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Quantitative Multi Target Screening (MTS) using liquid chromatography-tandem mass spectrometry with MS/MS library based identification for forensic toxicology

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Alan Barnes[1]; Tiphaine Robin[2]; Simon Ashton[1];
Neil Loftus[1]; Pierre Marquet[2]; Sylvain Dulaurent[2];
Franck Saint-Marcoux[2];
[1]Shimadzu, Manchester, UK;
[2]CHU Limoges, Limoges, France.

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Introduction

Multi Target Screening (MTS) has been applied to systemic toxicological analysis to reduce false positive and negative reporting using MS/MS spectral library based identification. MTS methods uses threshold triggered multiple reaction monitoring (MRM) and MS/MS product ion scans at three collision energies to confirm the compound identification based on mass spectral library searching. The MS/MS library was created using certified reference materials and included electrospray spectral data from over 1200 compounds relevant to clinical and forensic toxicology in both positive and negative ion modes. The MTS approach was applied to screening whole blood samples at three concentration levels to evaluate screening at therapeutic, overdose and toxic concentrations.

Methods and Materials

MTS methods were developed to screen whole blood spiked with a range of commonly observed compounds including antidepressant compounds, anxiolytic drugs, analgesics and antipsychotic agents. Samples were prepared by QuEChERS method with inclusion of ten internal standard compounds to normalise sample matrix effects. Data acquisition parameters were set to a single MRM per compound with threshold triggered MS/MS at 3 collision energies (10, 35, 55V) enabling confirmation of parent ion (low) and fragment ions at medium and high CE voltages. Library searching was performed on all CE spectral data in addition to a merged-CE spectrum.

Table 1. LC-MS/MS data acquisition conditions. The method included full scan and MRM data acquisition in both positive and negative ion mode. 10 internal standard compounds were also included in the method.

Liquid chromatography

UHPLC	: Nexera LC system
Analytical column	: Restek Raptor Biphenyl 2.7 µm 100 x 2.1 mm
Column temp.	: 50°C
Injection cycle	: 5 µL injection volume
Flow rate	: 0.3 mL/min
Solvent A	: Water + 2mM ammonium formate + 0.002% formic acid
Solvent B	: Methanol + 2mM ammonium formate + 0.002% formic acid
Binary Gradient	:

Time (mins)	%B
1.00	5
2.00	40
10.50	100
13.00	100
13.01	5
17.00	Stop
11-14.2	0.5 mL/min

Mass spectrometry

LC-MS/MS	: LCMS-8060
Ionisation mode	: Heated ESI
Scan speed	: 30,000 u/sec
Polarity switching time	: 5 msec
MRM Dwell time	: 5 msec
Pause time	: 3 msec
Interface temp.	: 300°C
Heating block	: 400°C
Desolvation line	: 250°C
Heating gas	: 10 L/min
Drying gas	: 10 L/min
Nebulising gas	: 3 L/min
CID gas pressure	: 250kPa
Interface voltage	: 4 kV

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LC-MS/MS method set up for simultaneous full scan and MRM data acquisition with polarity switching

Type	Event	Polarity	Name | m/z	Time (0-13mins)
MRM	5	+	Target | 7-aminonitrazepam 252.10>121.10	
Product Ion Scan	6	+	> CE: -10, 30.00-1000.00	
Product Ion Scan	7	+	> CE: -35, 30.00-1000.00	
Product Ion Scan	8	+	> CE: -50, 30.00-1000.00	
MRM	9	+	Target | 7-aminoclonazepam 286.05>121.10	
Product Ion Scan	10	+	> CE: -10, 30.00-1000.00	
Product Ion Scan	11	+	> CE: -35, 30.00-1000.00	
Product Ion Scan	12	+	> CE: -50, 30.00-1000.00	
MRM	13	+	Target | 3-Hydroxybromazepam 322.00>287.00	
Product Ion Scan	14	+	> CE: -10, 30.00-1000.00	
Product Ion Scan	15	+	> CE: -35, 30.00-1000.00	

Spectral Library >1200 compounds

Each library spectrum was acquired by authentic standard flow injection at collision energies 10-60V. Compounds that ionised efficiently with more than one adduct state were saved resulting in 1476 Library entries from 1207 compounds (1278 positive mode, 229 negative mode). Spectral Library information was registered for CE 10, 35 and 55V. Optimised MRM transitions were determined for all compounds with chromatographic retention time and peak area measured to enable reference ion-ratio calculation. RT analysis included internal standard compounds for relative RT calculation. Compound information was populated including: CAS number, formula, synonyms, compound class/properties, ChemSpider URL and ID number, mol file, InChI and InChIKey.

