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Note d'application

# Detection and Characterization of Drug Metabolites in Biofluids Using Survey Scan MS/MS Functionality on Waters™ Tandem Quadrupole Mass Spectrometers

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## Abstract

The ability to rapidly screen biofluid samples for drug metabolites helps researchers identify metabolic soft spots, detect potentially toxic metabolites, and improve pharmacokinetic characteristics of candidate medicines. This can be a complicated and time-consuming process requiring the acquisition and analysis of MS and MS/MS data. Tandem quadrupole mass spectrometers are used extensively for drug concentration determination due to their exquisite sensitivity in MRM mode, but they have other functionalities that can be applied to drug metabolism characterisation studies. Survey Scan acquisition mode on Waters tandem quadrupole mass spectrometers allows for the rapid detection and characterization of drug biotransformation's using diagnostic fragment ions and constant neutral loss. Survey Scan LC-MS/MS was applied to the analysis of urinary metabolites of methapyrilene. Over 30 drug related metabolites were detected using Survey Scan mode with precursor ion monitoring, by monitoring multiple common fragment ions of the dosed compound facilitated the localization of the site of metabolism.

## Benefits

- Unbiased data dependent acquisition
- Simple, easy to use MS/MS functionality
- Feature detection based on common fragment ion or constant neutral loss
- Precursor and product ion acquisition in a single analysis

## Introduction

The detection and characterization of drug metabolites plays a key role in drug discovery and development, from ADME profiling and in-vitro screening to the monitoring of in-vivo toxicokinetic studies.[1] The information derived from these studies allows for the identification of metabolic soft – spots from in-vitro screening, determination of the route and rate of metabolism in early animal studies and comparison of the metabolic profiles between, dose groups, genders, and species helping to establish toxicological coverage.[2]

Mass spectrometry combined with liquid chromatography has established itself as the platform of choice for DMPK studies due to it's sensitivity, selectivity, rapid methods development, information rich spectral data, and compatibility with biological samples. Tandem quadrupole mass spectrometers are extensively employed in bioanalytical laboratories to provide high sensitivity quantitative data to measure drug exposure and determine pharmacokinetic (PK) parameters of candidate medicines.[3] Along with the need to generate PK information there is a need to monitor and profile biofluids for changes in drug biotransformation's. This ensures sufficient metabolic coverage from safety assessment studies, monitors changes in relative metabolite abundance between species and dose levels and detect potentially toxic metabolites. The unique design of the tandem quadrupole mass spectrometers facilitates the rapid switching between MS and MS/MS mode to collect high quality fragment ion spectra in the time frame of a narrow LC peak. Survey Scan acquisition has been employed for the high sensitivity profiling of biological fluids for drug related biotransformation's based on either the diagnostic common fragment ions or the constant neutral loss of metabolic conjugates such as glucuronides or glutathiones.

## Experimental

## Sample Description

Urine samples were derived from the repeat oral administration of methapyrilene Figure 1, to the male wistar rat.[4] The samples were collected over 24-hours on days 2, 4, and 6 of dosing. Full particulars of the animal study and sample preparation are detailed in Waters application note 720008016.[5] Control wistar rat urine was obtained from BioIVT, NY, USA. Biofluid samples were prepared by mixing 50 µL of urine with 200 µL ice cold acetonitrile, the sample was vortex mixed then stored at -20 °C for one hout. The resulting samples were then centrifuged at 9,000 g for 5 minutes and the supernatant transferred to a total recovery vial for analysis by LC-MS/MS.

