Toxicology/DAU Testing by Mass Spectrometry

AACC Conference: Mass Spectrometry in the Clinical Lab
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Disclosures

Research Support (equipment):

Mass Spectrometry instrumentation provided to our laboratory for collaboration projects or on consignment for a period of time:

Agilent
Thermo Fisher
AB SCIEX
Bruker

Overview

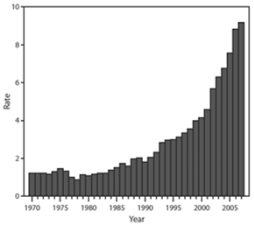
- Importance of toxicology testing
- Traditional drug screening approaches
- Limitations of Immunoassays for drug screening
- Mass spectrometry methods for drug screening
- Comparison of published drug screening methods
- Advantages and disadvantages of GC-MS
- Advantages and disadvantages of LC-MS/MS
- Advantages and disadvantages of LC-HRMS

Why is toxicology testing important?

- More than 2.3 million toxic exposures reported annually (AAPCC)
- Exposures involve more than 2.7 million agents (half are pharmaceuticals)
- Top reasons for seeking emergency care (trauma, resp. or cardiac distress, neurological changes, seizures, comas) could all result from drug exposure
- Accurate history of exposure is often not possible
- The illicit drug market is constantly evolving
- Knowledge of the substance facilitates the most effective care – shortest hospital stay – and prevents further medical workups

Rates of unintentional overdose / exposure

Rate of unintentional Rx overdose deaths (CDC)



Death rate per 100,000

Substances with greatest rate of exposure increase
AAPCC 2011 annual report

Table 17B. Substance categories with the greatest rate of exposure increase (Top 25).

Substance (Major Generic Category)	Increase in exposures per year* Mean	95% CI*	All substances in 2011
Analgesics	10,134	[12435, 7833]	322,016
Sedative/Hypnotics/Antipsychotics	7,959	[9083, 6835]	168,416
Cardiovascular Drugs	4,795	[5087, 4503]	102,766
Antihistamines	3,531	[4103, 2959]	94,159
Alcohols	2,129	[3163, 1095]	74,484
Vitamins	2,118	[2509, 1727]	70,195
Hormones and Hormone Antagonists	1,860	[2143, 1578]	60,234
Anticonvulsants	1,846	[2133, 1558]	49,607
Gastrointestinal Preparations	1,840	[2540, 1140]	50,414
Antidepressants	1,521	[2360, 682]	107,528
Other/Unknown Nondrug Substances	1,496	[2118, 873]	28,035
Cosmetics/Personal Care Products	1,312	[2604, −70]	218,269
Stimulants and Street Drugs	1,231	[2136, 326]	66,540
Topical Preparations	1,223	[2519, −72]	102,692
Muscle Relaxants	1,168	[1533, 1002]	28,857
Dietary Supplements/Herbals/Homeopathic	1,074	[1507, 641]	35,565
Anticholinergic Drugs	1,051	[1309, 792]	11,352
Miscellaneous Drugs	1,034	[1478, 590]	23,489
Antimicrobials	773	[1213, 333]	65,856
Unknown Drug	754	[861, 647]	21,184
Essential Oils	638	[695, 581]	10,906
Deodorizers	607	[842, 371]	24,255
Cleaning Substances (Household)	603	[2559, −1353]	192,771
Foreign Bodies/Toys/Miscellaneous	511	[1770, −749]	112,562
Anticoagulants	488	[521, 454]	8,315

*Increase and confidence intervals are based on least squares linear regression of the number of calls per year for 2000–2011.

Traditional Drug Screening Approach

Urine Drug Screen (utox) or "Complete" Drug Screen (CDS)
Immunoassay + GC-MS + LC-MS/MS

UTOX or CDS ordered
- What about emergency cases? What about other matrixes?
- urine is not indicative of the current clinical status of a patient (exposure history)

Immuno-assay Screen
- IAs or ELISAs are not available for all drugs of clinical interest
- sensitivity and specificity – prone to false positives and false negatives

Confirm-ation
- GC/MS and/or LC-MS/MS confirmation testing not always available
- Long turn-around times
- Usually targeted in nature limiting detection of "all" or "new" compounds

Drug Screening Panels by IA: Not "comprehensive"

