Toxicology Screening Workflows on QTRAP® Instruments

Targeted Screening and General Unknown Screening

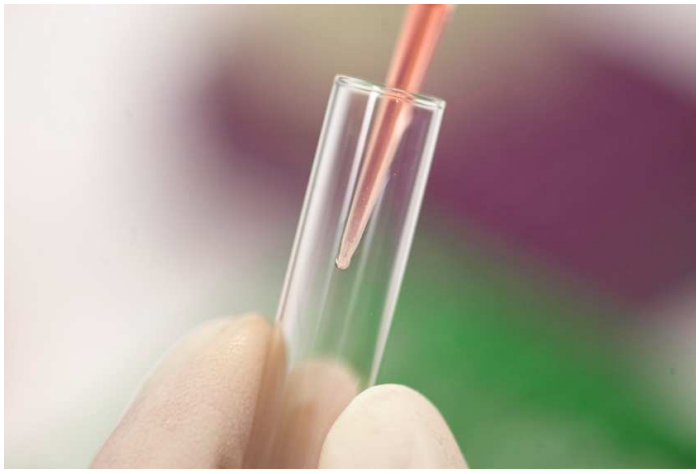
Introduction

Due to the widespread use and abuse of drugs, comprehensive screening for the detection of pharmaceuticals and illicit drugs is an important part of toxicological analysis and is often divided into two categories: targeted screening and general unknown screening.

Targeted screening is a directed screening approach that analyzes samples for a specific list of drugs. This method is often referred to as "multi-target screening", or MTS, and currently constitutes the majority of the screening tests performed. The types of drugs used or abused are often limited to a few hundred compounds; therefore, most MTS methods are focused on detecting a subset of the most commonly used drugs. Restricting the analysis in this way allows the use of sensitive and selective workflows, providing detection of low concentrations of drugs in complex biological matrices. Since this approach detects only those compounds selected, *a priori*, it will not reveal the presence of a compound not included in the target drug list.

While the majority of screening tests are targeted, interest in general unknown screening (GUS) is continuing to grow. GUS does not use a target analyte list, so the analysis is sensitive to detection of unexpected pharmaceuticals, nutritional supplement-based analytes, and designer drugs. The trade off for GUS is a slight compromise in the level of detection. In many applications, this limitation is minor given the benefit of identifying unpredicted analytes.

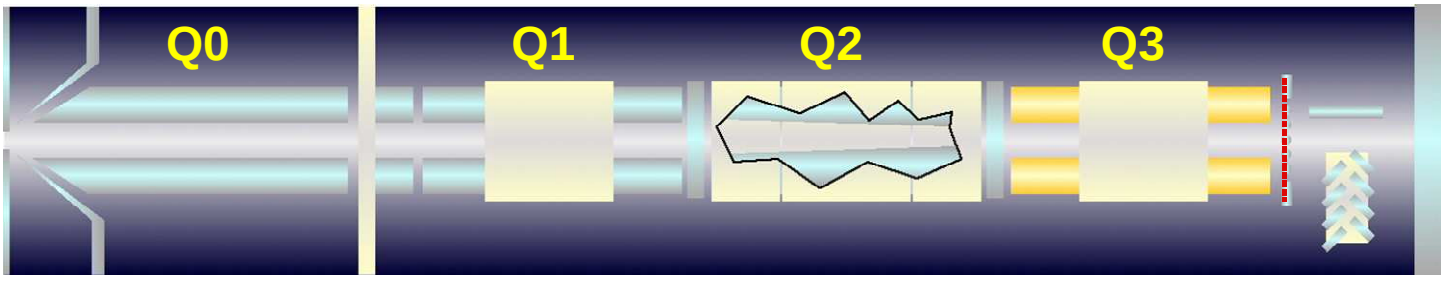
Screening has traditionally been performed using immunoassay, liquid chromatography with ultra-violet detection (LC/UV), or gas chromatography-mass spectrometry (GC/MS).



Immunoassays are widely used because they are inexpensive, sensitive, and easy to implement. However, these tests are only available for a limited number of drug classes, suffer from a lack of specificity due to cross reactivity, and are not easily adapted to detect new drugs. Furthermore, multiple analyses must be performed for complete compound coverage when screening for drugs across several classes.

LC/UV based methods also suffer from a lack of specificity. UV spectra are derived from the UV absorption of specific functional groups so compound identification still requires confirmation against a pure standard. Unambiguous compound identification and quantification also requires the target analyte to be completely resolved from neighboring components, which may necessitate long LC runtimes. Unfortunately, long runtimes are not compatible with the high throughput demands of most drug screening laboratories.

Figure 1. QTRAP® system ion path.



The instrument operates as a true triple quadrupole instrument, using Q1 and Q3 as mass filters and Q2 as a collision cell. In this hybrid instrument, Q3 functions as a linear ion trap, allowing rapid acquisition of sensitive full-scan MS and MS/MS spectra.

