

An Ultra-high Resolution Accurate Mass LC/MS solution to Forensic Toxicology Screening in Serum

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Overview

Purpose: The purpose of this work is to evaluate an ultra-high resolution accurate mass LC/MS solution for forensic toxicology screening in serum.

Methods: Screening and detection were carried out on a Thermo Scientific Exactive benchtop LCMS Orbitrap mass spectrometer coupled with an Accela™ UHPLC system.

Results: Parameters like mass accuracy, resolution, and HCD fragmentation were evaluated for screening with a serum containing 40 drugs (mainly isobars and isomers).

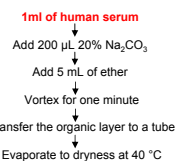
Introduction

In recent times, LC/MS has become the technology of choice for screening of illicit drugs. Two main approaches for tandem MS have been used in this area. The first one is called MTS¹: Multi-Target-Screening, and the second one is GUS²: General Unknown Screening. In both cases, these two approaches are limited by the number of entries available in the MS² library. In this work, we will present a completely new approach based on accurate mass. Confirmation is made using accurate mass detection of the analyte (below 5 ppm) and retention time. Data obtained from real samples will be presented and extra parameters used for confirmation of the results will be discussed.

Methods

Sample Preparation

The extraction procedure was performed using liquid/liquid extraction (LLE). Details of the procedure are described below.



Reconstitute in 400µl of 70/30 of A/B (A: water containing 10 mM ammonium acetate and 0.1% formic acid; B: acetonitrile containing 0.1% formic acid).

HPLC

Chromatographic analyses were performed using the Thermo Scientific Accela UHPLC system. The chromatographic conditions were as follows:

- **Column:** Thermo Scientific Hypersil GOLD PFP 5 µm, 150 x 2.1 mm
- **Flow rate:** 0.2 mL/min
- **Mobile phase:** A: water containing 10 mM ammonium acetate and 0.1% formic acid; B: acetonitrile containing 0.1% formic acid
- **Injection volume:** 10 µL
- **Gradient:** The gradient starts at 95% of A and ends at 95% of B in 27 minutes.

Mass Spectrometry

MS analysis was carried out on the Exactive™ mass spectrometer with an electrospray ionization (ESI) source. The MS conditions were as follows:

- **Ion polarity:** Polarity-switching
- **Mass range:** 100 – 800 amu
- **Resolution:** 10K, 50K, 100K
- **Fragmentation:** HCD MS/MS after every MS scan

Results

A serum sample was spiked with a mixture of 40 different molecules (see Figure 1). The concentration for each of the analytes was 1.25mg/L. Then successive dilutions were made in 80/20 A/B (A: water containing 10 mM ammonium acetate and 0.1% formic acid; B: acetonitrile containing 0.1% formic acid) in order to go down to 0.4 µg/L and evaluate the sensitivity of the instrument. Most of the selected molecules are isobars or isomers. The goal is to evaluate how we can properly identify all these molecules under our screening conditions. As an example, Amitriptyline and EDDP are isomers. They have exactly the same mass. Bromazepam and Clonazepam are isobars. Their masses differ by few milli-amu.

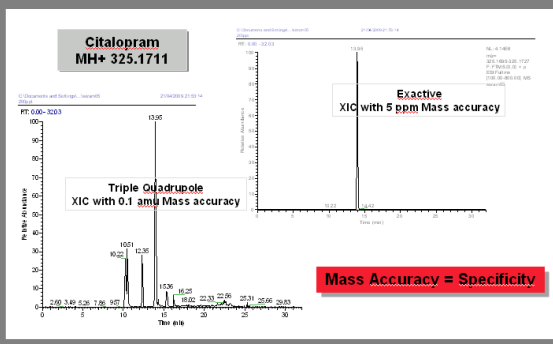
FIGURE 1. Molecules spiked into a serum sample

Amitriptyline	278.1903	LSD	324.2070	Phenobarbital	231.0764
Bromazepam	316.0080	Maprotiline	278.1903	Prazepam	325.1102
Buprenorphine	468.3108	Methadone	310.2165	Quinine	325.1911
Citalopram	325.1711	Nadolol	310.2012	Quinidine	325.1911
Clobazam	301.0738	Norbuprenorphine	414.2638	THC COOH	345.2060
Clomipramine	315.1623	Norlobazam	287.0582	THC Delta 9	315.2319
Clonazepam	316.0483	Norcyamemazine	310.1372	Thiopental	241.1062
Cyamemazine	324.1529	Nordiazepam	271.0633	Tramadol	264.1958
Declomipramine	301.1466	Norflouxetine	296.1257	Venlafaxine	278.2115
Diazepam	285.0789	NorLSD	310.1914	Verapamil	455.2904
EDDP	278.1903	Normaprotiline	264.1747	Zolpidem	308.1757
Fluoxetine	310.1413	Nortriptyline	264.1747	Zopiclone	389.1123
Glibenclamide	494.1511	Norvenlafaxine	264.1958		
Hydroxyzine	375.1834	Oxazepam	287.0582		