Drugs commonly in "Drug of Abuse" and/or "Pain Management" Panels
Amphetamines
Opiates
Benzodiazepines
Cocaine
Barbiturates
Methadone
Phencyclidine (PCP)
Marijuana (THC)
Oxycodone

IA Drug Screening Panels do not include all possible toxicological exposures

Drug Screening by MS

- Opioids
- Benzodiazepines
- Amphetamine-type stimulants
- Psychoactive drugs
- Antidepressants
- Sedative-hypnotics
- Analgesics
- Muscle relaxants
- Anticonvulsants
- Antipsychotics
- Anesthetics
- Antihistamines
- Other Rx and illicit drugs

Immunoassay Limitations: Opioids

Opiate Immunoassay Cross-reactivity

	Online DAT opiates II[1] assay	EMIT II+ opiate assay[2]	TDx/TDx-flex opiate opiate assay[3]	Architect/ Aeroset	AxSym opiate[3]	CEDIA opiate[4]	DRI opiate[4]	DRI oxycodone[4]
Morphine	100	100	100	100	100	100	100	<29
Codeine	134	98	>3.6	167	>3.6	125	167	<20
Ethyl morphine	101		<10		>100			
Diacetyl morphine (heroin)	82					53	86	<33
6-Acetylmorphine	78	69	>20	67	<30	81	79	<200
Dihydrocodeine	69	103	>3.6	106	>3.6	50	67	<100
Morphine-3-glucuronide	54	48	>57	47	>57	81	50	<11
Morphine-6-glucuronide			>5.7		<8.6	47	100	
Hydrocodone	28	121	>8.0	158	>12	48	18	<133
Hydromorphone	21	60	>4.4	54	>6.7	57	7.5	<333
Norcodeine	2							<10
Normorphine							0	<10
Oxycodone	0	12	>1.1	11	<1.7	3.1	1.9	100
Oxymorphone		1.5	<10	0	<15	1.9	0.7	103
Noroxycodone								<0.1
Noroxymorphone								<0.1
Meperidine	0	<0.6	<2.0	0	<3.0	0.2	0	
Levallorphan		<4	<6.0	13	<6.0			
Levorphanol		29	>6.0	27	>6.0		2.1	<50
Nalorphine		3	<20	2.3	<30			
Naltrexone	0	0.04	<20	0	<30		0	<50
Imipramine	0					1.6		
Ranitidine						0	0	
Thebaine	25		<20		<30		<15	
Naltrexone	0						0	<20
Fentanyl			<40		<60			

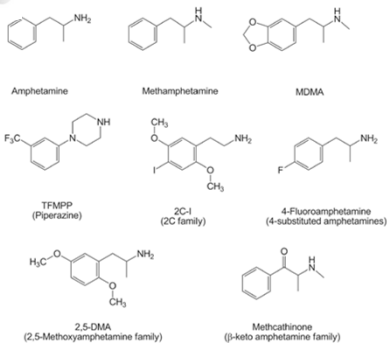
Immunoassay Limitations: Benzodiazepines

Benzodiazepines

- Limited immunoassay cross-reactivity
- Prone to false negatives
- Do not detect atypical benzodiazepine receptor ligands: eszopiclone (lunestra), zaleplon (sonata), zolpidem (ambien), etc.

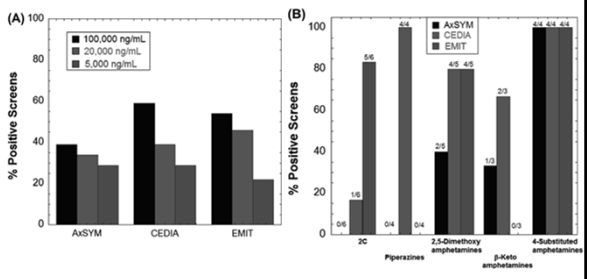
Compound	Without ß-Glucuronidase Tested ng/mL	Without ß-Glucuronidase %Cross-Reactivity	With ß-Glucuronidase Tested ng/mL	With ß-Glucuronidase %Cross-Reactivity
7-NH$_2$-Flunitrazepam	-	-	200	99
7-NH$_2$-Nitrazepam	-	-	250	83
α-OH-Alprazolam	163	188	115	167
α-OH-Triazolam	150	193	125	155
Alprazolam	138	205	100	220
Alprazolam glucuronide	-	-	200	100
Bromazepam	300	110	190	104
Chlordiazepoxide	2083	13	1200	16
Clobazam	400	62	300	59
Clonazepam	188	140	225	71
Clonazepate	325	84	300	75
Delorazepam	150	184	100	197
Demoxepam	1900	14	1000	19
Desalkylflurazepam	138	210	115	173
Diazepam	110	247	125	154
Estazolam	125	220	95	239
Flunitrazepam	188	135	175	109
Flurazepam	150	189	100	195
Haloazepam	200	145	200	101
Lorazepam	208	122	175	115
Lorazepam glucuronide	10000	1	400	45
Lormetazepam	163	165	150	137
Medazepam	200	135	150	118
NH$_2$-Clonazepam	-	-	200	96
Nitrazepam	300	100	200	100
Nordazepam	150	211	120	173
Oxaprozin	10000	2	10000	2
Oxazepam	275	107	165	125
Oxazepam glucuronide	10000	1	800	25
Prazepam	150	184	160	116
Temazepam	175	144	180	93
Temazepam glucuronide	10000	1	750	25
Triazolam	138	191	90	217

