desalkylflurazepam	
Haloxazolam	Haloxazolam RAZ-609	
Lorazepam	-	
Lormetazepam	Lorazepam	
Medazepam	Desmethyldiazepam	Oxazepam
Mexazolam	Delorazepam	
Midazolam	a-Hydroxymidazolam	
Nimetazepam	7-Aminonimetazepam	7-Acetamidonimetazepam
Nitrazepam	7-Aaminonitrazepam	
Oxazepam	-	
Oxazolam	Desmethyldiazepam	Oxazepam
Prazepam	3-Hydroxyprazepam	Oxazepam
Quazepam	Desalkylflurazepam	Quazepam M-4
Rilmazafone	Rimazafone M-4	
Temazepam	Oxazepam	
Tofisopam	-	
Triazolam	a-Hydroxytriazolam	4-Hydroxytriazolam
Zopiclone	Zopiclone-N-oxide	N-desmetylzopiclone
Zolpidem	Zolpidem-COOH [main]	Zolpidem-COOH [minor]

Table 2. Analytical conditions

Accelerated Nexera LC method	
Column	Shim-pack XR-ODS Ⅲ (2.0 mmI.D. x 50 mmL., 1.6 um)
Mobile phase A	10 mM Ammonium formate
Mobile phase B	Methanol
Gradient program	5 %B (0 min) - 95 %B (5-6 min); 5 %B (6.01 - 10 min)
Flow rate	0.3mL/min
Column temperature	40 °C
Injection volume	5 uL
Cycle time	10 mins
Conventional LC conditions	
Column	Shim-pack FC-ODS (2.0 mmI.D. x 150 mmL., 3 um)
Gradient	5 %B (0 min) - 95 %B (15-20 min); 5 %B (20.01 - 30 min)
Cycle time	30 mins
LCMS-8030 Triple quadrupole analysis	
Ionization	ESI
Polarity	Simultaneous positive/negative switching (15msecs)
Gas flows	Nebulising 1.5L/min; Drying 10L/min
Source temperatures	Capillary 250 °C; Block 400 °C

System design options	
Nexera UPLC	Pressure range up to 130 MPa high-speed injection, overlap injection, near-zero carryover, precise solvent delivery and excellent reproducibility
LCMS-8030	Ultra fast polarity switching of 15 msec & ultra fast scan speed of up to 15,000 u/sec UFsweeper® technology dramatically minimizes cross talk and delivers excellent linearity with a wide dynamic range.

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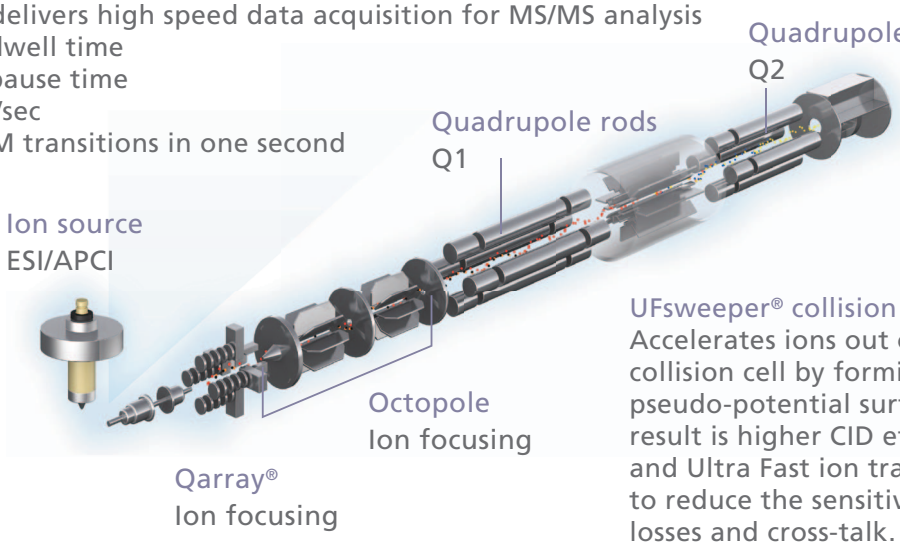
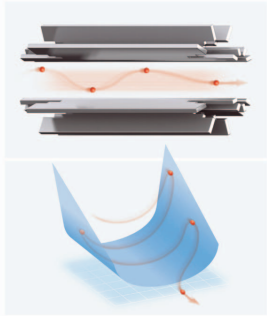
LCMS-8030 ion optics
System delivers high speed data acquisition for MS/MS analysis
1 msec dwell time
1 msec pause time
15,000u/sec
500 MRM transitions in one second



