Application Note 10495

High Confidence, Non-Targeted Screening for Drugs of Abuse in Urine

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Key Words
Drugs of abuse, GC-MS, urine, screening, high resolution, Q Exactive GC, Orbitrap mass spectrometry, accurate mass

Introduction

Drug abuse is a condition that is characterized by a destructive pattern of using a substance, usually through self-administration, which leads to significant problems or distress. Almost any substance, that on consumption can cause a euphoric feeling, can be abused. Examples of drugs of abuse (DoA) include depressants (opioids, barbiturates, benzodiazepines, alcohol), stimulants (amphetamines, cocaine), hallucinogens (LSD, mescaline, phencyclidine), and cannabinoids (marijuana). The reasons for DoA testing are diverse. For example, DoA screening can be performed for criminal and other forensic investigations, high-risk employment functions, clinical toxicology, or rehabilitation programs. For such tests, urine is generally accepted as the sample of choice as it is non-invasive, reliable, economical, widely utilized, and strictly regulated.[1] One limitation of testing in urine is that it will usually only provide information about current or recent substance use as a specimen is likely to be negative after a period of 2-3 days after drug administration. There are some compounds and their metabolites that remain detectable for longer periods, e.g., THC-COOH and some benzo diazepines.[1] To prolong the detection period as long as possible, sensitive analytical techniques are important.

Laboratory testing of urine for DoA is a challenging application. This is primarily due to the high number of compounds and metabolites that need to be screened for in a sample that has a variable chemical background. Furthermore, depending on exposure, the levels of such compounds can be present at both trace and very high concentrations. Gas chromatography coupled with mass spectrometry (GC-MS) is well-suited for DoA screening and confirmation as it provides excellent chromatographic resolution, peak capacity and extensive spectral libraries to aid in identification.[2,3] However, higher sensitivity and specificity would increase the confidence in positive results and improve the robustness of the system, especially in a high throughput routine laboratory environment. One further challenge is that the evaluation of information rich electron impact spectra generated using GC-MS is difficult if performed manually, especially when analyte peaks are often overlapped by matrix and background ions.

In this application note, the performance of the Thermo Scientific™ Q Exactive™ GC Orbitrap™ GC-MS/MS system was evaluated for the screening of DoA in urine. This work aims to demonstrate the application of a non-targeted workflow using the Q Exactive GC Orbitrap GC-MS/MS system to detect and identify DoA. This work focuses on analyzing real case urine samples using a full-scan, non-targeted acquisition and high-mass resolving power to obtain accurate mass measurements in support of spectral library matching. The evaluation of scan speed in combination with high in-scan dynamic range and high sensitivity will be made for the detection of low and high intensity components. In addition, unique software algorithms for automated deconvolution and identification were also assessed for routine screening.

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Experimental Conditions

Sample Preparation

Six urine samples (A-F) from real case investigations, provided by the Zurich Institute of Forensic Medicine of the University of Zurich, were analyzed. Sample extracts were prepared as follows: 0.5 mL urine was adjusted to a pH of 5.2 with the addition of 100 µl acetate buffer 0.5 M. After the addition of 50 µl glucuronidase/arylsulfatase conjugate cleavage was performed over 3 h at 60 °C. 200 µl of ammonium buffer was added and the mixture liquid-liquid extracted with 1 mL of ethyl acetate/isopropanol/dichloromethane mixture (3:1:1, v/v/v). 800 µl of the organic layer was evaporated to dryness. The residue was acetylated with 100 µL of an acetic anhydride-pyridine mixture (3:2 vol/vol) at 60 °C for one hour. The acetylated mixture was evaporated and the residue then redissolved in 100 µL of ethyl acetate, which was directly used for GC-MS analysis.

Instrument and Method Setup

In all experiments, a Thermo Scientific Q Exactive GC Orbitrap GC-MS/MS system was used. Sample introduction was performed using a Thermo Scientific™ TriPlus™ RSH autosampler, and chromatographic separation was obtained using a Thermo Scientific™ TRACE™ 1310 GC system and a Thermo Scientific™ TraceGOLD™ TG-1MS 30 m × 0.25 mm I.D. × 0.25 µm (P/N 26099-1420) film capillary column. Additional details of instrument parameters are displayed in Tables 1 and 2.