Immunoassay Limitations: Designer Drugs



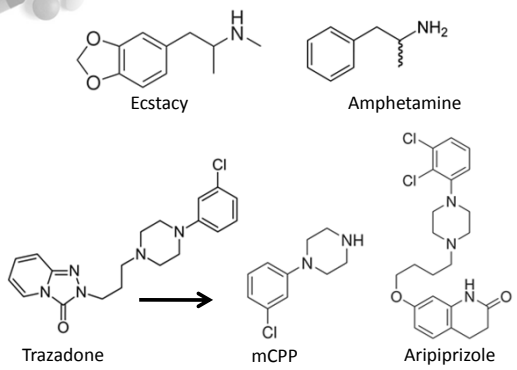
Petrie MS, et al. Clinical Toxicology 2013

Immunoassay Limitations: Designer Drugs



Petrie MS, et al. Clinical Toxicology 2013

Immunoassay Limitations: Rx cross-reactivity



Immunoassay Limitations: Rx cross-reactivity

Trade Name	Generic Name	Immunoassay	LC-MS
Adderall, Dexedrine, Dextrostat, Vyvanse	d-amphetamine	+	+AMPH
Desoxyn	methamphetamine	+	+METH +AMPH
Anipryl, Zelapar	Selegiline	+	+METH +AMPH
Various Rx Drugs	Phentermine, Bupropion, Trazadone, Sildenafil	+ (at high [ ])	-
Ritalin, Concerta, Daytrana, Metadate, Methylin	methylphenidate	-	-
Focalin	dexmethylphenidate	-	-
Ephedrine, Pseudoephedrine		-	-

Other Testing Methodologies

Testing Methods	Abbreviation
Point-of-care assays	POC
Radioimmunoassay	RIA
Enzyme-linked immunosorbent assay	ELISA
Enzyme-multiplied immunoassay technique	EMIT
Cloned enzyme donor immunoassay	CEDIA
Fluorescence polarization immunoassay	FPIA
Liquid chromatography with ultraviolet detection	HPLC-UV
Gas chromatography mass spectrometry	**GC-MS**
Liquid chromatography tandem mass spectrometry	**LC-MS/MS**
Liquid chromatography time-of-flight mass spectrometry	**LC-TOF**
Liquid chromatography high resolution mass spectrometry	**LC-HRMS**

GC-MS Advantages and Disadvantages

Advantages:
- Increased sensitivity compared to IA and LC-UV
- High-reproducibility in generated mass spectra
- Large transferable mass spectral libraries available
- Coupled to a headspace autosampler – ideal for volatile analysis
- Long standing "gold-standard" for drug testing

Disadvantages:
- Cannot detect non-volatile, polar and thermally labile compounds
- Requires lengthy sample preparation (hydrolysis/ derivatization)

LC-MS Advantages and Disadvantages

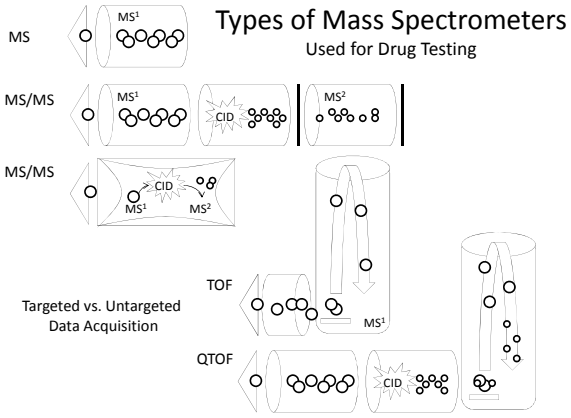
(compared to GC-MS)

Advantages:
- Increased sensitivity and specificity
- Minimal sample preparation (sample already in aqueous matrix)
- Can differentiate co-eluting compounds more easily
- Can detect non-volatile, polar and thermally labile compounds
- The "new gold standard" for toxicology testing?