Mass Accuracy

Mass accuracy was evaluated at different concentrations and at different resolution settings. For levomepromazine, the mass accuracy goes from 3 to 4.4 ppm and is not affected by the concentration or the resolution. In average, when using external calibration the mass accuracy for all the molecules is around 2-3 ppm. With internal calibration it is around 1 ppm. The mass accuracy will directly impact the specificity of the instrument. In Figure 2, we have made a comparison of the Extracted Ion Chromatogram for Citalopram on the Exactive with 5ppm mass accuracy and with a 0.1 amu (300 ppm) window which corresponds to the mass accuracy of a triple quadrupole instrument. As reported in this figure, it is easier to identify without compromise the citalopram on the Exactive chromatogram even without any retention time information as we only have a single peak in the chromatogram. Regarding the triple quadrupole approach, there are many peaks on the chromatogram coming from the matrix or the mobile phase which make the identification mode difficult.

FIGURE 2. Impact of mass accuracy on sensitivity



Sensitivity

All molecules have been identified at 1.25 mg/L except thiopental, which does not give good sensitivity in LCMS.

Figure 3 reports the percentage of molecules that were identified at different concentrations and at different resolution settings. Identification was confirmed for a mass accuracy below 5 ppm. When going down to 0.4 µg/L, 65% of the compounds are still identified at a resolution of 100,000 and 62.5% at 10,000 resolution. Overall, the percentage of molecules that have been identified is higher at high resolution. In low resolution conditions, some molecules coming from the matrix may interfere with the analyte peaks and therefore increase the mass accuracy of the analyte above the threshold of 5 ppm.

FIGURE 3. Percentage of molecules identified

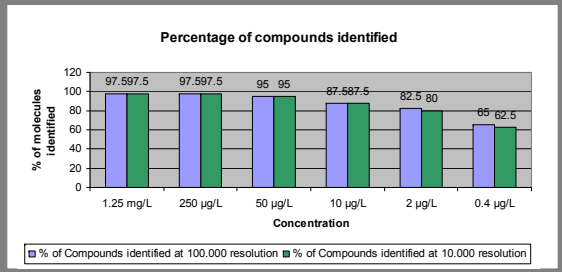
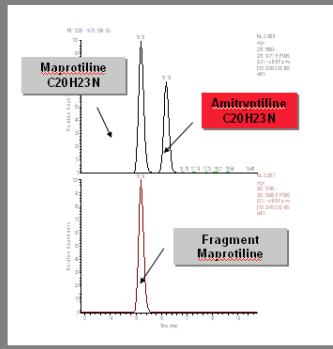


FIGURE 4. HCD fragmentation



HCD Fragmentation

In the case of isomers that elute at very close retention times, another criteria has to be selected to differentiate and properly identify the analytes. This criteria can be the use of a fragment ion generated under gas collision dissociation in the HCD collision cell. Figure 4 shows an example with maprotiline and amitriptyline. Both have the same mass as they are isomers (Formula: C₂₀H₂₃N) and they have very similar retention times under our LC conditions. The only difference is that maprotiline generates a fragment ion at 250.158 amu that is not seen with amitriptyline. Using fragment ion is, in general, mandatory to confirm the presence of an analyte.

Resolution Settings

The analysis was performed under different resolution settings (R=10,000 and R=100,000). Figure 5 shows an example of the impact of the resolution on the sensitivity. The compound analyzed is cyamemazine. Under HCD conditions, it gives a specific fragment at 279.09 amu (lower traces). Both settings have been compared: 100,000 resolution and 10,000 resolution. The signal-to-noise for the fragment ion is much higher under high resolution conditions (614 versus 19). The reason for that is that at 100,000 resolution, the instrument is able to separate the fragment of the analyte from other components available in the matrix or the mobile phase. This is not the case at 10,000 resolution where the trace of the fragment being monitored is contaminated by another molecule coming from the mobile phase (in that case, probably a phthalate). For this reason, the background in this lower resolution setting is high, which results in a lower signal-to-noise.