Table 1. GC and injector conditions.

TRACE 1310 GC Parameters	
Injection volume (µL)	1
Liner	Split
Inlet (°C)	250
Carrier gas (mL/min)	He, 1.2
Split ratio	20:1
Oven Temperature Program	
Temperature 1 (°C)	50
Hold time (min)	1
Temperature 2 (°C)	325
Rate (°C/min)	10
Hold time (min)	8.5

Table 2. Mass spectrometer conditions.

Q Exactive Mass Spectrometer Parameters	
Transfer line (°C)	250
Ionization type	EI
Ion source (°C	250
Electron energy (eV)	70
Acquisition mode	Full scan
Mass range (Da)	50-600
Resolving power (FWHM at *m/z* 200)	60,000
Lockmass, column bleed (*m/z*)	207.03235

Data Processing

Data was acquired using the Thermo Scientific™ TraceFinder™ software. This single-platform software package integrates instrument control, method development functionality, and qualitative and quantitative-focused workflows. TraceFinder also contains spectral deconvolution and spectral matching functionality. The Maurer/Pfleger/Weber mass spectral library of drugs, poisons, pesticides, pollutants and their metabolites for 2011 was used for spectral matching.[4] Further filtering of library hits was performed using accurate mass and high-resolution filtering (HRF) for intelligent compound identification.

Results and Discussion

The objective of this study was to analyze the acetylated urine samples using a non-targeted full-scan experiment and to screen the samples against a spectral library. The complete workflow is summarized in Figure 1. Having acquired full-scan data with high sensitivity and wide-spectral dynamic range, we are able to screen for an almost unlimited number of compounds. This allows for retrospective analysis of the data and the search for additional compounds that were not necessarily screened for at the time of the initial investigation. The success of such a broad-scope screening lies entirely on the quality of the spectral library against which searches are made. In this case, a nominal mass library containing 8670 spectra of drugs, drug metabolites, contaminants and other biomolecules was used to search the data file for compounds with speed and confidence to generate a list of candidate compounds. The high-resolution data is then used to intelligently filter the list of hits based on accurate mass.

Isolate Features and Make Confident Identifications

Each data file was deconvoluted to extract the individual peaks from the total ion chromatogram (TIC). The full-scan data obtained from the urine extracts is complex with a high chemical background as demonstrated in the total ion chromatogram of a sample injection (split 20:1) (Figure 2). The complexity was reduced by the automatic deconvolution within TraceFinder that extracts all of the features from the background in the TIC. The deconvolution results in a cleaned spectrum for each peak, containing only the ions that maximize at the same retention time. The cleaned spectrum can then be searched against the Maurer/Pfleger/Weber spectral library for tentative compound identification. The list of tentative hits returned for each peak are scored based on a combination of a classical search index (SI) score and high-resolution filtering (HRF) value. The HRF value is the percentage of the spectrum that can be explained by the chemical formula proposed from the best library match result.