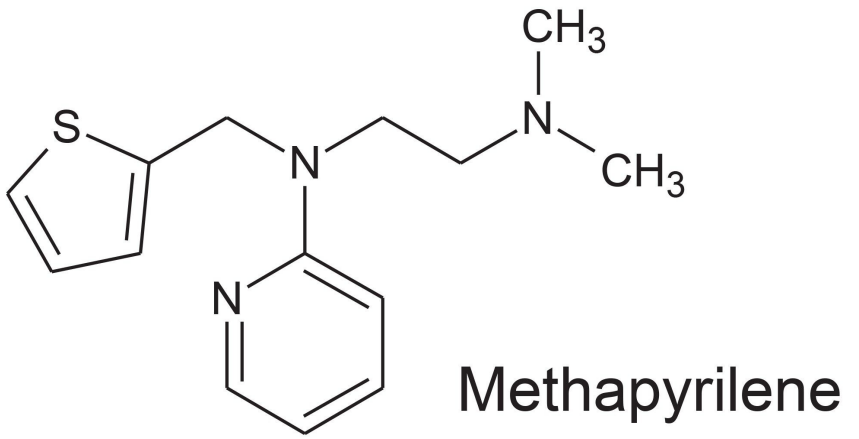


*Figure 1. Methapyrilene.*

## Method Conditions

Following sample extraction, a 2 µL aliquot of sample was loaded onto a 2.1 x 100 mm Cortecs™ $C_{18}$ 2.7 µm Column chromatography column. The column was maintained at 40 °C and eluted with a linear reversed – phase gradient over 10 minutes at 600 µL/min, using aqueous 0.1% formic acid as mobile phase A and 0.1% formic acid in 95:5 (v/v) acetonitrile : water as mobile phase B, Table 1. The column effluent was monitored by positive ion ESI mass spectrometry operating in survey scan mode, with either precursor ion scanning or constant neutral loss acquisition triggering, Table 2.

## LC Conditions

| | |
|---|---|
| LC system: | ACQUITY™ I Class UPLC™ |
| Detection: | Xevo™ TQ-XS |
| Vials: | Waters total recovery vials (p/n: 186004631) |
| Column(s): | CORTECS Premier T3 Column, 2.7 µm, 2.1 x 100 mm (p/n: 186010473) |
| Column temperature: | 40 °C |
| Sample temperature: | 8 °C |
| Injection volume: | 2 µL (urine) |
| Flow rate: | 600 µL/min |
| Mobile phase A: | 0.1% (v/v) aqueous formic acid |
| Mobile phase B: | 95% acetonitrile, 5% waters, 0.1% (v/v) formic acid |
| Gradient: | *See gradient table* |

| Time (min) | Flow (mL/min) | %A | %B | Curve |
|---|---|---|---|---|
| 0 | 0.6 | 95 | 5 | initial |
| 1 | 0.6 | 95 | 5 | 6 |
| 10 | 0.6 | 60 | 40 | 6 |
| 10.1 | 0.6 | 5 | 5 | 6 |
| 12 | 0.6 | 5 | 5 | 6 |
| 12.1 | 0.6 | 95 | 5 | 6 |

*Table 1. Gradient Table.*

## Table 2. MS Conditions

| | |
|---|---|
| MS system: | Xevo TQ-XS |
| Ionization mode: | Positive Ion |
| Acquisition range: | ESI |
| Capillary voltage: | 2.0 kV |
| Collision energy: | 30 eV |
| Cone voltage: | 30 V |
| Acquisition: | Survey scan using precursors of *m/z*=97.1, 119.2, 133.2, or constant neutral loss 80.1 and 176.1 |

## Data Management

| | |
|---|---|
| Chromatography software: | MassLynx™ Ver 4.2 |
| MS software: | MassLynx Ver 4.2 |
| Informatics: | MassLynx Ver 4.2 |

**Note 5. Specify version for each software.*

## Results and Discussion

Metabolite identification in biofluids by LC-MS/MS requires first the detection of drug related material in matrices such as urine and plasma, followed by the acquisition of precursor and product ion spectral data for analysis. This data is then rationalized using the precursor mass and fragmentation pattern of the dosed compound, precursor, and product ion spectra of the detected metabolites and known metabolic transformations (functionalization and conjugation). In order for an analyte to be accepted as a drug related metabolite it must fulfil the following criteria:[6]

1. Metabolite can be explained by plausible biotransformation's
2. Metabolite has at least one fragment ion in common with the parent compound or has at least one neutral loss in common with the parent
3. The analyte is not observed in the control or pre dose sample and the compound must elute within a justifiable retention range compared to the parent compound
4. All components eluting in the solvent front should be eliminated to avoid false positives

Survey Scan acquisition takes advantage of the flexibility of the Waters tandem quadrupole MS ion optics to screen for drug related analytes using various MS(MS) acquisition modes, such as constant neutral loss (CNL) or common fragment ions. Upon detection of these features (above a user specified intensity threshold) the instrument automatically switches to obtain targeted MS/MS data for the base peak of the detected feature. This data directed mode of analysis (DDA) facilitates a sensitive and selective mode of metabolite screening followed

by targeted MS/MS data acquisition, which results in high quality MS spectra with reduced background noise.