GC/MS is a powerful tool for complex mixture analysis. However, because GC/MS analysis is limited to volatile compounds and organic solvents, extensive sample extraction and/or derivatization is often necessary. These sample preparation protocols are often specific to a compound class, limiting the utility of GC/MS for screening across several drug classes and especially for GUS. This limitation makes GC/MS incompatible with efficient GUS workflows.

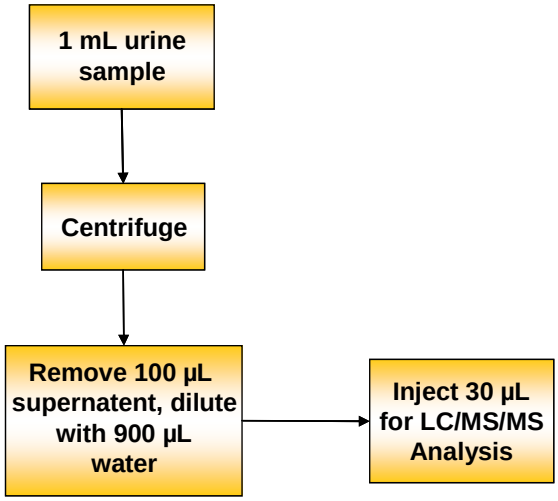
Liquid chromatography-tandem mass spectrometry (LC/MS/MS) is being increasingly adopted in forensic and clinical laboratories. Although most often used for quantification and confirmation, LC/MS/MS is proving to be a powerful technique for comprehensive drug screening. LC/MS/MS allows detection of low level analyte concentrations using short chromatographic run times due to the sensitivity and selectivity of the technique. Sample preparation is also simpler because most biologically active compounds are readily ionized via LC/MS/MS, without any derivatization. A single-step protein precipitation or sample dilution is often sufficient prior to analysis. In addition to being convenient, LC/MS/MS sample preparation procedures are generic, making them ideal for comprehensive drug screening – both MTS and GUS.

The 3200 QTRAP® instrument is a hybrid triple quadrupole/linear ion trap mass spectrometer – a unique, flexible LC/MS/MS system that can accommodate a wide variety of both quantitative and qualitative workflows. A schematic of the QTRAP® ion path is shown in Figure 1. This instrument is based on a triple quadrupole platform, using Q1 and Q3 as mass selective filters and Q2 as a collision cell for fragmentation. Because it is a triple quadrupole, true MRM experiments – the gold standard in highly selective and sensitive quantitative analysis– can be performed. In addition to triple quadrupole functionality, the QTRAP® instrument incorporates linear ion trap capabilities in Q3, allowing ion accumulation for full scan MS or MS/MS analysis, which provides high sensitivity qualitative spectra. Ion trap enhanced product ion (EPI), or full scan MS/MS, spectra can be acquired and searched against a spectral library for compound identification and/or structural information. It is the ability to use both triple quadrupole and linear ion trap scan functions on a single platform – and even within a single LC/MS/MS run – that makes the QTRAP® LC/MS/MS system adaptable to a wide variety of both screening and quantitative tests. A comparison of MTS and GUS for detection and identification of drugs in urine using a QTRAP® LC/MS/MS system is presented.

Experimental

Several drug-positive urine samples were obtained from a local hospital toxicology lab. Sample preparation is outlined in Figure 2. Because true quantitative results were not necessary, the samples were not hydrolyzed in order to simplify sample preparation for high throughput analysis.

Figure 2. Sample preparation protocol.



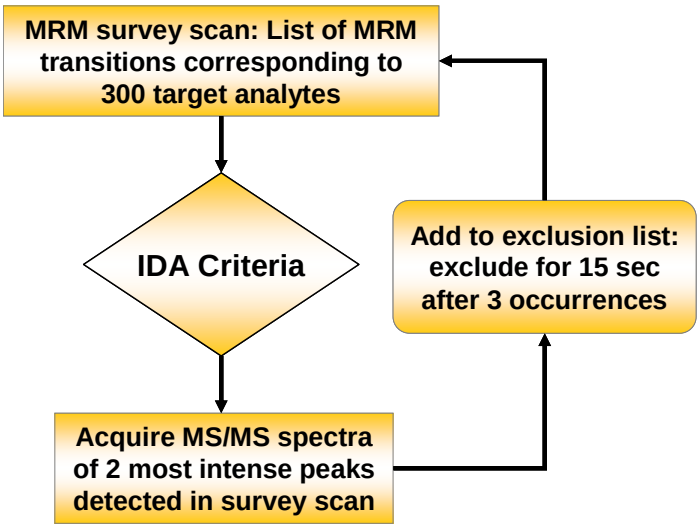
Since absolute quantitative results were not required, the lengthy hydrolysis step was omitted for simplicity and increased throughput. The generic sample preparation is also beneficial when screening for drugs across several compound classes.