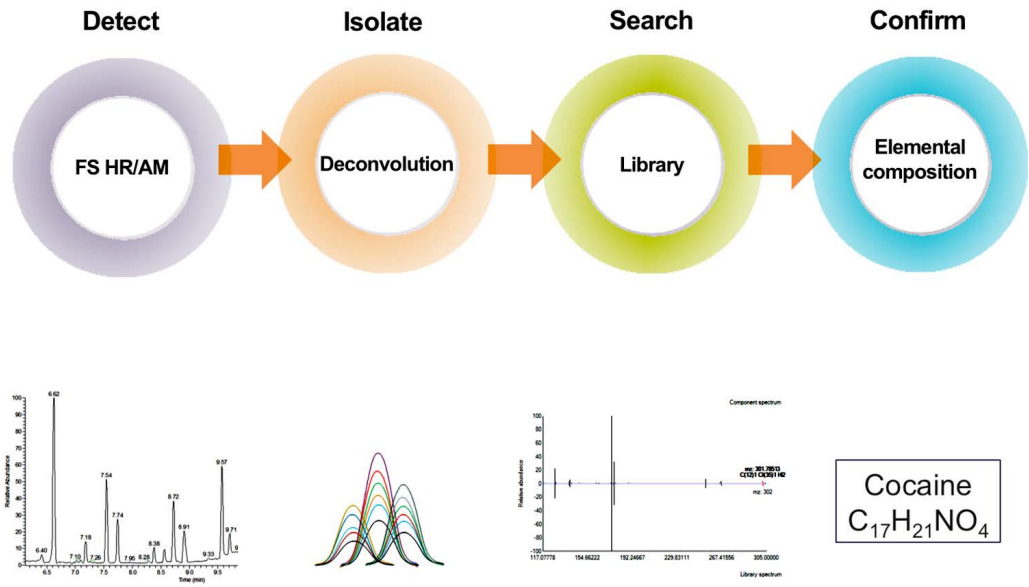

Figure 1. Workflow for the Q Exactive GC system for screening

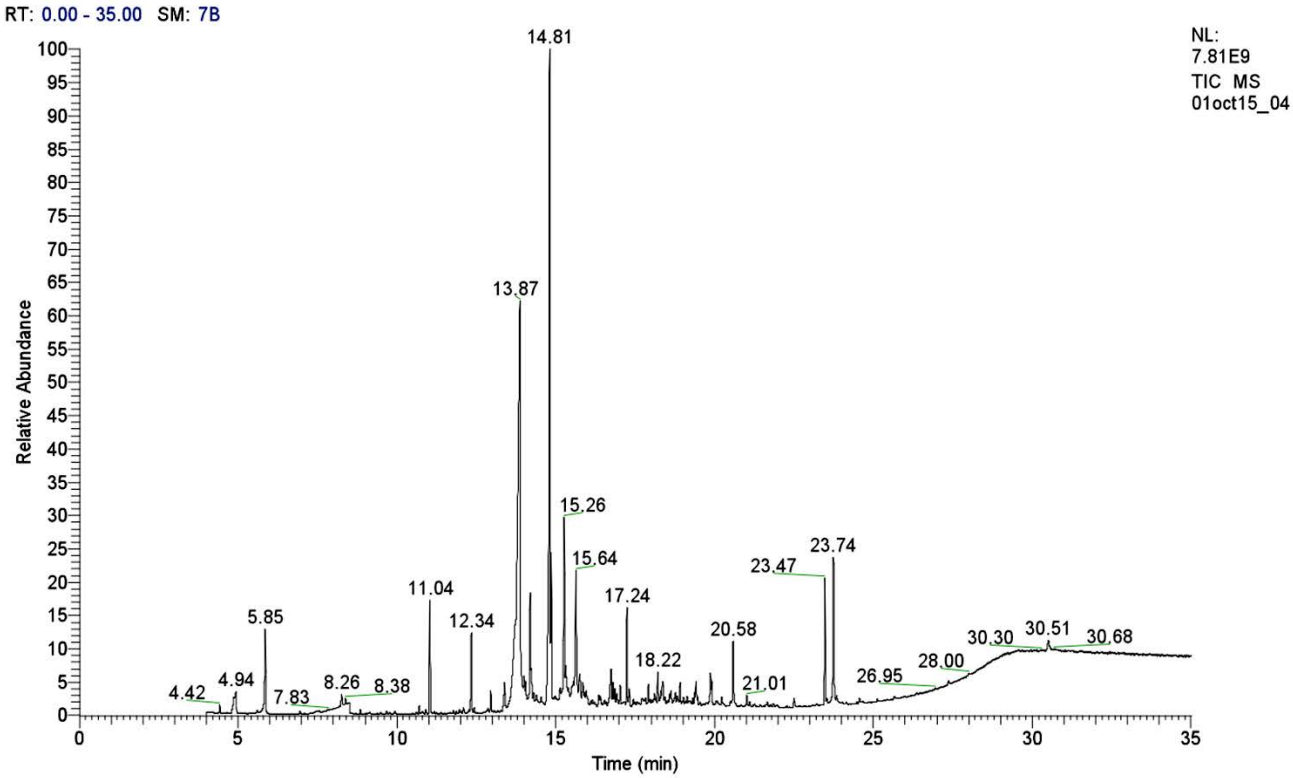

Figure 2. GC-MS total ion chromatogram of a urine sample (split 20:1).