To illustrate the application of Survey Scan acquisition for drug metabolite screening and characterization urine samples were analysed following the oral administration of the antihistamine and anticholinergic drug Methapyrilene to the rat. Methapyrilene has the chemical formula $C_{14}H_{19}N_3S$, positive ion MS/MS analysis of the authentic standard of methapyrilene (*m/z*=262.23) gave rise to the diagnostic fragment ions *m/*z=96.88, 119.17, 121.17, 133.07, and 217.09. These fragment ions can be rationalized as the thiophene ring fragment (*m/*z=96.88), loss of thiophene ring, aliphatic chain, and tertiary amine group from pyridine ring (*m/*z=119.17), loss of thiophene ring, and aliphatic chain to leave tertiary amine and pyridine group (*m/*z=121.17), loss of thiophene ring and tertiary amine to leave pyridine ring and aliphatic chain (*m/*z=133.07), and finally loss of tertiary amine (*m/*z=217.09), Figure 2.

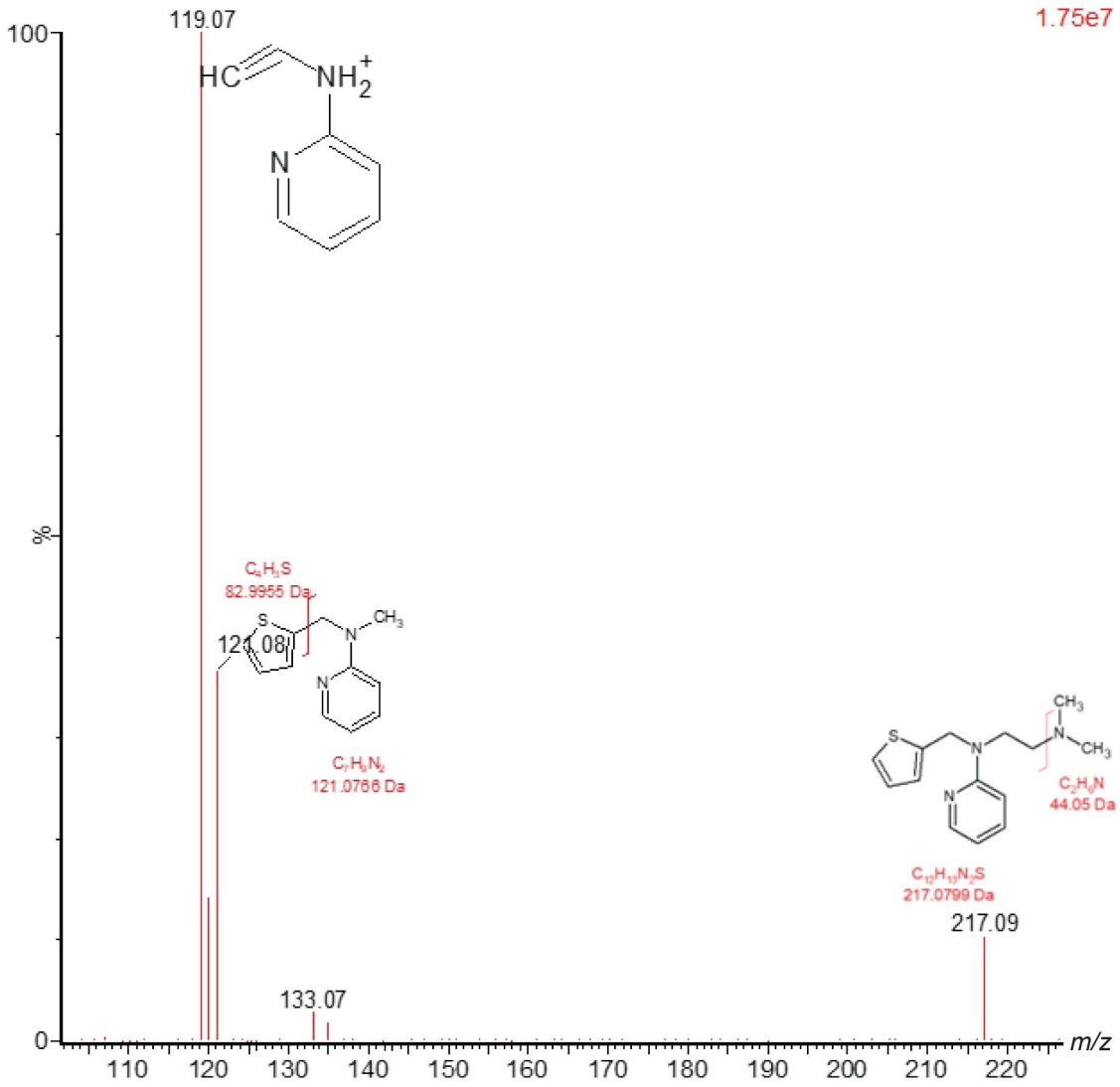


*Figure 2. Product ion MS/MS analysis of methapyrilene authentic standard, m/z=262.23.*