Disadvantages:
- Large transferable mass spectral libraries do not exist
- Matrix effects can be an issue and need to be investigated and defined in method validation

Types of Mass Spectrometers

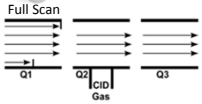
Used for Drug Testing



Targeted vs. Untargeted Data Acquisition

Drug Testing by MS and MS/MS: scan modes

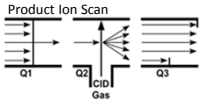
Full Scan



Untargeted: scan all sample components in a defined mass range

Output: nominal mass of all compounds

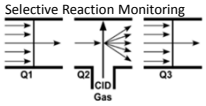
Product Ion Scan



Targeted : scan and select compounds of a given mass - fragment them and scan all product ions in a defined mass range

Output: nominal mass of compounds of interest and product ion spectrum

Selective Reaction Monitoring



Targeted : scan and select compounds of a given mass - fragment them and scan for a single product ion

Output: nominal mass of compounds of interest and selected product ion

Drug Testing by MS: Harmonization?

Table 2. Broad spectrum drug screens detecting greater than 200 drugs/metabolites

Compounds	Matrix	Sample Prep	Stationary Phase	Ion Source	Mass Analyzer	Targeted/ Untargeted	Acquisition Mode	Identification Criteria	Run Time	Year, Reference
400 drugs	Serum	SPE	C8 (125x2mm, 5µm)	ESI +	MS	untargeted	full scan, in-source CID	RT, nominal mass, library search	32 min	1999, 12
~1300 drugs	Serum	SPE	C18 (150x1mm, 5µm)	ESI +/-	MS	untargeted	full scan, in-source CID	RT, nominal mass, library search	50 min	2000, 13
238 drugs	Blood	LLE	C18 (100x2.1mm, 4µm)	ESI +	MS/MS (QqQ)	targeted	SRM	RT, SRM	8 min + post time	2003, 14
301 drugs	Serum	LLE or SPE	C18 (150x2mm, 4µm)	ESI +	MS/MS (QqLIT)	targeted	SRM-IDA-EPI	RT, SRM, library search	33 min	2005, 15
700 drugs	Urine, Serum	dilute and shoot or LLE	PFP (50x2.1mm, 5µm)	ESI +	MS/MS (QqLIT)	targeted	SRM-IDA-EPI	RT, SRM, library search	17.5 min	2010, 16
1000 drugs	Serum, Urine	SPE	C18 (100x2.1mm, 3.5µm)	ESI +/-	MS/MS (QqLIT)	untargeted	full scan-IDA-EPI	RT, nominal mass, library search	25.5 min	2006, 17
365 drugs	Serum, Urine	On-line SPE	C18 (125x4mm, 3µm)	APCI +/-	MS/MS (LIT)	untargeted	full scan-DDA-PI	RT, nominal mass, library search	23 min	2010, 18
392 drugs	Urine	SPE (enzymatic cleavage)	C18 (100x2mm, 3µm)	ESI +	TOF	untargeted	full scan	RT, accurate mass	24 min	2003, 21
735 drugs	Urine	SPE (enzymatic cleavage)	C18 (100x2mm, 3µm)	ESI +	TOF	untargeted	full scan	RT, accurate mass, isotope pattern	21 min + post time	2006, 22
~300 drugs	Urine	LLE	HSS T3 (100x2.1mm, 1.8µm)	ESI +	TOF	untargeted	full scan, in-source CID	RT, accurate mass, isotope pattern, library search	17 min	2009, 23

Drug Testing by MS: More than one method needed?