The combination of accurate mass, library matching and percentage of explained ions observed in the spectrum provides a fast and confident route to the identification of compounds in the sample. An example of compound detection and identification is shown in Figure 3, where myristicin was detected. In Figure 3, we show a close up of the peak identification table showing three possible matches for the peak at 16.15 minutes. If only the SI was used there are a number of acceptable matching compounds. However, when we include HRF and sort the list based on the combined score, the top hit (myristicin) has a score of 74.3%. The other five hits are all <51%, and can be eliminated. Further confirmation of the matched compound can be obtained by looking at the mass accuracy of the ions, where the base peak is *m/z* 180.07799 with 0.6 ppm. Having sub 1 ppm mass accuracy enables the analyst to make fast and robust compound identifications.

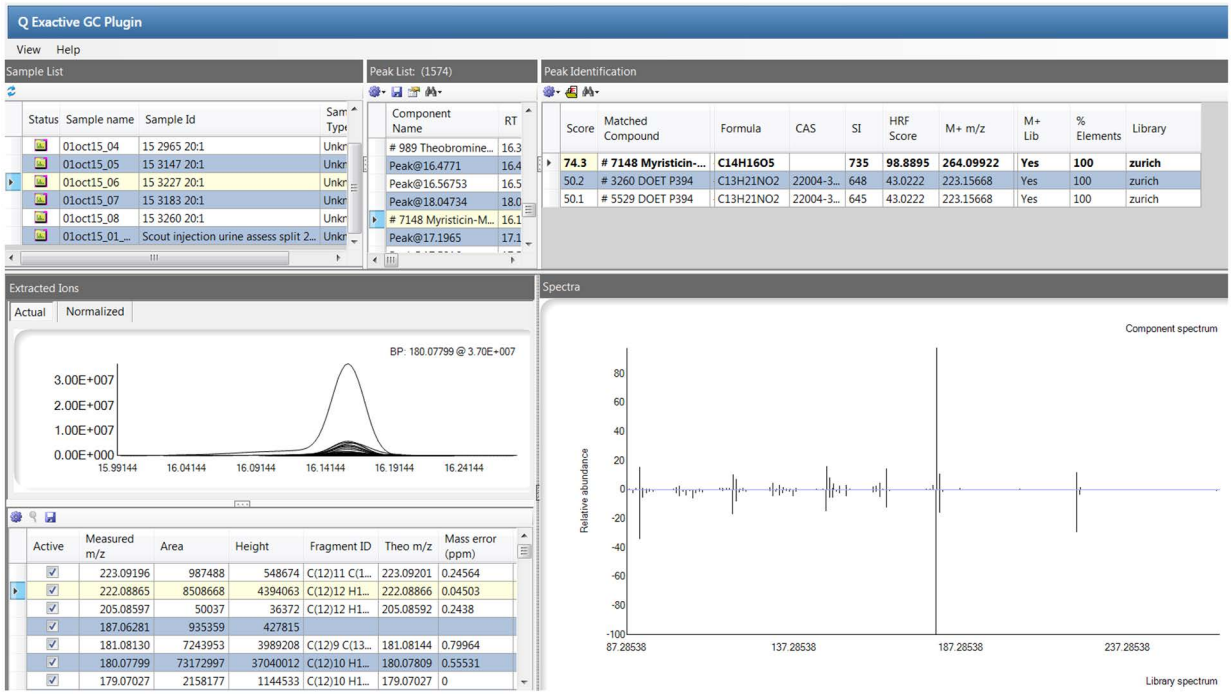

Figure 3. Identification of myristicin using TraceFinder. Spectral deconvolution of the TIC and library search index and the HRF score together with additional criteria are used for intelligent compound identification. The list of matched compounds (upper) is sorted based on a combined search index and high resolution filtering (HRF) score.