FIGURE 5. Impact of the resolution on the signal-to-noise

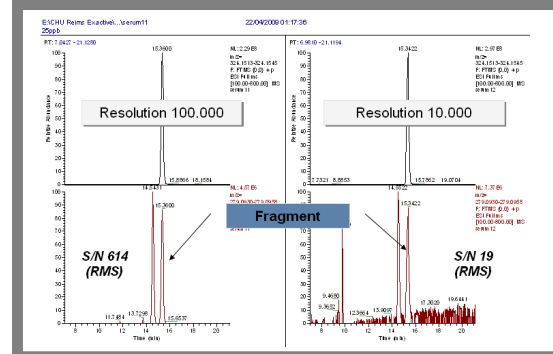


FIGURE 6. Identification of coeluted compounds

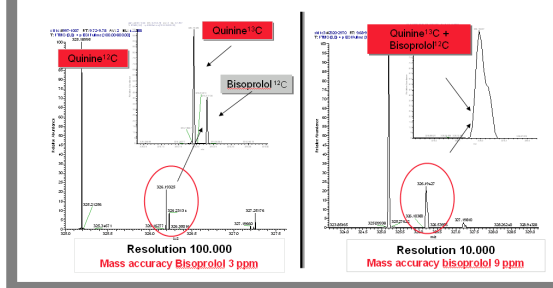
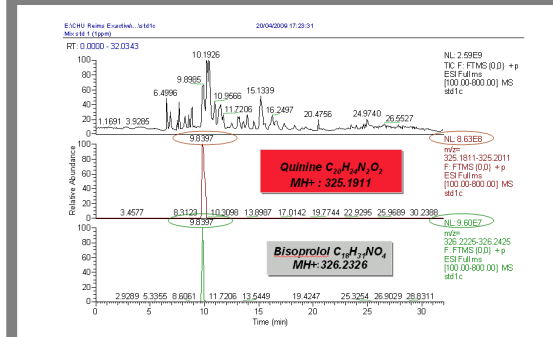
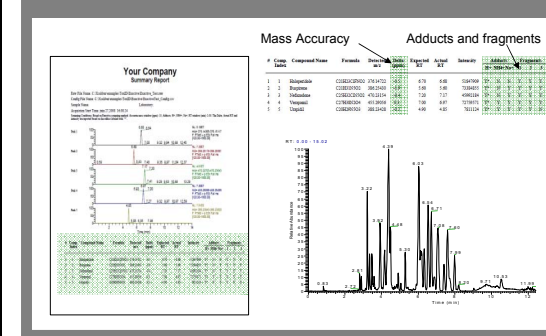


FIGURE 7. Thermo Scientific ToxID report, short summary style



Ultra-high resolution is necessary in some cases to differentiate two analytes having the same retention, or an analyte from an interference from the matrix. Figure 6 shows an example with Quinine and Bisoprolol. These two compounds have the same retention time. Their molecule weight differ by 1 amu, which means that the ¹³C isotope mass of quinine will match with the ¹²C of the bisoprolol. Figure 6 shows the spectra obtained under different resolution settings. On the left side, the data was acquired at 100,000 resolution and on the right side, data was acquired at 10,000 resolution. Under ultra-high resolution, the ¹³C isotope of quinine and ¹²C isotope of bisoprolol are clearly separated and it is easy to identify in that case bisoprolol with only 3 ppm mass accuracy. At a lower resolution (R=10,000) the two peaks cannot be separated and therefore the ¹³C isotope of the quinine interferes with the ¹²C of the bisoprolol. The mass accuracy is then affected and bisoprolol cannot be identified as the mass accuracy of the peak is then 9 ppm (above the 5 ppm accepted threshold).

Data Processing

All data acquired was reprocessed using ToxID™ software. An example of the automatically generated report can be seen in Figure 7. This report contains the list of molecules that have been identified, and also the mass accuracy and the detection or no of fragment ions. The retention time is also used as a criteria for confirmation.

Conclusions

- Limits of Detection (LODs) for most drugs below 1 µg/L
- ToxID software is ideally suited for library searching and reporting of results
- Exactive benchtop LCMS Orbitrap MS is an instrument of choice for forensic/toxicology screening.

References

- (1) Mueller, C.A.; Weinmann, W.; Dresen, S.; Schreiber, A.; Gergov, M. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 1332–8.
- (2) Sauvage, F.-L.; Saint-Marcoux, F.; Duret, B.; Deporte, D.; Lachatre, G.; Marquet, P. *Clin. Chem.* **2006**, *52*(9), 1735–1742.

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