UFsweeper® collision cell
Accelerates ions out of the collision cell by forming a pseudo-potential surface. The result is higher CID efficiency and Ultra Fast ion transport to reduce the sensitivity losses and cross-talk.



Results

Benzodiazepine analysis has been accelerated by bringing together high resolution LC separations and high speed data acquisition LC/MS/MS system with fast polarity switching.

The method uses Synchronized Survey Scan® (in this mode MS/MS scanning is triggered by MRM signals) generating a full-product ion mass spectrum and MRM data.

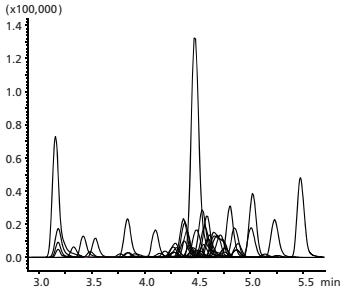


Figure 1.
Accelerated benzodiazepine analysis
MRM chromatograms obtained from analysis of a standard solution of 35 benzodiazepines, Zopiclone, Zolpidem and their metabolites (each 100 ng/mL). Using high resolution LC conditions and high speed MS/MS acquisitions the sample analysis time is reduced to 10 minutes compared to 30 minutes for the standard method.

It is also important to note the near zero carry over on sample injection plays a key part in minimizing errors and helping to improve sensitivity and precision.

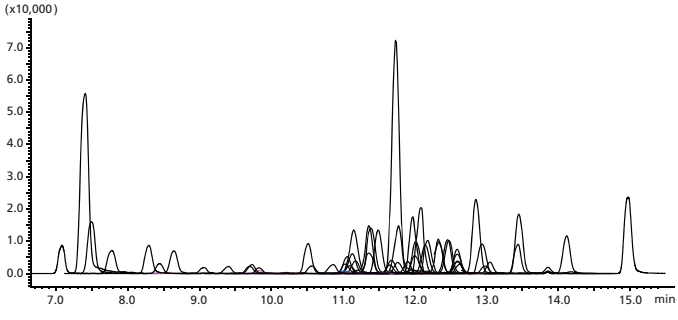


Figure 2.
Standard benzodiazepine analysis
MRM chromatograms obtained from analysis of a standard solution of 35 benzodiazepines, Zopiclone, Zolpidem and their metabolites (each 100 ng/mL) using a low resolution LC separation Shim-pack FC-ODS. The analysis time was 30 minutes.

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MS/MS library matching

Using a polarity switching speed of 15msec and a scan speed of 15,000u/sec, product ion spectra were generated in both positive and negative ionization which could be matched against a user library of 70 compounds an automated aid to screening and compound identification. Fast polarity switching helps to provide information rich product ion spectra resulting in better detection and identification for each benzodiazepine.