Applying High Resolution for Reliable Drug Screening

The six samples were screened against the Maurer/Pfleger/Weber library and the results are summarized in Table 3. The total number of deconvoluted peaks extracted from the samples, with a signal to noise greater than 100, ranged from 824 to 1574. The number of peaks with at least one matched compound in each sample ranged from 169 to 263. This is a relatively high number of matched peaks and is expected when we screen against a library of over 8000 entries. These matched compounds include not only DoA but also contaminants and biomolecules that are expected in a complex sample such as urine. For clinical and forensic screening it is vital to be able to comprehensively characterize a sample and understand what defines a typical chemical profile, so that abnormal peaks can be identified. If the analyst is only interested in screening for drugs of abuse then a smaller library containing only these compounds can be used to screen against. However, DoA screening is complex and it is highly beneficial to capture a more complete chemical profile of a sample so that conclusions can be made on which drug has been consumed, and how long it has been resident in the individual's system.

Table 3. Total number of deconvoluted peaks and the number matched to a compound in the spectral library containing 8670 entries.

Sample	Total Deconvoluted Peaks	Matched Peaks
Urine A	824	169
Urine B	1523	223
Urine C	1574	263
Urine D	745	174
Urine E	1008	197

For the six samples the list of matched compounds can be searched for the drugs that are of most concern and these are summarized in Table 4. The compounds were automatically identified based on spectral matching to the Maurer/Pfleger/Weber library entry and confirmation by the accurate mass of the molecular ion (if present) and additional fragment ions. In addition to the accurate mass of ions in the spectrum, we can match the isotopic pattern of the molecular ion cluster to the theoretical pattern. For example, in Figure 4 the anticonvulsant drug lamotrigine was identified in urine sample C by a positive match with lamotrigine 2AC. When the measured molecular ion cluster was compared with the theoretical, an almost identical match was observed with outstanding mass accuracy for each isotope. This spectral fidelity leads to strong evidence of the compounds presence in the urine sample. Further confirmation can be obtained by assessing the retention time information with a chemical standard, although for many metabolites certified standards are not commercially available. In these cases, the accurate mass and isotope information are very powerful criteria for compound identification. The major advantage in having this information is it reduces the evaluation time, so the time to result is significantly faster compared to nominal mass data.

Table 4. Summary of compounds tentatively identified in the six urine samples .

Sample	Compound	Base Peak (*m/z*)	HRF (%)	Base Peak Mass Accuracy (ppm)	Molecular Ion Mass Accuracy (ppm)
Urine A	Cocaine	82.06512	100.0	1.5	—
	Methadone	72.08077	99.7	0.5	—
	Morphine	268.1332	99.9	0.8	0.2
	Paracetamol	109.05221	99.0	0.6	0
Urine B	Tramadol	114.09134	99.7	0.8	—
Urine C	Mirtazapine	195.09167	98.2	0.2	—
	Lamotrigine	184.97935	100.0	0.4	0.3
Urine D	Morphine	268.1332	96.7	0.8	0.2
	Paracetamol	109.05221	98.9	0.9	0.5
Urine E	Myristicin	180.07809	98.9	0.6	—
Urine F	Butyrfentanyl	146.09646	—	0.4	0.0