All samples were analyzed using a Shimadzu Prominence HPLC system interfaced to an AB SCIEX 3200 QTRAP® mass spectrometer. Both targeted and general unknown screening experiments were performed on each sample.

For MTS experiments, independent data acquisition (IDA) using an MRM survey scan and two dependent EPI scans was utilized. This experiment is outlined in Figure 3 and representative data is shown in Figure 4.

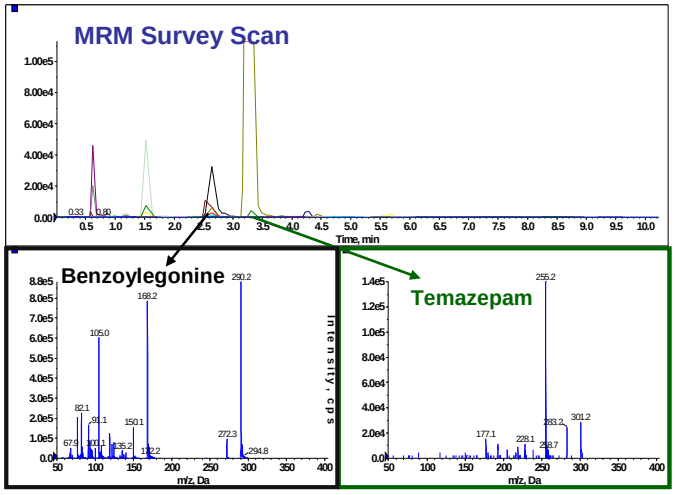
A survey scan consisting of 300 targeted MRM transitions is used to identify potential drugs. Because MRM scans are extremely sensitive and selective, this survey scan can detect drugs present at low concentrations in complex biological matrices. When a signal in an MRM transition is detected, the precursor ion is submitted for an enhanced product ion (EPI) scan, or linear ion trap full scan MS/MS. The EPI spectrum can be searched against a spectral library for compound confirmation, as shown in the example report displayed in Figure 5.

Figure 3. MRM-IDA-EPI Workflow.



Multiple Reaction Monitoring-Information Dependent Acquisition-Enhanced Product Ion (MRM-IDA-EPI) workflow used for multi-target screening (MTS).

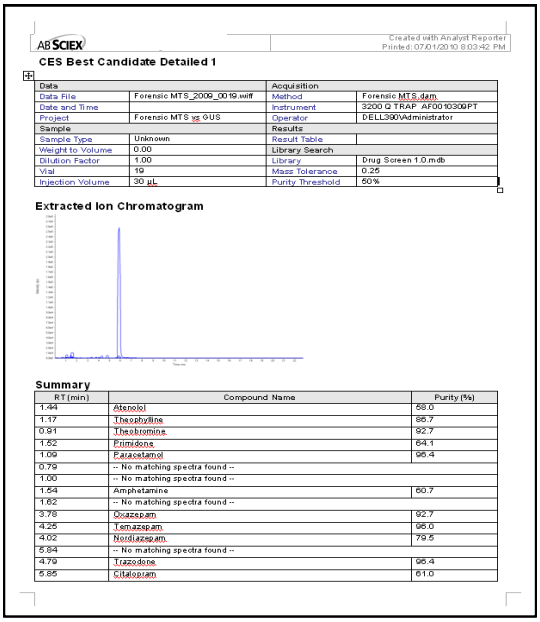
Figure 4. Multi-target screening (MTS) example data.



This sample shows the presence of several drugs, including benzoylecgonine and temazepam. Drug identities were confirmed by matching the EPI spectrum to reference library spectra.

The use of GUS has gained interest in recent years because of its ability to detect unexpected compounds. GUS experiments are based on the use of a full scan single MS survey scan to detect the major peaks. Pharmaceuticals, metabolites, designer drugs, and supplement derived ingredients can all be detected with GUS based workflows. When using the QTRAP® instrument, this enhanced MS (EMS) survey scan is acquired in linear ion trap mode for optimal sensitivity. Dependent EPI spectra are collected on the three most intense peaks in the EMS survey scan. Figure 6 shows a chart of the workflow and representative data is presented in Figure 7. When using single MS as a survey scan, matrix/background interferences can be present at high levels. To maximize the likelihood that dependent EPI spectra are acquired of potential exogenous drugs, dynamic background subtraction (DBS) is used. DBS is an algorithm that subtracts background signals in real time to minimize acquisition of dependent EPI spectra of background signals. The effectiveness of DBS is demonstrated in Figure 7, which shows m/z 276 as the first signal submitted for MS/MS

Figure 5. Sample MTS Report.