## Precursor Ion Scanning Acquisition

Urine data was acquired in Survey Scan mode using precursor ion scanning of *m/*z=96.88, 121.17, and 137.20, (*m/*z=137.20 represents the hydroxylation of the 121.17 moiety) with MS/MS data of the precursor ion collected over the mass range of *m/*z=50–600. Data was acquired in centroid mode using a scan time of 0.5 seconds with a collision energy of 30 eV, upon switching to MS/MS mode using a scan time of 0.2 seconds. Acquisition returned to precursor scanning mode when the signal intensity fell below 1,000 counts. The data obtained for the urine

analysis using the precursor of *m/*z=96.88, for vehicle only and the D6 150 mg/kg sample are displayed in Figure 3. The data shows that the Survey Scan acquisition of precursors *m/*z=96.88 detected multiple peaks in the dosed sample that were absent in the vehicle only sample. Additionally the overlaid trace the vehicle only sample shows no evidence of drug related material, illustrating the selectivity of the acquisition process. The data displayed in Figure 4, compares the TIC chromatograms obtained for the analysis of the same D6 150 mg/kg urine sample using each of the three precursor ions *m/*z=96.88, 121.17, and 137.20. As can be seen from this data some analytes are detected with all three precursor masses, ***e.g.,*** peak at $t_R$=1.35 minutes whereas the majority of peaks are detected with just the *m/*z=96.88, 121.17 acquisitions. This illustrates the selectivity of this acquisition mode. The asymmetrical peak shapes are an artifact produced from the DDA acquisition process.

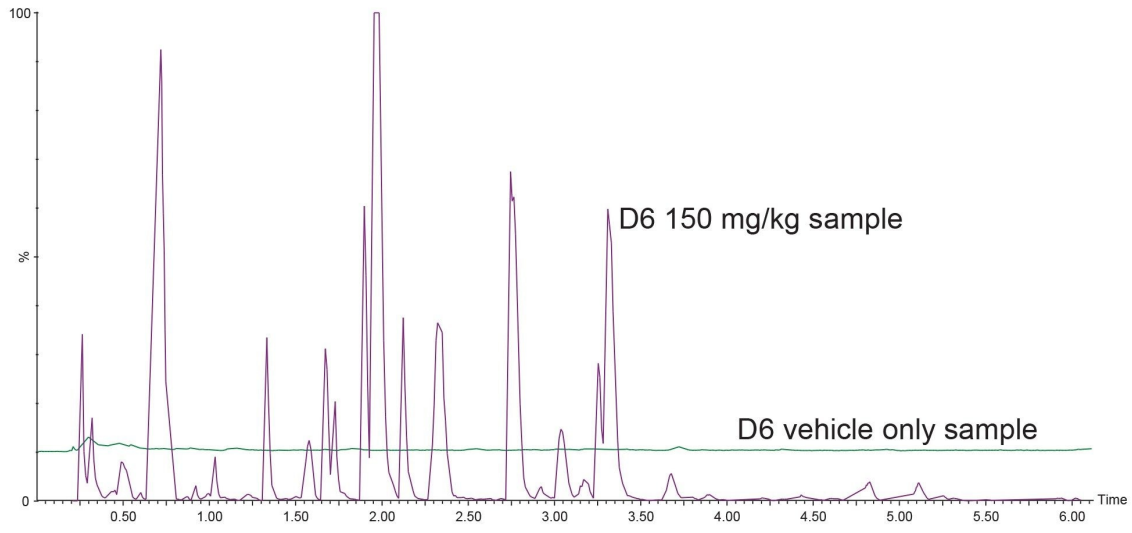


*Figure 3. Analysis of D6 vehicle only urine and 150 mg/kg sample using Positive ion LC-MS/MS Survey Scan acquisition precursors m/z=96.88.*