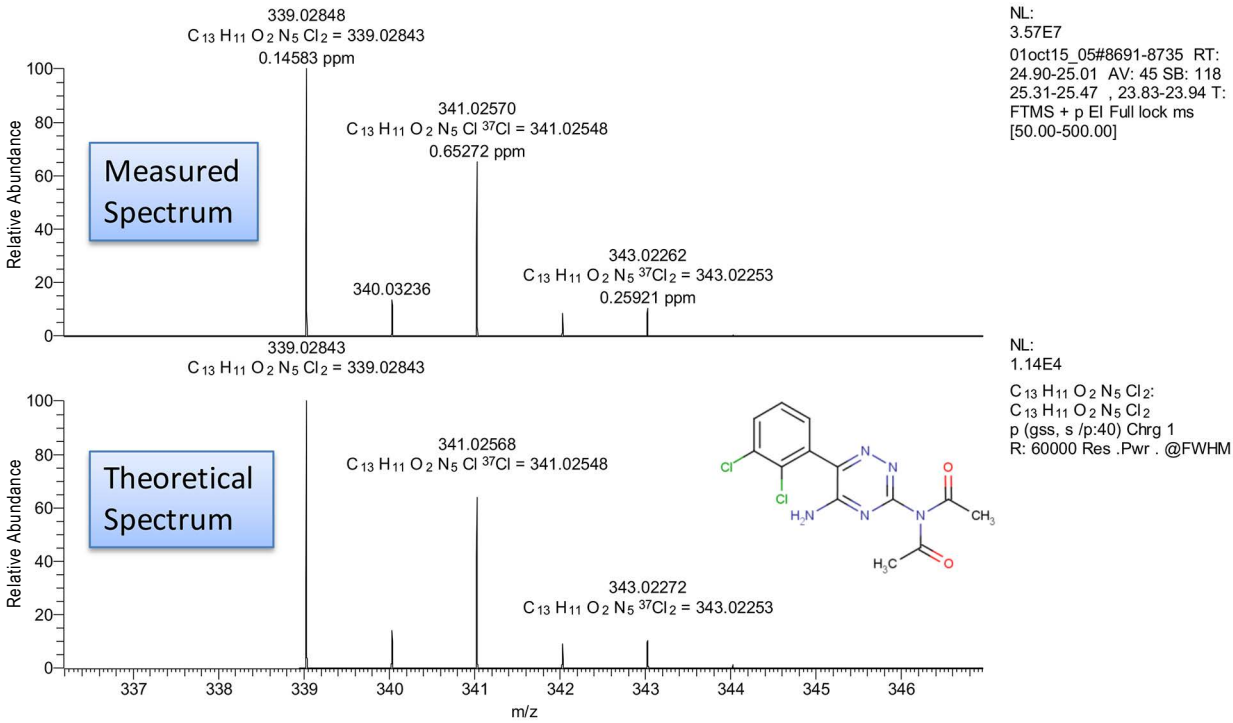

Figure 4. Measured and theoretical isotope molecular ion cluster of lamotrigine 2AC. Elemental formula for each isotope is displayed with in each case ‹ 1 ppm mass accuracy.

As mentioned above, when screening for drugs in urine the compounds of interest can be present at both very high and ultra trace concentrations. For screening to be effective and to avoid false negatives the detection system needs to be able to provide the same level of performance regardless of analyte response. This work demonstrated that the Q Exactive GC Orbitrap GC-MS/MS system has a wide-dynamic range that enables this to be achieved routinely. An example is shown in Figure 5 where paracetamol and homo-vanillic acid peaks have been extracted from the TIC. The paracetamol peak area close to three orders of magnitude higher than that of homo-vanillic acid, but vitally the accurate mass performance remains at 0.5 ppm for both compounds.

