An ultra-high resolution accurate mass LC/MS solution to Forensic Toxicology screening in serum

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Introduction

LC-MS/MS has fast become the technology of choice for the screening of illicit drugs. Two main approaches for tandem MS have been used in this area. The first one is called MTS[1]: Multi-Target-Screening, and the second one is GUS[2]: General Unknown Screening. In both cases, these two approaches are limited by the number of entries available in the MS^2 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 (information in **Figure 1**) will be presented and extra parameters used for confirmation of the results will be discussed**.**

FIGURE 1. Molecules and their protonated molecular mass, which were spiked into a serum sample prior to a LLE procedure

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	Norclobazam	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	Norfluoxetine	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	Norventlafaxine	264.1958		
Hydroxyzine	375.1834	Oxazepam	287.0582		

1ml of human serum

Add 200 µL 20% Na_2CO_3

Add 5 mL of ether

Vortex for one minute

Transfer the organic layer to a tube

Evaporate to dryness at 40 °C

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

Methods

HPLC

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

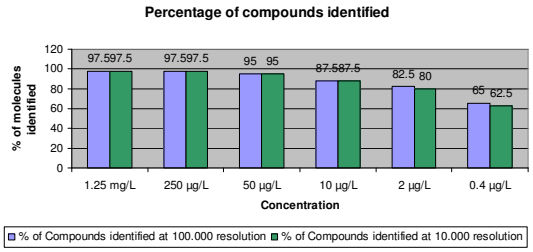
- *Column:* Thermo Scientific Hypersil GOLD PFP 5 µm, 150 x 2.1 mm
- *Flow rate:* 0.2 mL/min
- *Mobile phase:* A: water containing 10 mM ammonium acetate and 0.1% formic acid; B: MeCN containing 0.1% formic acid
- *Injection volume:* 10 µL
- *Gradient:* The gradient starts at 95% of A and ends at 95% 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 (positive and negative)
- *Mass range*: 100 – 800 *m/z*
- *Resolution*: 10K, 50K, 100K
- *Fragmentation*: HCD MS/MS after every MS scan

FIGURE 2. Percentage of molecules identified with 10K and 100K resolution

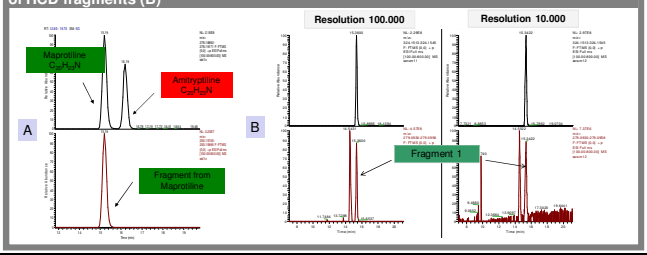


Results

A serum sample was spiked with a mixture of 40 different molecules (see **Figure 1**). The concentration for each of the analytes was 1.25 mg/L. Then successive dilutions were made in 80/20 A/B (see Methods for A and B) down to 0.4 µg/L to evaluate the sensitivity of the instrument. Most of the selected molecules are isobars or isomers. As an example, Amitryptiline and EDDP are isomers. They have exactly the same mass. Bromazepam and Clonazepam are isobars. Their masses differs by few milli-amu.

Mass accuracy was evaluated at different concentrations and at different resolution settings. On average, when using external calibration the mass accuracy for all the molecules was ~ 2-3 ppm and with internal calibration it was ~ 1 ppm. **Figure 2** reports the percentage of molecules that were identified at different concentrations and at two different resolution settings (10K and 100K). Identification was confirmed for a mass accuracy below 5 ppm. When going 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. Example of HCD fragmentation used to confirm presence of isomeric analytes (A) and an example of the impact of resolution on the signal-to-noise of HCD fragments (B)