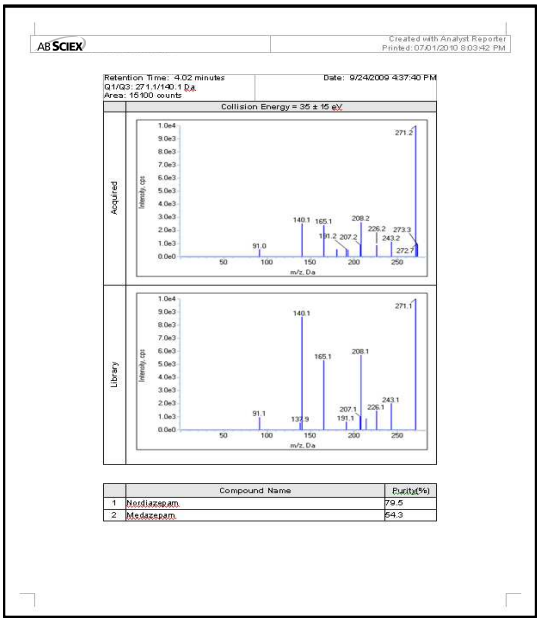

CES Best Candidate Detailed 1

Data		Acquisition	
Data File	Forensic MTS_2009_0019.wiff	Method	Forensic MTS.dam
Date and Time		Instrument	3200 Q TRAP AF0010309PT
Project	Forensic MTS vs GUS	Operator	DELL360\Administrator
Sample		Results	
Sample Type	Unknown	Result Table	
Weight to Volume	0.00	Library Search	
Dilution Factor	1.00	Library	Drug Screen 1.0.mdb
Vial	19	Mass Tolerance	0.25
Injection Volume	30 µL	Purity Threshold	50%

Extracted Ion Chromatogram

Summary

RT (min)	Compound Name	Purity (%)
1.44	Atenolol	58.0
1.17	Theophylline	85.7
0.91	Theobromine	92.7
1.62	Primidone	64.1
1.09	Paracetamol	96.4
0.79	-- No matching spectra found --	
1.00	-- No matching spectra found --	
1.54	Amphetamine	60.7
1.62	-- No matching spectra found --	
3.78	Oxazepam	92.7
4.25	Temazepam	96.0
4.02	Nordiazepam	79.5
5.84	-- No matching spectra found --	
4.79	Trazodone	96.4
5.85	Citalopram	61.0



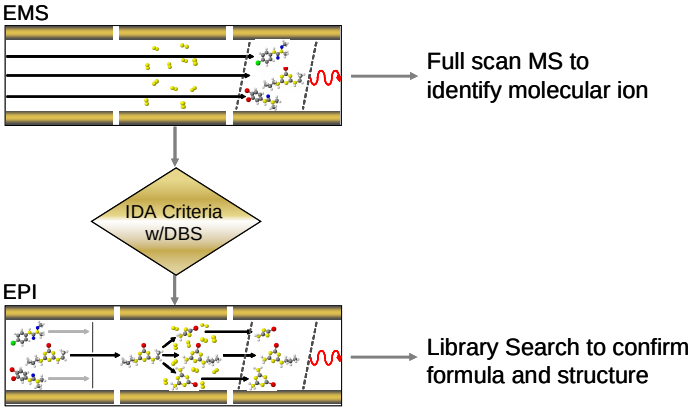
	Compound Name	Purity(%)
1	Nordiazepam	79.5
2	Medazepam	64.3

Example MTS report showing detection and confirmation of many compounds, including nordiazepam.

analysis. Note that there are several low mass signals with a higher intensity than 276. However, DBS identifies these as background signals and 276 is recognized as the most intense non-background signal and submitted for MS/MS analysis.

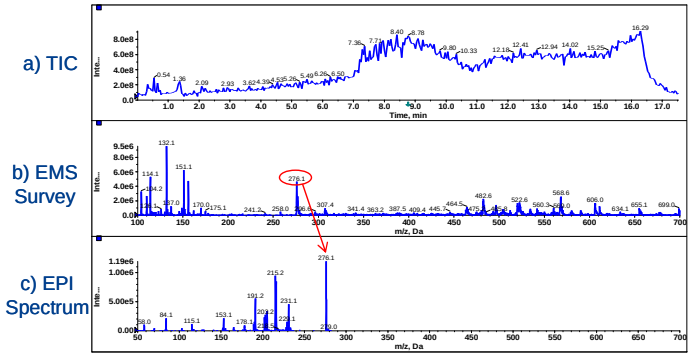
GUS experiments generate a great deal of complex data that requires sophisticated software to streamline the workflow. Cliquid® software can simplify the acquisition, processing, and reporting of GUS results. The software generates extracted ion chromatograms at every mass, identifies chromatographic peaks, and determines if an MS/MS spectrum for that peak was obtained. If an MS/MS spectrum exists, it will be searched against a spectral library and any match reported, as shown in the example GUS report in Figure 8.

Figure 6. EMS-IDA-EPI Workflow.