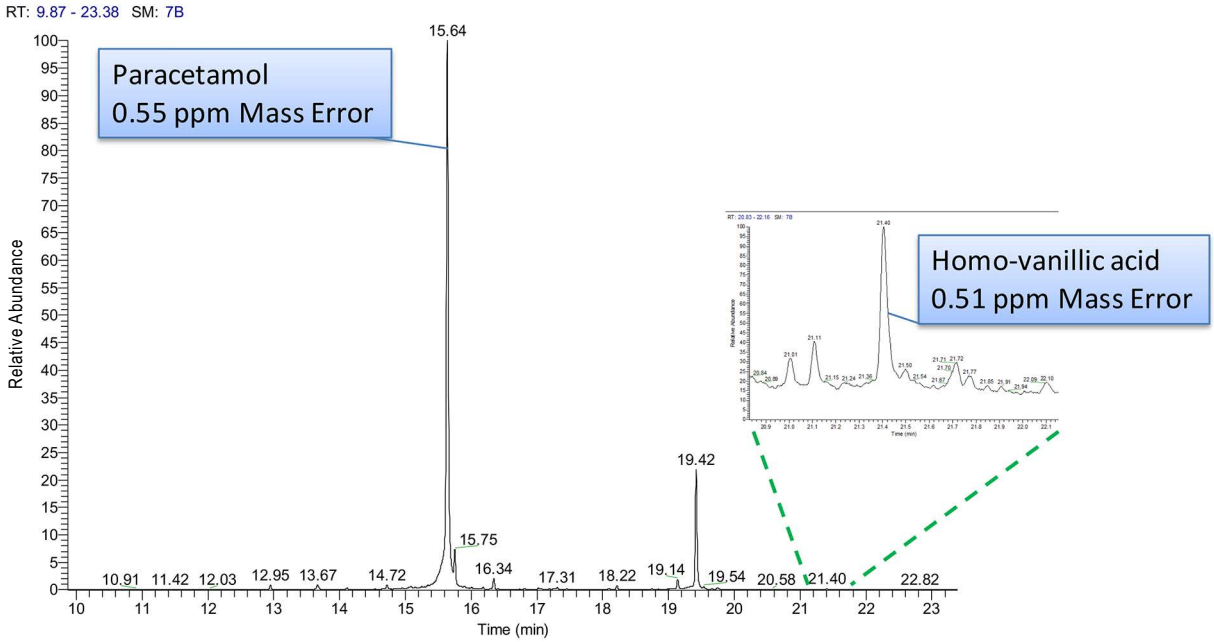

Figure 5. Mass accuracy at sub 1 ppm regardless of compound concentration. Paracetamol (0.5 ppm for BP *m/z* 109.05221) peak area 930 times higher than homo-vanillic acid (0.5 ppm for BP *m/z* 137.0597).

Identifying the Unexpected

In sample F, a deconvoluted peak was identified, but with no match to a compound in the library. However, this peak did have a base peak ion, *m/z* 146.09643, which is typically observed in fentanyl type spectra. It was possible to quickly rule out both alpha and beta methylfentanyl as there was no spectral match with the library. The accurate mass spectra of both the EI and CI data (Figure 6) were used to identify the $[M+H]^+$ ion as *m/z* 351.24309 and a formula $C_{23}H_{31}N_2O$ was proposed with a mass difference of 0.03 ppm. This is a critical stage in the process and it is where excellent mass accuracy can be used to limit the