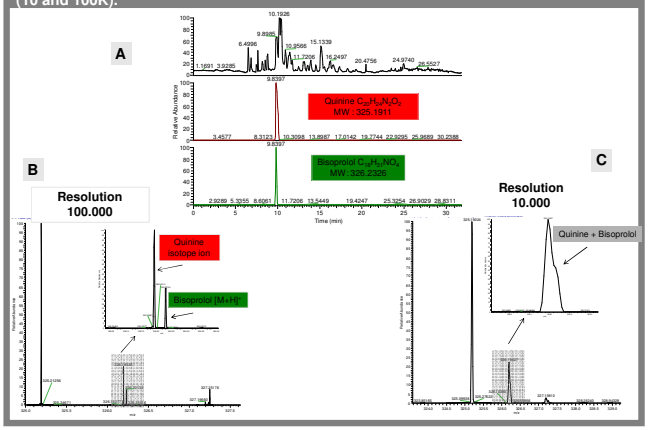
HCD Fragmentation

When isomers elute at very close retention times, another criteria has to be selected to differentiate and properly identify the analytes. Fragment ions generated under gas collision dissociation in the HCD collision cell can be used to fulfill this criteria. **Figure 3 A** shows an example with maprotiline and amitryptiline. Both have the same mass as they are isomers (Formula: $C_{20}H_{23}N$) and they have very similar retention times under our LC conditions. The only difference is that maprotiline generates a fragment ion at 250.158 *m/z* that is not seen with amytriptiline. Using fragment ions is, in general, mandatory to confirm the presence of an analyte.

Resolution Settings

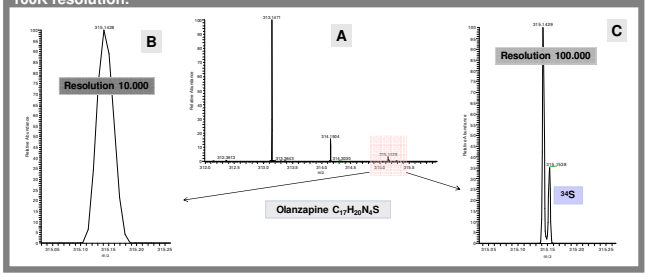
The analysis was performed under different resolution settings (R=10.000 and R=100.000). **Figure 3 B** shows the compound cyamemazine. Under HCD conditions, it gives a specific fragment at 279.09 *m/z* (lower traces). Both settings have been compared: 100.000 resolution and 10.000 resolution. The signal-to-noise for the fragment ion is much higher under high resolution conditions (614 versus 19). 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 (probably a phthalate). For this reason, the background in this lower resolution setting is high, which results in a lower signal-to-noise.

FIGURE 4. A: TIC and XICs of co-eluting compounds Quinine and Bisoprolol. B and C are expanded regions of the spectrum observed with two resolution settings (10 and 100K).



Ultra-high resolution is necessary in some cases to differentiate two analytes having the same retention time or from interfering matrix ions. **Figure 4** shows the example of Quinine and Bisoprolol, two compounds that have the same retention time (**Fig. 4A**). Their molecular weight differ by 1 amu, thus the ^{13}C isotope peak of quinine is not resolved from the ^{12}C isotope peak of bisoprolol at 10K resolution (**Fig. 4C**). Using ultra-high resolution, the ^{13}C isotope of quinine and ^{12}C isotope of bisoprolol are clearly separated, and thus, allows easy identification of bisoprolol with 3 ppm mass accuracy (**Fig. 4B**).

Figure 5. A: Mass spectrum of Olanzapine using ultra high resolution. B: expanded region of the spectrum where an olanzapine isotope ion is found using 10K resolution. C: expanded region of the spectrum where an olanzapine isotope ion is found using 100K resolution.



Ultra high resolution can also be used to further confirm and identify an analyte's presence. In **Figure 5A** a spectrum of olanzapine has a region around one of the isotope peaks highlighted in pink. At 10K (**Fig. 5B**) the peak provides no additional information than it's accurate mass, however, when 100K is utilised (**Fig. 5B**) the isotope pattern of the sulfur compound within Olanzapine is visible. This isotope pattern information further aids unique identification of this analyte in this sample.

Data Processing

All data acquired was reprocessed using ToxID™ software. ToxID automatically generates reports that contain the list of molecules that have been identified, and also the mass accuracy and the presence/absence of fragment ions. The retention time is also used as a criteria for confirmation.

Conclusions

- Limits of Detection (LODs) obtained for most drugs in the anlayte panel were below 1 ug/L.
- Ultra high resolution (100K) was utilised to solve a number of screening issues such as co-eluting and isobaric ions. Additional isotope pattern information can be obtained using this setting too.
- HCD fragmentation can be utilised to provide additional ions for analyte confirmation.
- ToxID software is ideally suited for library searching and reporting of results.

References

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(2) Sauvage, F-L.; Saint-Marcoux, F.; Duretz, B.; Deporte, D.; Lachatre, G.; Marquet, P. *Clin. Chem.* **2006**, *52(9)* , 1735–1742.