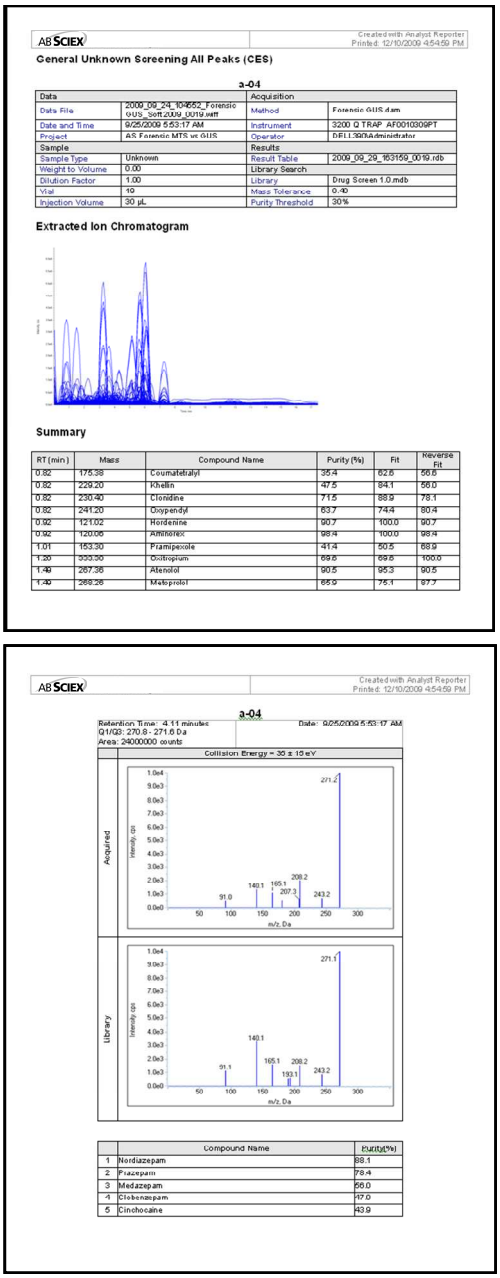
Enhanced Mass Spectrum-Information Dependent Acquisition-Enhanced Product Ion (EMS-IDA-EPI) workflow used for general unknown screening (GUS). A linear ion trap EMS scan identifies intense signals to submit for acquisition of dependent EPI spectra. Dynamic background subtraction (DBS) is used to subtract background signals in real time, significantly improving the likelihood that low level drugs of interest will be detected and identified.

Figure 7. Representative GUS data.



The total ion chromatogram (TIC) is shown in Figure 7a. The enhanced MS – or full scan MS – spectrum is used as a survey scan and shown in Figure 7b. EPI spectra are collected on the 3 most intense ions in the survey scan. The EPI spectra can be interpreted for structural elucidation or searched against a library for identification or classification. Although m/z 276 is not among the three most intense ions, it is submitted for EPI acquisition because most of the low mass, intense signals are background and DBS prevented them from being submitted for MS/MS analysis.

Figure 8. Sample GUS Report.


General Unknown Screening All Peaks (CES)

a-04

Data		Acquisition	
Data File	2009_09_24_104552_Forensic GUS_Soft 2009_0019.wiff	Method	Forensic GUS.dam
Date and Time	9/25/2009 5:53:17 AM	Instrument	3200 Q TRAP AF0010309PT
Project	AB Forensic MTS vs GUS	Operator	DFL1\SCIXAdministrator
Sample		Results	
Sample Type	Unknown	Result Table	2009_09_29_153159_0019.rdb
Weight to Volume	0.00	Library Search	
Dilution Factor	1.00	Library	Drug Screen 1.0.mdb
Vial	10	Mass Tolerance	0.40
Injection Volume	30 µL	Purity Threshold	30%

Extracted Ion Chromatogram

Summary

RT (min)	Mass	Compound Name	Purity (%)	Fit	Reverse Fit
0.82	175.38	Coumatetralyl	35.4	62.6	56.6
0.82	229.20	Khellin	47.5	84.1	56.0
0.82	230.40	Clonidine	71.5	88.9	78.1
0.82	241.20	Oxypendyl	63.7	74.4	80.4
0.92	121.02	Hordenine	90.7	100.0	90.7
0.92	120.06	Amfepramone	98.4	100.0	98.4
1.01	153.30	Pramipexole	41.4	50.5	68.9
1.20	333.20	Oxitropium	69.6	69.6	100.0
1.49	267.36	Atenolol	90.5	95.3	90.5
1.49	268.26	Metoprolol	66.9	75.1	87.7

a-04

Retention Time: 4.11 minutes
Q1/Q3: 270.8 - 271.6 Da
Area: 24000000 counts
Collision Energy = 35 ± 15 eV