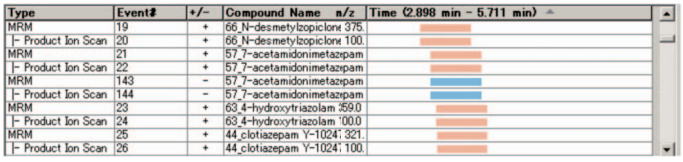
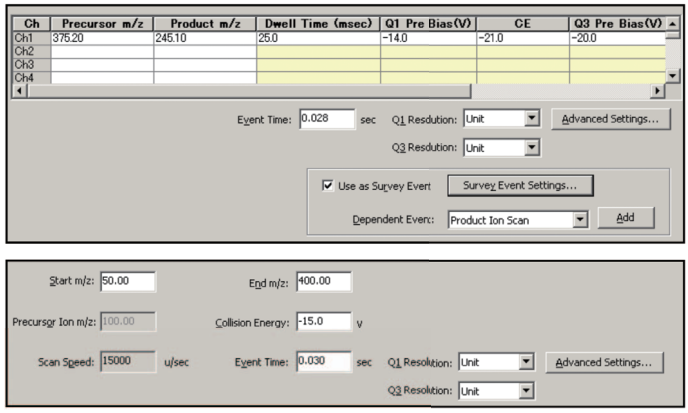


Figure 3.
Method set-up for accelerated benzodiazepine analysis using overlapping MRM/product ion scan acquisitions in both positive and negative mode (17 of 70 compounds were set both positive and negative mode).



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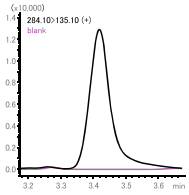
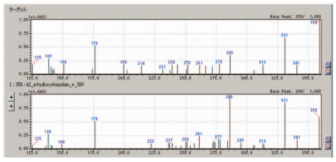
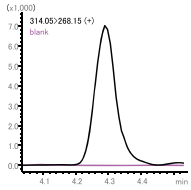
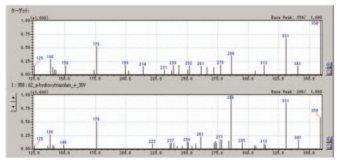
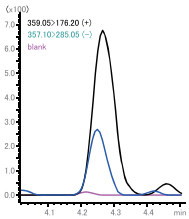
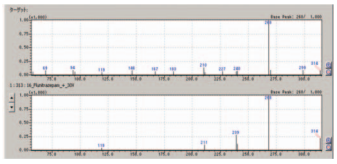
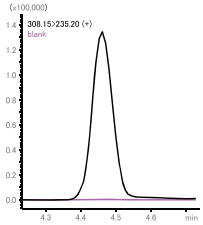
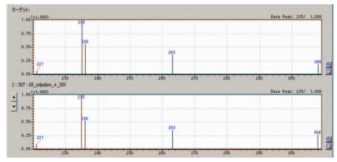
Compound	Library Result	Compound	Library Result
7-Amino flunitrazepam Rt 3.42 mins 	284.10 > 135.10 ; Positive ion 	Flunitrazepam Rt 4.29 mins 	314.05 > 268.15 ; Positive ion 
α-Hydroxy triazolam Rt 4.26 mins 	359.05 > 176.20 ; Positive ion 357.10 > 285.05 ; Negative ion 	Zolpidem Rt 4.46 mins 	308.15 > 235.20 ; Positive ion 

Figure 4. MRM chromatograms of 4 compounds (each 100 ng/mL) spiked into urine and analyzed by Nexera using Shim-pack XR-ODS III coupled to LCMS-8030 after sample preparation. As the LC/MS/MS system has a high speed of data acquisition, the assay generates both MRM and Product Ion Scan (MS/MS) spectra resulting in quantitative data and library searching/product matching to help product confirmation. Fast polarity switching helps to provide information rich product ion spectra resulting in better detection and identification for each benzodiazepine.

Conclusion

- With Nexera using Shim-pack XR-ODS III coupled to LCMS-8030 provides significant advantage over other method for benzodiazepine analysis: fast analysis time (all compounds are eluted in a retention window less than 3 minutes) and detected in both positive and negative ion using a single analytical run.
- To help forensic chemists this high speed MRM triggered automatic MS/MS and a new method package and database enabled simultaneous screening and quantification of benzodiazepines and their metabolites.
- This method will be applied to the forensic analysis of urine samples taken to confirm administration in cases of benzodiazepine abuse.

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