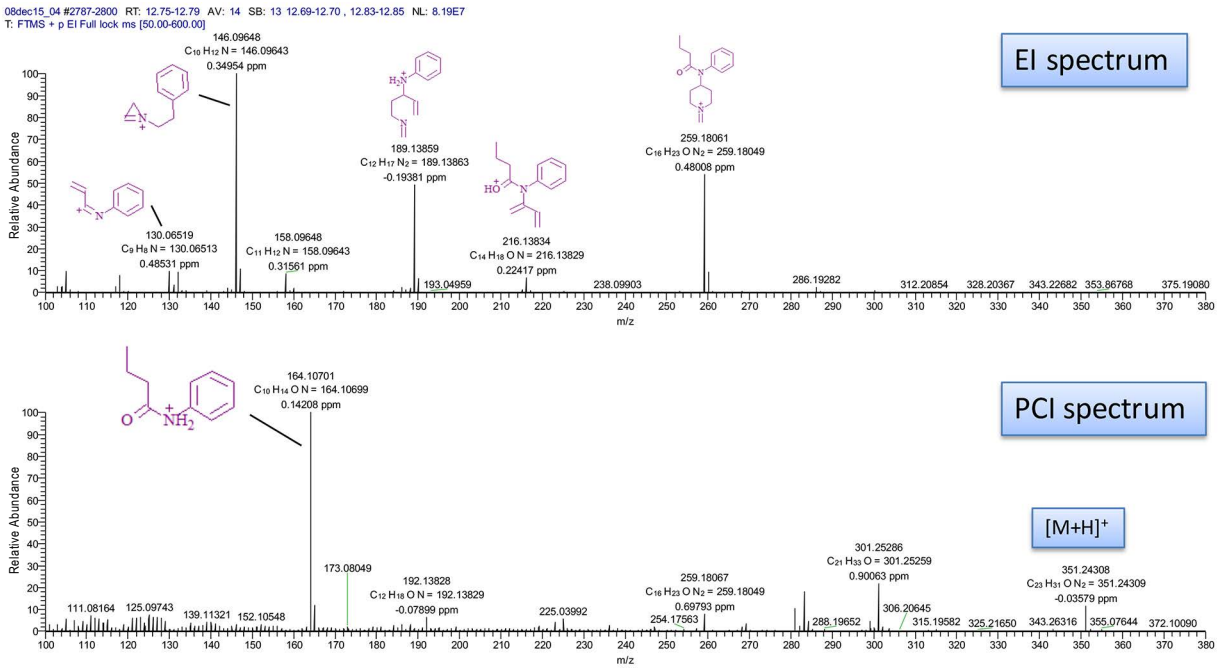

Figure 6. Butyrfentanyl ($C_{23}H_{30}N_{20}$) EI and PCI spectra at 12.77 minutes sample F. PCI data supports identification of parent with formula above with sub 1 ppm mass accuracy. EI Fragments annotated with structures from MassFrontier 7.0.

number of possible chemical formulae. This level of mass accuracy significantly reduces the number of formulae that need to be investigated and also increases the confidence in any proposed assignment. The proposed chemical formula for the compound $C_{23}H_{30}N_2O$ was searched using the online chemical database Chemspider™ and suspecting it was an analogue of fentanyl, the compound butyrfentanyl was proposed as an identity. This fentanyl analogue did not have an entry in the spectral library, hence there being no spectral match. Finally MassFrontier 7.0 was used to theoretically fragment butyrfentanyl and match these to the measured fragments with sub 1 ppm mass accuracy in the EI and CI spectra to confirm the identification. The spectrum can be added to the library for future sample screening.

A total of 16 Metabolites of butyrfentanyl were also quickly identified by filtering the list of deconvoluted peaks for only those peaks that contained the fragments *m/z* 146.09647 and 189.13865. The largest metabolite peak (retention time 14.4 minutes) was identified from the CI spectra as having an elemental composition of $C_{25}H_{33}O_3N_2$ derived from the $[M+H]^+$ ion with a mass difference of 0.8 ppm. It is proposed that this is an oxidation reaction acetylated during the described sample preparation. The metabolites of butyrfentanyl can also be added to the spectral library for future screening.

Conclusions

The results of this study demonstrate that the Thermo Scientific Q Exactive GC Orbitrap GC-MS/MS system in combination with TraceFinder software is a powerful tool for the routine screening of drugs of abuse and chemical profiling of complex urine samples.

- The Orbitrap mass spectrometer delivers excellent mass accuracy of all components, regardless of concentration, enabling fast and accurate identifications.
- The six urine samples were screened for drugs of abuse using the unique automatic peak detection, spectral deconvolution and compound identification workflow. Several drugs of abuse were detected, with compounds of interest identified based on spectral library matching and fragment rationalization using the accurate mass information. This approach reduces the evaluation time and enables a faster and more confident reporting of results.
- The consistent sub 1 ppm mass accuracy in combination with good sensitivity makes confident identification of known and unknown components in a sample possible. Using the accurate mass EI/CI data, it was possible to propose that butyrfentanyl was present in sample F.
- Routine resolving power of 60,000 FWHM and a wide-dynamic range allows for the necessary selectivity in difficult matrices eliminating isobaric interferences and ultimately increasing confidence in results when compounds are present at either very high or trace concentrations in complex matrices.

References

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