Toxicological Screening

Compounds were spiked into whole blood, prepared in triplicate at a concentration range 1-1000 µg/L (calibration curves typically ranged 5-500 µg/L). Quality control samples were prepared (5x) at three concentrations (20, 100, 500 µg/L). Two MTS methods were prepared, the first measuring benzodiazepines (36 compounds), the second measuring antiepileptics, antipsychotics, barbiturates and cannabinoids (35 compounds).

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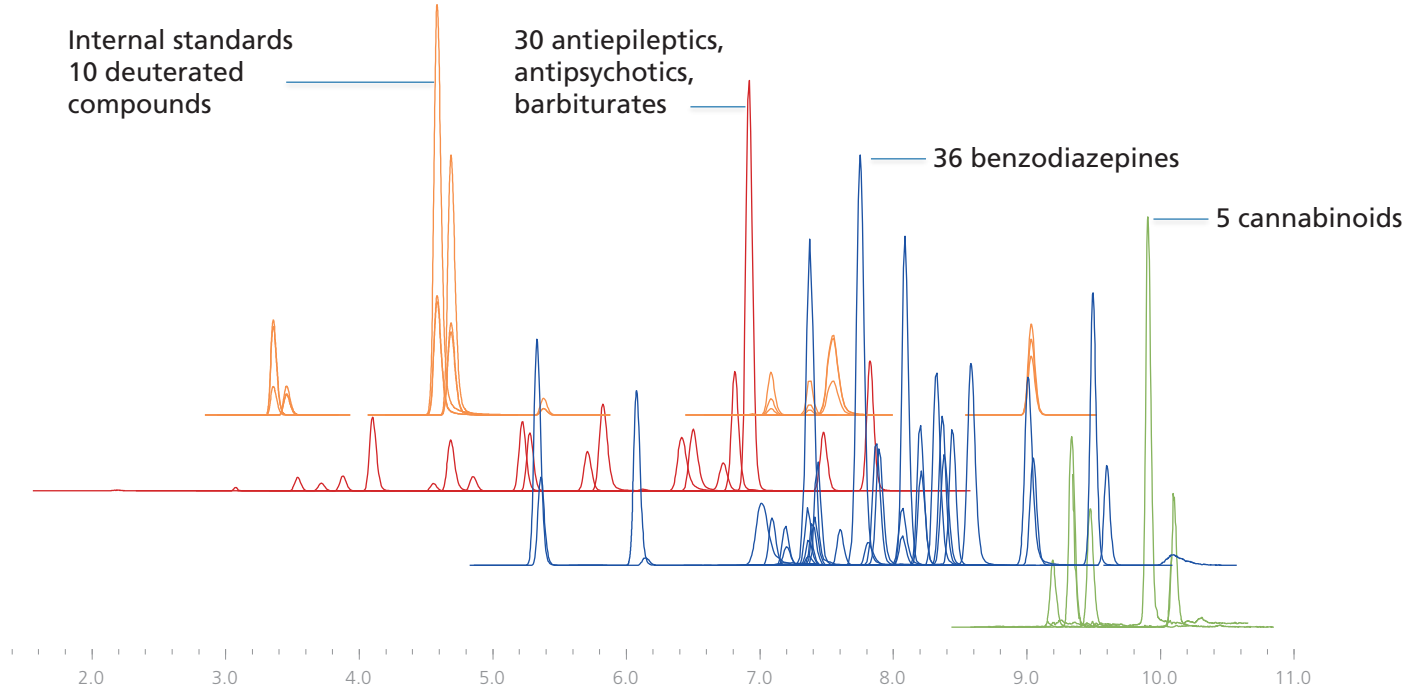