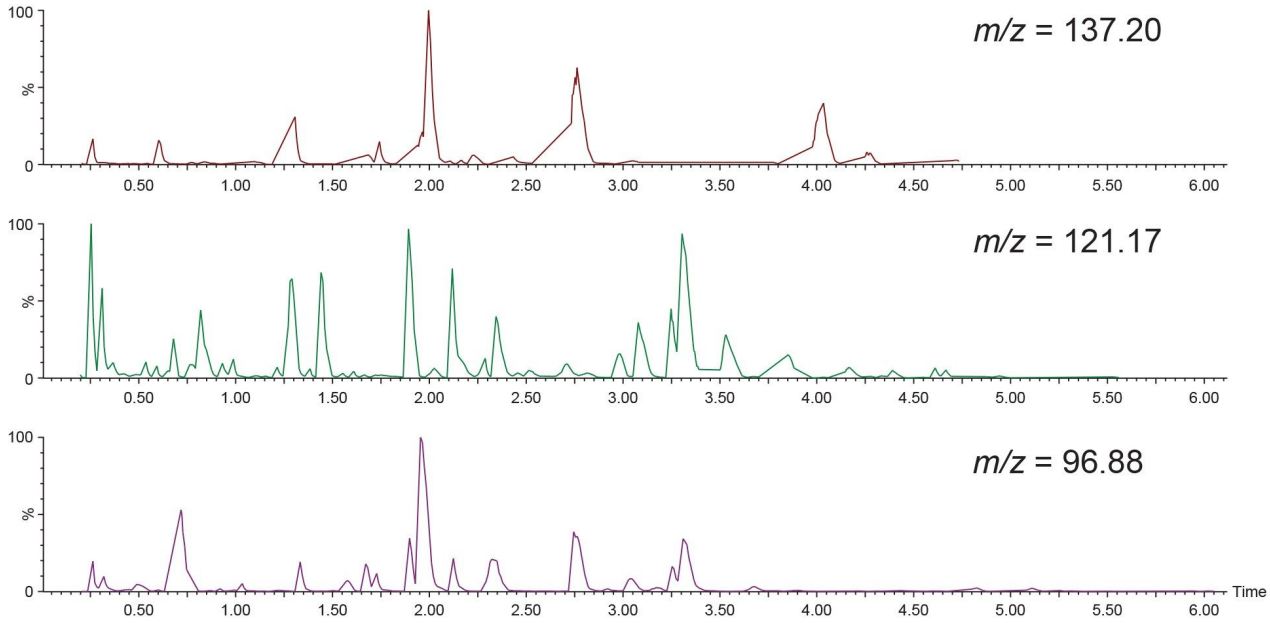


*Figure 4. Analysis of 150 mg/kg sample using Positive ion LC-MS/MS Survey Scan acquisition precursors m/z =96.88, 121.17, and 137.20.*

Survey scan is a DDA acquisition mode that utilizes a scanning acquisition, in this case precursor ion, to collect both MS and MS/MS spectral data in one single analysis. These MS and MS/MS spectra were used for metabolite classification and characterization. A total of 34 drug related metabolites were detected using this mode of analysis. Examples of the MS and MS/MS spectra obtained from the precursors of *m/z*=96.88 are given below in Figure 5. The data shown illustrates the MS and MS/MS spectra obtained for the N-oxide ($t_R$=3.3 minutes), hydroxylation ($t_R$=3.0 minutes) and dihydroxylation ($t_R$=2.7 minutes), metabolites of methapyrilene. It is interesting to note that, due to the specificity of the DDA acquisition, the MS/MS spectra produced show an absence of extraneous spectral noise and are thus simpler to interpret.