Table V. Drug and Metabolite Detection Rate and Positive Predictive Value for Individual and Combined Screening Methods

	GC–MS	UPLC–MS	UPLC–MS–MS	GC–MS + UPLC–MS	GC–MS + UPLC–MS–MS	UPLC–MS UPLC–MS–MS
Drugs detected	796	819	856	1011	1033	1060
Detection sensitivity	71.0%	73.1%	76.4%	90.20%	92.20%	94.60%
No. of screen-positive confirmation tests performed	785	649	674	816	816	805
No. of confirmed screen-positive tests	774	646	665	804	799	794
Positive predictive value	98.60%	99.50%	98.7%	98.50%	97.90%	98.60%

Rosano TG, et al. J Anal Tox 2011;35:411-423

- Which instrument combination is best? LC-MS/MS + GC-MS or LC-MS/MS + LC-HRMS or just LC-HRMS
- Which approach is capable of identifying the most drugs and metabolites in routine clinical samples?
- Is a targeted or untargeted approach better?

Drug Testing by MS: More than one method needed?

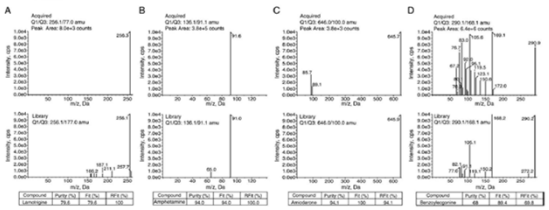
Table 3
Example data obtained with six authentic urine samples; verification with conventional screening techniques.

Sample ID	Analytes	UPLC-TOF	REMEDi	GC–MS	HPLC-DAD	UPLC-MS/MS
07CT199733	Benzoylecgonine	+	--	--	+	+
	Cocaine	+	--	+	--	+
	Codeine,	+	+	+	+	+
	Codeine, N-desmethyl	+	--	+	+	NM
	Ecgonine methyl ester	+	+	NM	--	NM
	Phenylpropanolamine/pseudoephedrine, nor	+	--	+	--	NM
	Promethazine, hydroxydesmethyl	+	NM	NM	NM	NM
	Promethazine, sulfoxide	+	+	NM	NM	NM
	Pseudoephedrine/ephedrine	+	+	+	--	+
07CT232829	Amphetamine	+	+	+	--	+
	Benzoylecgonine	+	--	+	+	+
	Cetirizine	+	NM	--	--	+
	Cocaine	+	+	+	--	+
	Ecgonine methyl ester	+	--	NM	--	NM
	Ketamine	+	--	+	+	+
	Ketamine, dehydronor	+	NM	NM	NM	NM
	Ketamine, nor	+	NM	+	--	NM
	Methamphetamine	+	+	+	+	+
	Methamphetamine, hydroxy	+	NM	NM	NM	NM
	Pseudoephedrine/ephedrine	+	+	+	--	+
07CT157259	Diphenhydramine	+	--	--	+	+
	Diphenhydramine, N-desmethyl	+	--	NM	NM	NM
	Ketamine	+	+	+	--	+
	Ketamine, dehydronor	+	NM	NM	NM	NM
	Ketamine, nor	+	NM	+	+	NM
	Propoxyphene, cyclic dimer	+	NM	NM	NM	NM
	Propoxyphene, N-desmethyl	+	+	+	+	NM
	Ranitidine	+	+	+	--	NM
	Ranitidine, metab #1 (N-oxide/S-oxide)	+	NM	NM	NM	NM
07CT347768	Brompheniramine	+	+	NM	NM	+
	Brompheniramine, N-desmethyl	+	NM	NM	NM	NM
	Ephedrine/pseudoephedrine	+	--	+	--	+
07CT229104	Codeine	+	+	+	+	+
	Estazolam	+	--	+	+	+
	m-CPP (nte-trazodone/nefazodone)	+	NM	NM	+	NM
	Morphine	+	+	+	--	+
	Morphine, 6-O-monoacetyl	+	--	+	--	+
	Trazodone	+	--	NM	NM	+
	Trihexyphenidyl	+	NM	NM	NM	NM
	Trihexyphenidyl, metab #2 (hydroxylated)	+	NM	NM	NM	NM
	Zopiclone	+	+	+	+	+
	Zopiclone, desmethyl	+	+	NM	NM	NM

Lee HK, et al. Anal Chim Acta 2009;649:80-90

Drug Testing by MS/MS: importance of a trained eye

Drug Screening – False Positives



Analyzer	# drugs (before)	# drugs (after)	% false positive[†]	% false negative[‡]
LC-ZQ-MS	217	146	37.4	10.3
LC-QTRAP-MS/MS	146	196	36.4	49.3
LC-LIT-MS/MS	390	240	35.7	4.8

[†] drugs that did not meet the manual review criteria for positive identification.
[‡] drugs not detected by the search algorithms but identified upon manual review.