Figure 1. MRM chromatograms for a panel of drugs extracted from whole blood using a QuEChERS method corresponding to a concentration of 100 µg/L

Results

Quantitative analysis

The scope of the method was to ensure robust quantitation and a high level of confidence in the reported result. Using a MRM method followed by three product ion scans at different collision energies resulted in linear calibration curves over the concentration range of 5-500 µg/L (r2 >0.996 for all compounds). With regard to accuracy and precision; accuracy was between 80-120% and precision <20% throughout the calibration range.

Using a pause time of 3msecs and a dwell time of 5msec, the scan time was set to 50msecs (scanning from 30-1000u). As a result of fast data scanning, the peak sampling rate resulted in more than 20 data points across a peak.

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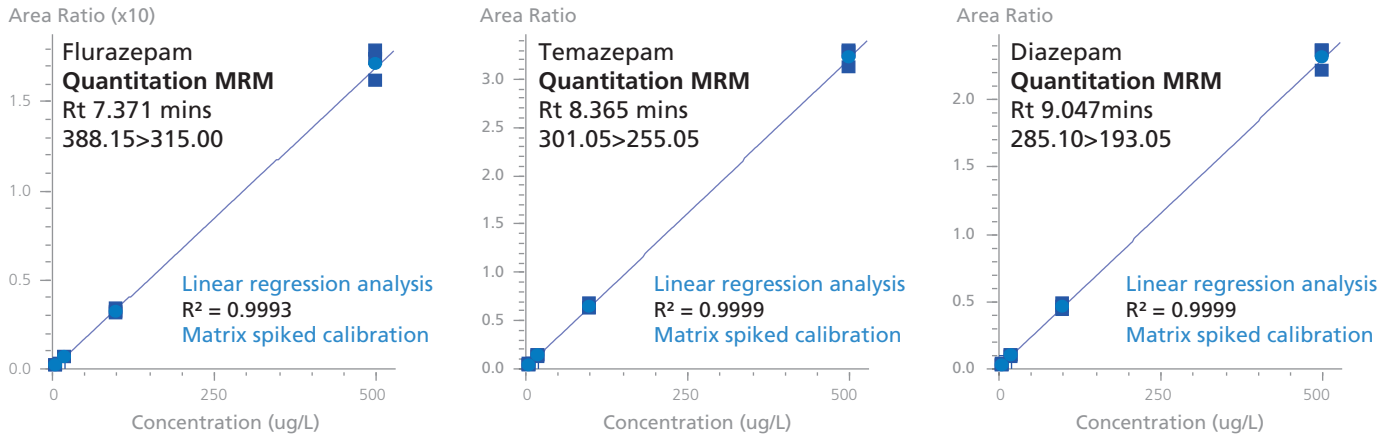
MRM quantitation



	Rt (mins)	MRM	Mean Accuracy				Repeatability			
			Calibration standards (n=3 for each calibration level)				20ug/L replicate (n=5)		100ug/L replicate (n=5)	
			5ug/L	20ug/L	100ug/L	500ug/L	Mean Conc (ug/L)	%RSD	Mean Conc (ug/L)	%RSD
Flurazepam	7.371	388.15>315.00	109.5	95.5	95.3	101.1	18.1	3.0	100.2	7.7
Temazepam	8.365	301.05>255.05	103.4	97.8	99.1	100.2	18.8	1.9	101.5	6.0
Diazepam	9.047	285.10>193.05	102.0	99.5	98.6	100.3	18.6	2.5	100.2	5.6

Figure 2. Calibration curve data for flurazepam, temazepam and diazepam spiked into whole blood and extracted using QuEChERS together with results for accuracy and reproducibility at two different concentrations (20 µg/L and 100 µg/L; n=5; %RSD less than 8%).

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MRM triggered product ion spectrum

MRM data was used to generate robust quantitation and also to help trigger product ion scans at three different collision energies.