Acquired

Library

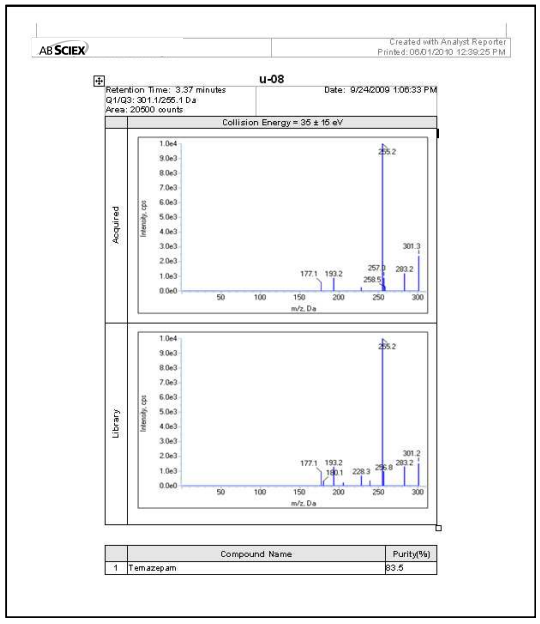
	Compound Name	Purity(%)
1	Nordiazepam	88.1
2	Prazepam	78.4
3	Medazepam	66.0
4	Clobenazepam	47.0
5	Cinchocaine	43.9

Several drugs were detected and identified, including nordiazepam, which is confirmed by spectral match.

Discussion

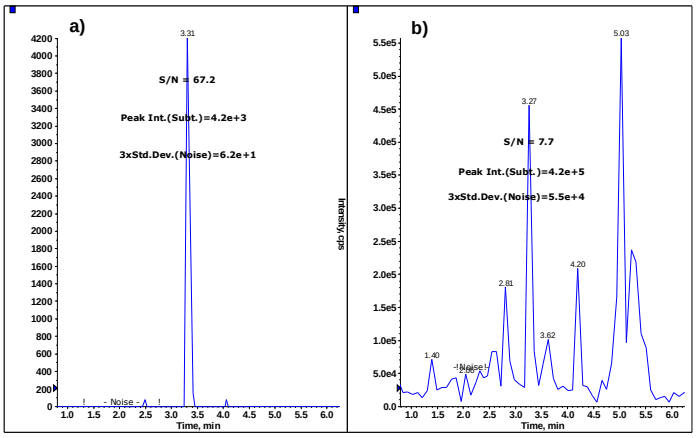
Targeted screening detected more drugs in the samples versus the general unknown approach, as expected. Figure 9 shows a library search match for temazepam that was identified in a sample when analyzed by MTS but not when analyzed using the GUS method. Figures 10a and 10b compare the extracted ion chromatograms (XICs) of the survey scans for the two methods. The XIC of temazepam's MRM transition (301/255) from MTS screening has a signal-to-noise ratio (S/N) of almost 70. The peak intensity is relatively low (4500 counts), suggesting that the concentration is on the lower end. A much lower S/N of 8 is observed for the XIC of m/z 301 from the EMS survey scan used in the GUS experiment. Several neighboring interferences are also observed in the full scan experiment. When the full scan EMS spectrum is examined (Figure 11), numerous peaks have intensities higher than the intensity of m/z 301 so dependent EPI spectra are not acquired, explaining the missed detection.

Figure 9. Temazepam confirmation.



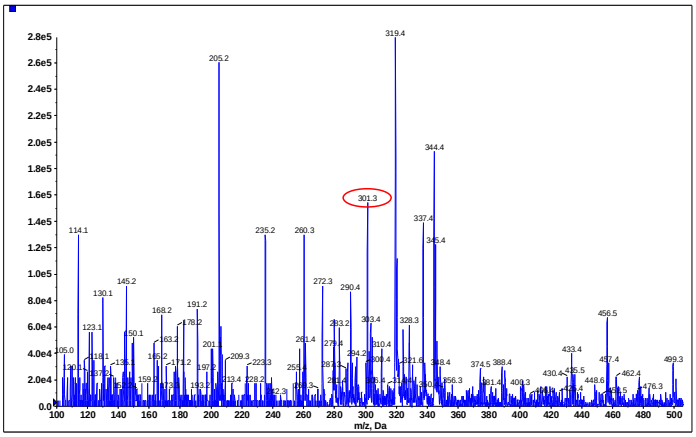
Temazepam identified in unknown sample #1 using MTS.

Figure 10. Extracted ion chromatograms for temazepam from analysis of sample #1.



a) XIC of 301/255 from the MRM survey scan of the MTS experiment. B) XIC of 301 from the EMS survey scan of the GUS experiment. The intensity of the MRM signal is low, suggesting a relatively low concentration of temazepam in the urine sample. However, because of the selectivity of MRM the signal-to-noise (S/N) is much greater than the extracted ion chromatogram in the GUS experiment.

Figure 11.EMS survey scan at 3.3 min from the GUS analysis of sample #1.