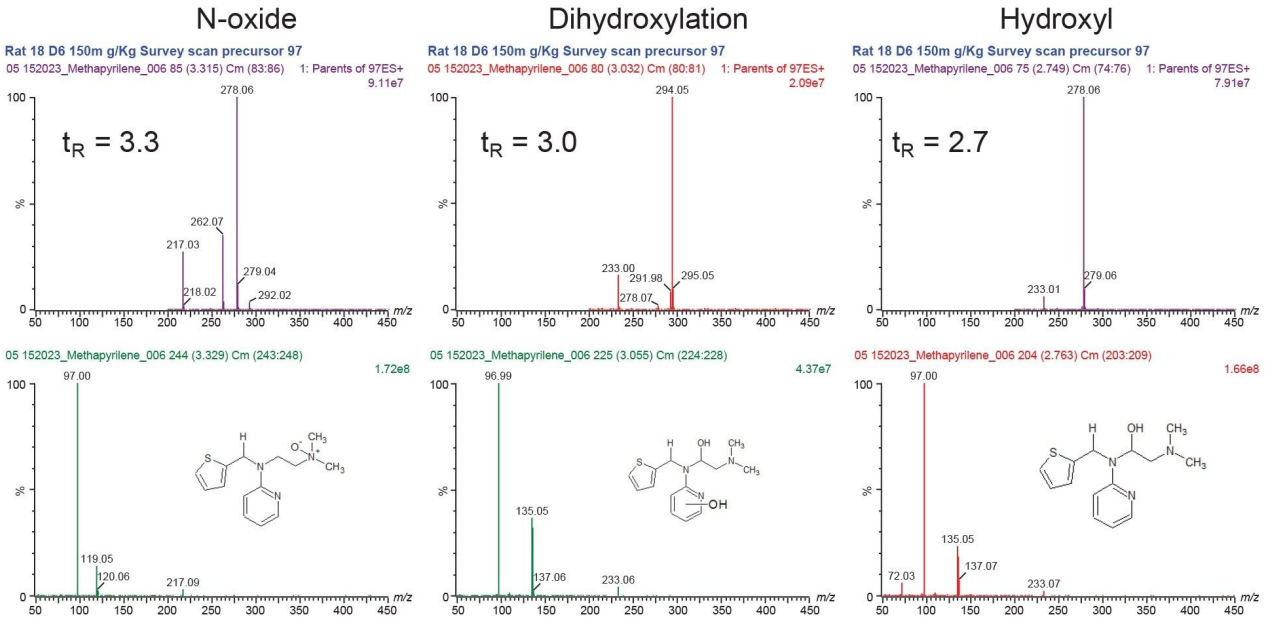


*Figure 5. MS and MS/MS spectra obtained from the positive ion ESI LC-MS/MS analysis of rat urine D6 150 mg/kg using Survey Scan acquisition of precursors of m/z=96.88.*

The data displayed in Figure 6 illustrates the MS and MS/MS obtained from the Survey Scan acquisition using the precursor of *m/z*=121.17. The example data shows the spectra obtained for methapyrilene (dosed compound), as well as the N-oxide ($t_R$=3.3 minutes), and the N-O-glucuronide ($t_R$=1.8 minutes), metabolites. As with the data shown in Figure 5 the MS/MS spectra obtained are very clear and show an absence of background noise, simplifying the process of structural elucidation. The fast acquisition capability of Waters tandem quadrupole mass spectrometers allowed multiple precursor ions to be scanned in one analytical run. This allowed for rapid, comprehensive metabolite detection and localization of the site of metabolism.

