Retrospective, Multi-evidence Veterinary Drug Screening Based on Drift Tube Ion Mobile Mass Spectrometry

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The retrospectivity (the ability to retrospect a previously unknown compound in raw data) is very meaningful for risk assessment when facing new emerging drugs. High resolution mass spectrometry (MS) provided retrospective accurate mass, but accurate mass alone may lead to false positives in real samples. More evidence such as retrospective tandem MS (MS/MS) was desirable. In this study, drift tube (DT) ion mobile (IM)-MS was applied on veterinary drug screening to provide accurate mass, retention time (RT), collision cross section (CCS) and retrospective MS/MS data. In a single experiment, a veterinary drug can be identified based on all the evidence and the raw data can be traced for previous unknown drugs.

**Reagents and Sample Preparation**

Methanol (MeOH) was purchased from Merck KGaA (Darmstadt, Germany). Formic acid (FA) was purchased from Agilent Technologies (Santa Clara, CA). The porcine urine samples were collected from local farms. The samples were prepared using Bond Elut QuEChERS EMR-Lipid kit purchased from Agilent Technologies (Santa Clara, CA).

**LC-MS Conditions**

Agilent 1290 Infinity HPLC series binary pump

Column: Agilent Zorbax Eclips Plus C18 (3.0x150mm, 1.8 μm)

Column Temperature: 40 °C

Injection Volume: 5 μL

Autosampler Temp.: 4 °C

Mobile Phase A: Water with 0.2% FA, 2 mM ammonium acetate

Mobile Phase B: MeOH with 0.2% FA

Flow Rate: 0.4 ml/min

Gradient:

- 0.5 min 5%B
- 3.0 min 15%B
- 10.0 min 40%B
- 18.0 min 100%B

**Agilent 6560 IM-Q-TOF**

Gas Temperature: 325 °C

Gas Flow: 11 L/min

Nebulizer: 35 psi

Sheath Gas Temperature: 250 °C

Sheath Gas Flow: 7 L/min

Capillary Voltage: 3500 V (positive)

Nozzle Voltage: 300 V (positive)

Fragmentor: 380 V

Mass Range: 100-1700
Results and Discussion

This study systematically investigated the performance of IM-MS in veterinary drug screening without and with urine matrix. The find by formula (FbF) function used with a database search algorithm was compared with the four-dimensional molecular feature extraction (4D MFE) and identification workflow. It was found that the sensitivity of the 4D MFE workflow was higher for all the samples investigated. This sensitivity advantage was especially true with matrix and at low concentration.

In short, the 4D MFE workflow tolerated interference better (Figure 1). More positive results were identified using the 4D MFE workflow, due to the additional CCS value or IM separation. Most of the positive FbF results were included in the 4D MFE results (Figure 2). The 4D MFE results were more reliable than those from FbF, with far fewer false positives.

The IM-MS also provided data-independent acquisition (DIA) mode for collecting retrospective MS/MS data, which was another important evidence for identification. By the help of IM separation, there was no loss caused by quadrupole selection. In the experiment, ~86% of the identified features were confirmed by the MS/MS spectra in the samples with matrix. A new retrospective, multi-evidence veterinary drug screening platform was developed.

Figure 1. IM improved the mass spectrum quality for better identification. (A) The Leuco Malachite Green (C23H26N2) was not identified because interference in matrix distorted the isotope abundance. The red boxes represented the theoretical peak distribution of C23H26N2. (B) The IM separated the interference from the peaks of the drug. The extracted mass spectrum of the 4D feature fit well to the theoretical peak distribution of C23H26N2.
Results and Discussion

Figure 2. Area-proportional Venn Diagram of the positive results from FbF and 4D MFE workflow. (A-D) Mixed standards without matrix from 1ppb to 50ppb. (E-H) Mixed standards from 1ppb to 50ppb in urine matrix.

Conclusions

- The sensitivity of the 4D MFE workflow was higher for all the samples investigated. This was especially true for samples in matrix and at lower concentrations.
- The 4D MFE workflow tolerated interference better. More positive results were identified using the 4D MFE workflow and because of the additional CCS value as evidence.
- 4D MFE workflow contained most positive results from FbF and most of the FbF unique results were false positive.
- The 4D MFE workflow was practical to be used as a veterinary drug screening method.

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