Lynch KL, et al. Clin Chem Acta 2010;411(19-20):1474-81

LC-HRMS for drug testing

- Identification dependent upon accurate mass, isotope pattern, retention time (if drug standard is available) and fragmentation pattern (if QTOF or Orbitrap)
- Detection range 100 – 3000 m/z
- Sensitivity – sub picogram level – varies for each compounds
- Resolution - ~2ppm
- Distinguishes compounds with the same nominal mass but different exact mass
- Development of protocols for multiple drugs by LC-MS/MS can be labor intensive and time-consuming
- HRMS enables detection of compounds without prior experience by the testing lab – in theory

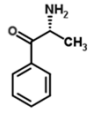
MS/MS vs. HRMS: Advantages/Disadvantages

	LC-MS/MS	LC-HRMS
Method Development	Development of compound dependent parameters for all compounds of interest, build method, establish RTs	Run an analytical standards to establish RTs
Acquisition Method	Targeted – SRM/product ion scan	Full Scan – Full TOF MS scan with triggered collection of product ion scans
Resolution	Nominal Mass	Exact Mass (~2ppm)
Forms of Identification	Nominal mass (MS/MS) Retention Time Fragmentation Pattern Library Search	Accurate Mass (MS/MS) Retention Time Fragmentation Pattern Isotope Pattern Database/Library Search
Data Analysis	Targeted (fixed number of drugs/metabolites)	Targeted (fixed number of drugs/metabolites) and/or untargeted (can identify additional compounds)

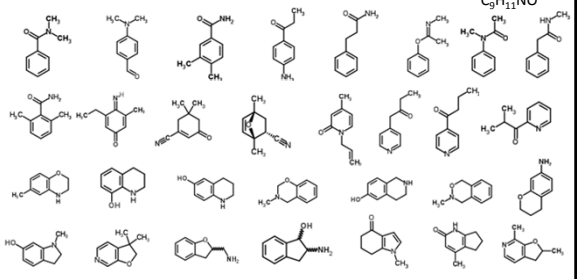
TOF vs. QTOF

Examples of structural isomers of cathinone

www.chemspider.com, 829 hits



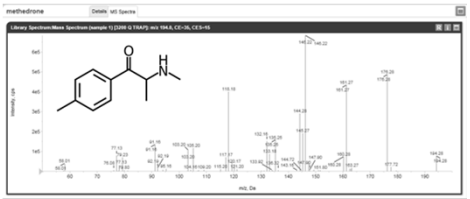
$C_9H_{11}NO$



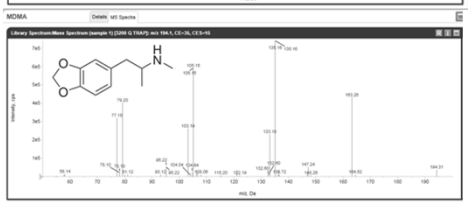
Broecker, Herre & Pragst PCDL

$C_{11}H_{15}NO$

Rt = 3.86

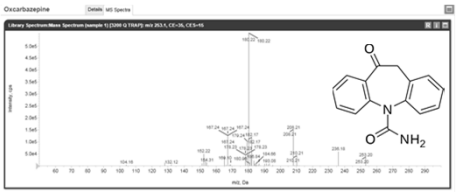


Rt = 3.92

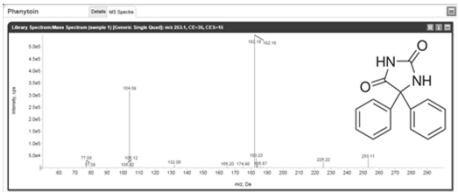


$C_{15}H_{12}N_2O_2$

Rt = 5.92



Rt = 5.96



Conclusions

- Immunoassays alone are not sufficient for toxicology/DAU testing
- There are advantages and disadvantages to GC-MS, LC-MS/MS and LC-HRMS for toxicology testing
- A combination of MS based methods is often used for toxicology drug testing
- There are advantages to using both a targeted and untargeted approach to data collection and data analysis
- LC-HRMS offers many advantages for the detection of new or novel compounds