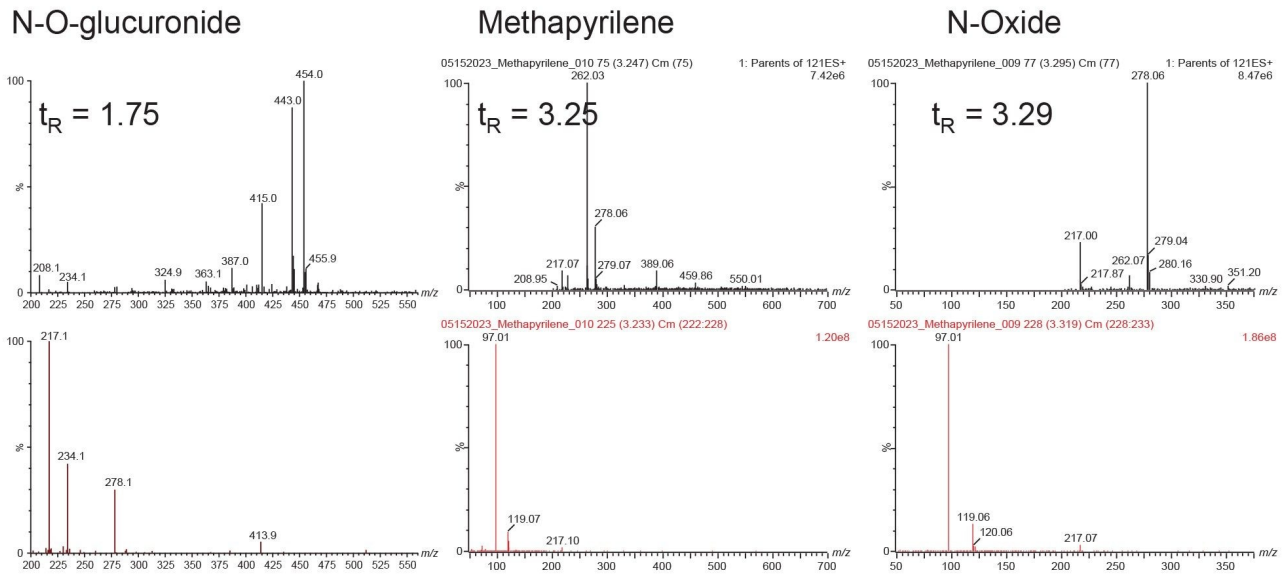


*Figure 6. MS and MS/MS spectra obtained from the positive ion ESI LC-MS/MS analysis of rat urine D6 150 mg/kg using Survey Scan acquisition of precursors of m/z=121.17.*

Constant neutral loss (CNL) is a mode of MS/MS data acquisition in which compounds are detected based on the loss of a neutral fragment. This mode of acquisition is commonly employed to screen for drug metabolites which are formed by conjugation with polar moieties such as sulphate, glucuronic acid, and glutathione. To illustrate this mode of acquisition, methapyrilene urine samples were analysed in positive ion ESI Survey Scan mode using the neutral loss of 176.12 Da to monitor for glucuronide metabolites (acquisition parameters were similar to that of precursor ion scanning mode). The data displayed in Figure 7 shows the data obtained for the D6 vehicle only and D6 150 mg/kg samples. As can be seen from the data there are several peaks detected in the vehicle only sample which showed a signal for the loss of the glucuronide conjugate (176.12 Da), these are most likely conjugates of endogenous compounds, ***e.g.,*** food and environment. Analysis of the D6 150 mg/kg sample showed significantly more glucuronide conjugates compared to the vehicle only sample.

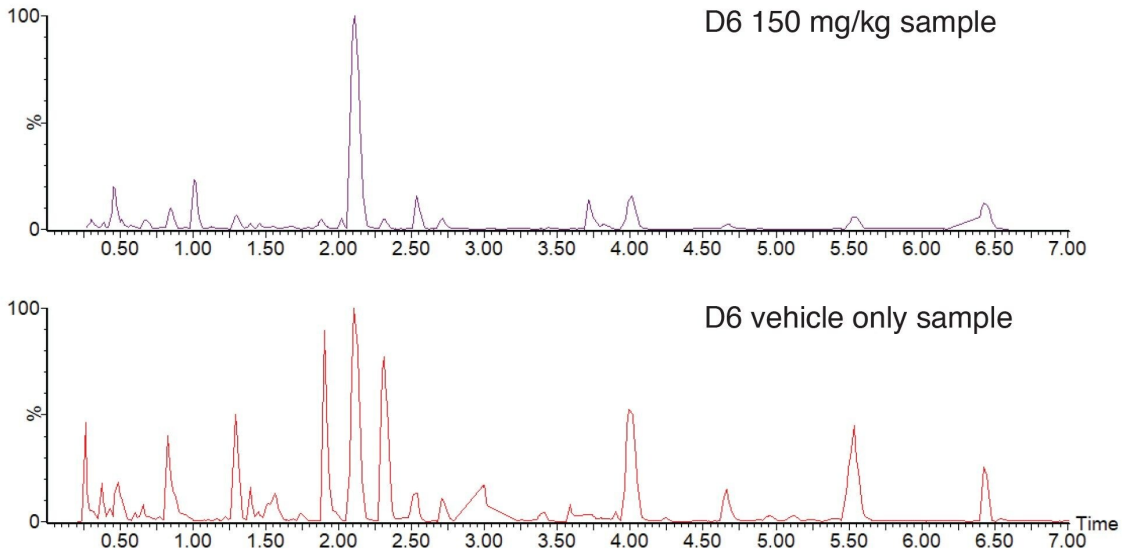


*Figure 7. Analysis of D6 vehicle only urine and 150 mg/kg sample using Positive ion LC-MS/MS Survey Scan acquisition constant neutral loss 176.12 Da.*