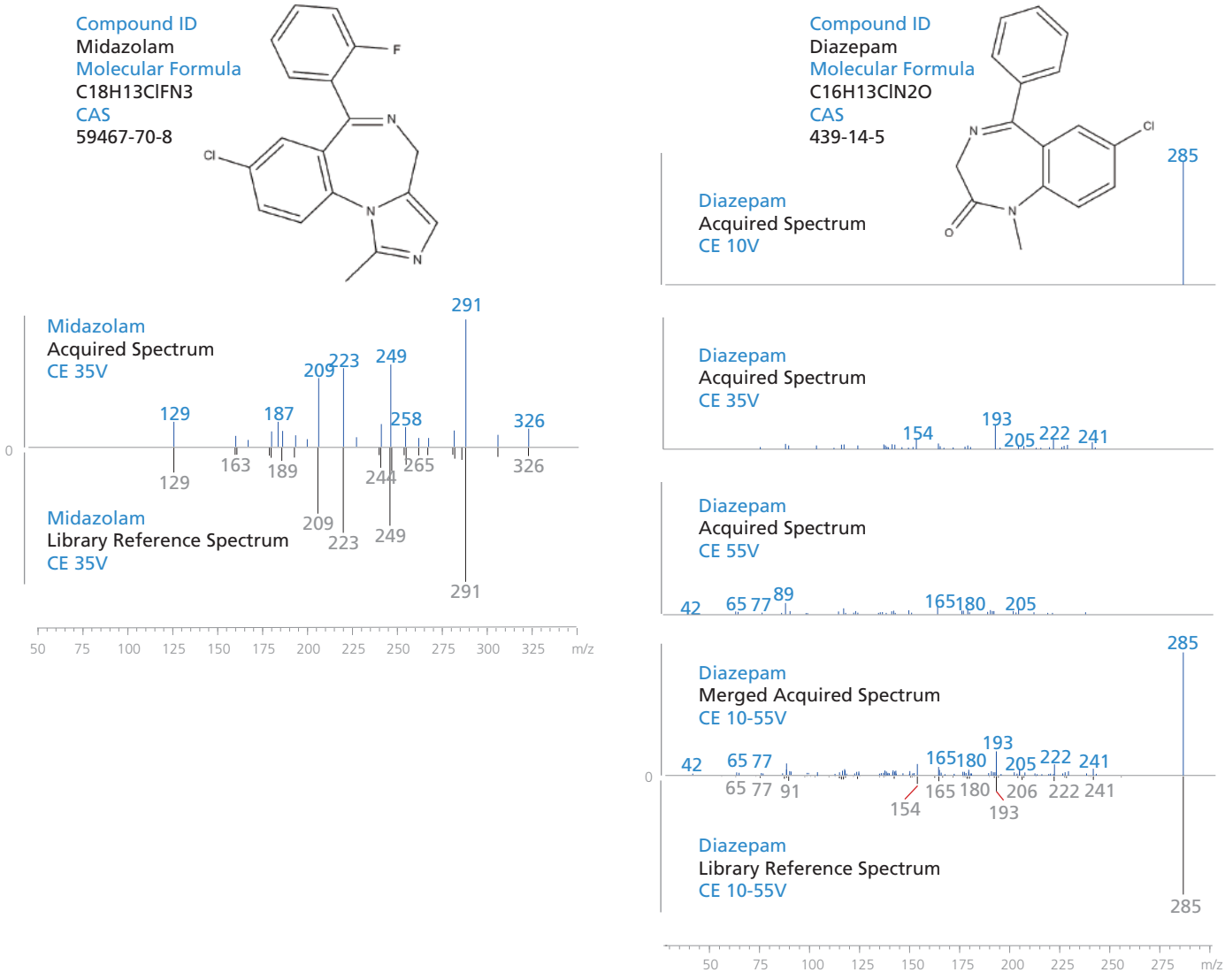

Figure 3. MRM triggered product ion spectrum data for midazolam and diazepam. The library included spectra for each collision energy and a separate library for merged spectra enabling match criteria to be set for a specific fragmentation voltage (as shown for midazolam) or to use a broad band fragmentation and merged spectra (as in the case for diazepam).