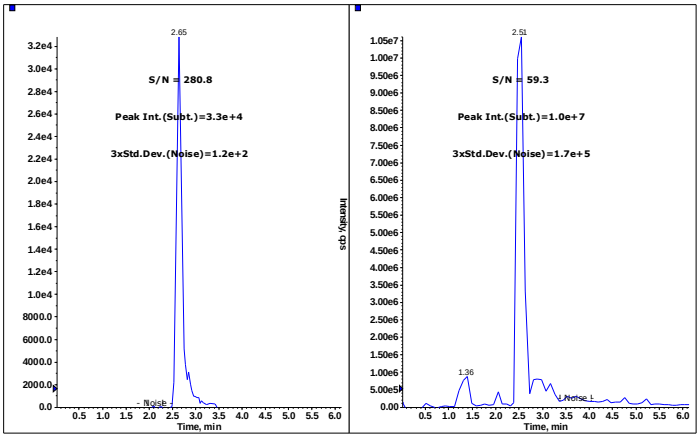


An intense peak is present at m/z 301, the M+H for temazepam, and several other signals with higher intensity are also present. As a result dependent EPI spectra are not acquired for m/z 301, and temazepam is not identified as being present in this sample.

Alternatively, benzoylecgonine (BZE) was identified using both screening techniques and is present at a very high concentration, as observed from the high intensity of the MRM XIC in Figure 12a. The S/N for the benzoylecgonine XIC (m/z 290) is still very high when analyzed by GUS (Figure 12b), however the S/N from the MTS experiment is 5x higher due to the selectivity of the MRM survey scan. A prominent peak at m/z 290 is observed in the EMS survey spectrum (Figure 13) and the spectral match and identification of BZE using GUS is shown in Figure 14.

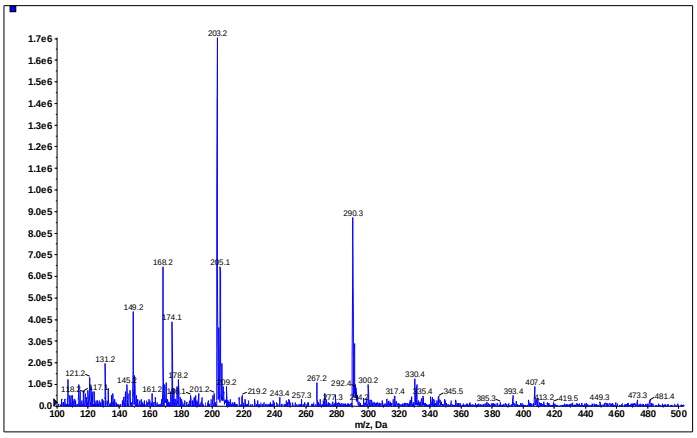
Although MTS detected more drugs in the urine samples, GUS has the advantage of detecting unexpected compounds and the possibility to classify a compound based on its fragmentation pattern. Figure 15 shows an MS/MS spectral match for hordenine, with a purity of >90%. This compound was not detected using the MTS method. Investigation into hordenine discovered that it is a biogenic amine that can increase blood pressure. Hordenine's structure (Figure 16) has a molecular weight of 165, which would yield an M+H ion of 166. In the GUS experiment, spectrum matching hordenine had a precursor m/z

Figure 12. Extracted ion chromatograms for benzoylecgonine (BZE) from analysis of sample #1.



a) XIC of 290/168 from the MRM survey scan of the MTS experiment. B) XIC of 290 from the EMS survey scan of the GUS experiment. The intensity of the MRM signal is high, suggesting a high concentration of BZE in the urine sample. The peak in the GUS XIC has few interferences and an adequate S/N, but the S/N for the MTS experiment is 5x higher due to the selectivity of the MRM survey scan.

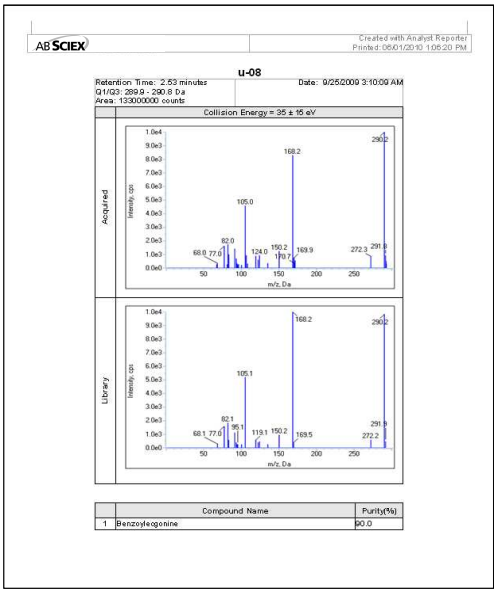
Figure 13.EMS survey scan at 2.5 min from the GUS analysis of sample #1.



An intense peak is present at m/z 290, the M+H for benzoylecgonine (BZE). An MS/MS spectrum was acquired for BZE and the library match and confirmation is presented in Figure 14.