The CNL glucuronide (-176 DA) analysis of the urine from the animals dosed with methapyrilene was further analysed for the presence of drug related glucuronide metabolites. This revealed that there were several drug related glucuronide conjugates of multiple functionalization's of methapyrilene, including O-glucuronide, N-O-glucuronide, desmethyl O-glucuronide, dihydroxyl glucuronide. The data shown in Figure 8, shows the extracted ion chromatogram (A) for the O-glucuronide metabolites of methapyrilene (*m/z*=454.1). The data showed the presence of four O-glucuronide metabolites. The derived MS and MS-MS spectra from the two peaks at $t_R$=1.94 and 1.54 minutes are shown in Figures 8 B, C. The MS spectra of the $t_R$=1.94 min peak (Fig. 8B) shows a base MS peak of *m/z*=454.1 and MS/MS fragment ion *m/z*=233.1, 137.08, and 96.88, this metabolite can be rationalized as glucuronide conjugate of the metabolite formed from hydroxylation of the pyridine ring. In contrast the MS/MS fragment ion derived from the peak at $t_R$=1.54 (Fig. 8C) gave fragment ions consistent with the parent compound, methapyrilene *m/z*=217.10, 121.08, and 96.99 suggesting that this is the glucuronide conjugate of the N-oxide metabolite.

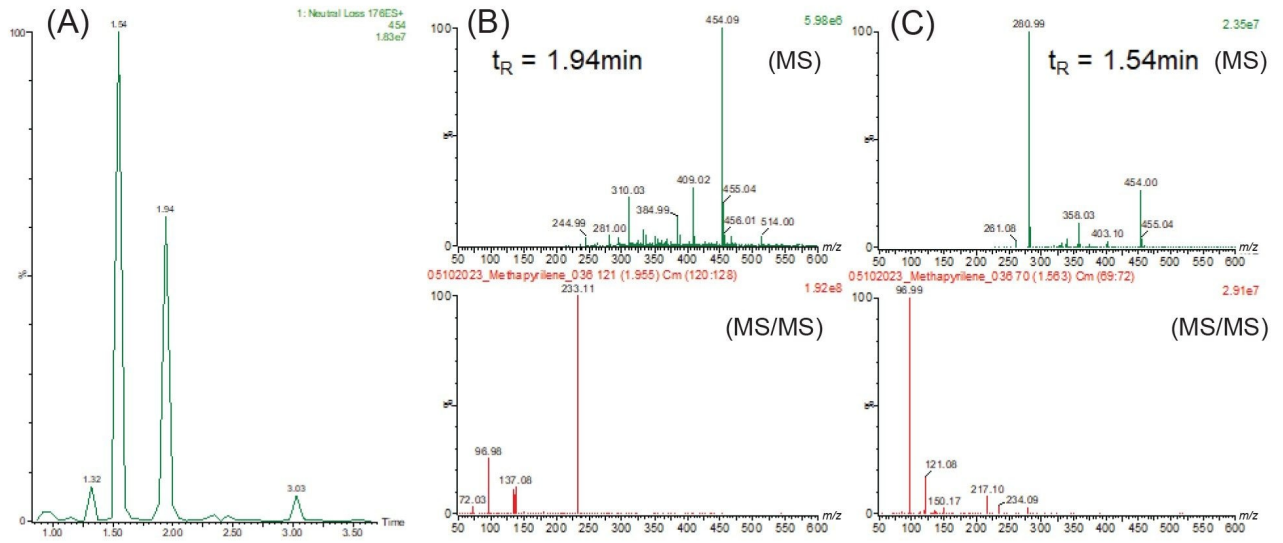


*Figure 8. Extracted ion chromatogram of precursor metabolites m/z=454 from the constant neutral loss m/z=176 of the D6 150 mg/kg urine sample (A). MS and MS/MS spectra of peak eluting at $t_R$=1.94 min (B) and MS and MS/MS spectra of peak eluting at $t_R$=1.54 min (C).*

## Conclusion

The detection and characterization of drug metabolites plays a critical role in the drug discovery and development process. Waters tandem quadrupole mass spectrometers are equipped with novel collision cell design allowing for the rapid switching between different acquisition modes. Survey Scan exploits this capability to selectively screen for drug related material via common fragment ion or constant neutral loss. This acquisition mode was used to investigate the urinary drug metabolites of methapyrilene following the oral administration to the rat. The Survey Scan acquisition mode provided a rapid sensitive and specific mode of data analysis producing clean MS/MS spectra, simplifying structural interpretation. The targeted MS/MS spectra obtained using this approach allowed for the rapid, simple characterization of the drug metabolites. Using this approach over 30 urinary drug related metabolites were detected and characterized. Previously unreported metabolites of methapyrilene such as N-O-glucuronide, were detected using this mode of acquisition.

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