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Library hits

A MTS procedure for clinical and forensic toxicology screening was developed for a single LC/MS/MS method following a QuEChERS extraction of whole blood. This approach results in robust quantitation using MRM data and enables a higher degree of confidence in compound identification as shown in Table 2.

Table 2. Library search results for a panel of drugs spiked into whole blood and extracted by QuEChERS from three QC levels (low, medium and high QC's correspond to 20, 100, 500 ug/L). Most compounds can be identified as the first hit in a spectral based library match (5 compounds are identified as the second candidate in the library; for 4 compounds the hit was not identified as either the first or second candidate).

Compound	RT (min)	Quality control level			Compound	RT (min)	Quality control level		
		Low	Medium	High			Low	Medium	High
		Library Hit					Library Hit		
		Merged CE spectrum					Merged CE spectrum		
Paracetamol	3.02	2	1	1	2-(2-amino-5-bromobenzoyl)pyridine	7.43	1	1	1
Levetiracetam	3.49	1	1	1	Dextropropoxyphene	7.44	1	1	1
Theophylline	3.67	1	1	1	Desalkylflurazepam	7.60	-	1	1
Scopolamine	4.05	1	1	1	Zolpidem	7.75	1	1	1
Felbamate	4.51	1	2	1	Hydroxyzine	7.80	1	1	1
Lamotrigine	4.81	2	2	1	Hydroxyalprazolam	7.82	1	1	1
Tramadol	5.18	1	1	1	4-hydroxymidazolam	7.87	1	1	1
10-hydroxycarbamazepine	5.24	1	1	1	Chlordiazepoxide	7.89	1	1	1
7-aminonitrazepam	5.33	1	1	1	1-hydroxymidazolam	8.07	1	1	1
7-aminoclonazepam	5.36	1	1	1	Nordiazepam	8.07	-	1	1
Ketamine	5.67	1	1	1	Clobazam	8.09	1	1	1
Niaprazine	5.77	1	1	1	Flunitrazepam	8.20	1	1	1
Norbuprenorphine	6.08	1	1	1	Lormetazepam	8.21	1	1	1
3-Hydroxybromazepam	6.15	1	1	1	Estazolam	8.32	1	1	1
Doxylamine	6.36	1	1	1	Temazepam	8.37	1	1	1
LSD	6.45	1	1	1	Triazolam	8.38	1	1	1
Diphenhydramine	6.78	1	-	1	Ethyl loflazepate	8.44	1	1	1
Carbamazepine	6.90	1	1	1	Alprazolam	8.58	1	1	1
Zopiclone	6.99	1	1	1	Midazolam	9.01	2	1	1
Desmethylflunitrazepam	7.09	1	1	1	Diazepam	9.05	1	1	1
N-desmethylclobazam	7.19	1	1	1	11-OH-THC	9.23	1	1	1
Lorazepam	7.20	1	1	1	THC	9.37	1	1	1
3-hydroxy-flunitrazepam	7.36	1	1	1	Clotiazepam	9.49	1	1	1
Oxazepam	7.37	-	1	1	THC-COOH	9.51	1	1	1
Flurazepam	7.37	1	1	1	Tetrazepam	9.60	1	1	1
Clonazepam	7.40	1	1	1	Cannabinol	9.94	1	1	1
Nitrazepam	7.41	1	1	1	Loprazolam	10.09	1	1	1

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Conclusions

A spectral based library of more than 1200 compounds has been created using certified reference materials acquired at three collision energies on a triple quadrupole mass spectrometry platform.
A MRM triggered product ion spectra method to quantify and identify a panel of compounds commonly found in clinical and forensic toxicology was successfully applied to whole blood samples spiked with a panel of compounds. All compounds were detected at highest concentration and positively identified using product ion scan MS/MS library based searching generating higher data quality for compound identification.

Disclaimer: The Shimadzu LCMS-8060 is intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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