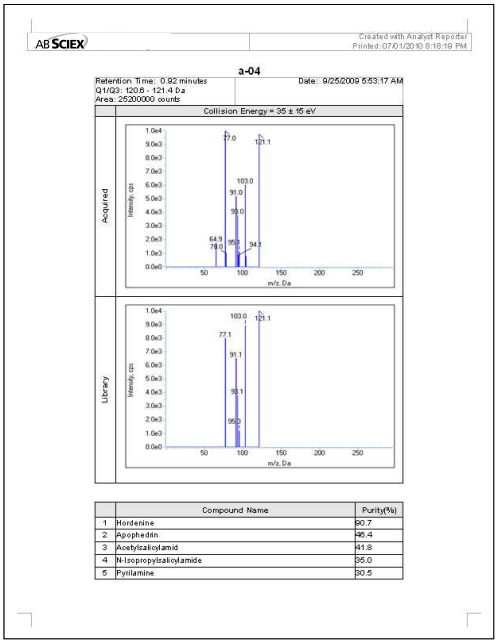
of 121, which is a difference of 45 amu. Figure 17 shows a possible fragmentation pathway that corresponds to the MS/MS spectrum of hordenine. Because GUS is not based on any a priori knowledge, such as molecular weight or retention time, similar spectral interpretation and structural elucidation can be implemented to identify drug analogs or metabolites that have similar MS/MS spectral patterns. From the data obtained in this GUS experiment, hordenine – or a structurally similar compound – was present in this sample.

Figure 14. Example GUS Report for Sample #1.



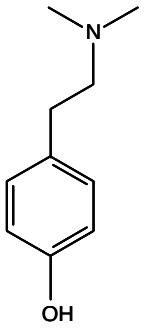
Benzoylecgonine was detected and identified with a spectral purity of 90%.

Figure 15. Confirmation of Hordenine.



Hordenine was identified in sample #2 using GUS.

Figure 16. Structure of hordenine.



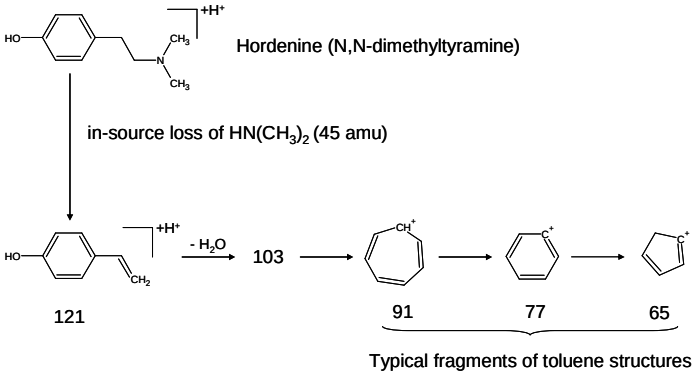
Hordenine

$C_{10}H_{15}NO$

$[M+H]^+$ =166.1226

Hordenine, which has a molecular weight of 165, yields a signal at M+H=166 in positive ion mode. Research discovered that hordenine is a biogenic amine that is isolated from barley and can cause increased blood pressure.

Figure 17. Proposed fragmentation pattern of hordenine.



The spectral match of hordenine had a precursor mass of 121, which is 45 amu less than the M+H for hordenine. A possible interpretation of hordenine's fragmentation pathway is depicted, suggesting that hordenine – or a structurally similar compound – was present in the sample.

Many experiments that claim to be a general unknown screen only extract ion chromatograms from a list of M+H values that correspond to known or suspected drugs. As a result, even though data for drug analogs or metabolites may be acquired, a corresponding XIC may not be obtained in the processing step ifthe M+H value is not expected. In reality, this type of data processing results in an experiment that more closely resembles a targeted rather than a general unknown screen. The processing approach used in the experiment described here extracts chromatograms at every mass and does not use a pre-determined list, so it is truly a general unknown screen.

Summary

LC/MS/MS has become a very valuable tool in toxicology screening. By using LC/MS/MS, sample preparation can be simplified and run times reduced, resulting in increased throughput and faster sample turnaround times. Sample preparation is generic, allowing for screening across a wide variety of compound classes using a single sample preparation procedure and chromatographic analysis.

Multi-target screening and general unknown screening are both useful workflows in toxicology. Typically, there is a relatively small subset of substances that are heavily used and abused, making a targeted approach the most useful because of its ability to detect drugs at low concentrations and relative ease of data processing and interpretation. If drugs have not been identified as frequently used or abused substances, they will not be on the target compound list and their use can remain undetected. General unknown screening is therefore a complementary screening technique. GUS has the ability to detect and identify unexpected drugs, drug analogs, and drug metabolites. The 3200, 4000, and 5500 QTRAP® LC/MS/MS systems combined with Cliquid® Software can successfully implement both workflows using a single instrument. True MRM scans allow for detection of analytes at the lowest concentrations and can also be used for true quantitation experiments. The linear ion trap capabilities allow acquisition of sensitive full scan MS spectra and high quality MS/MS spectra that can be used for library searching and/or structural interpretation. The versatility of the QTRAP® platform provides laboratories with the flexibility to utilize a wide variety of experiments, alone or in combination, to achieve their analysis goals.

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