Single Platform for Both Confident Identification and Quantitation Workflows in the Forensic Toxicology Lab

High Resolution, Targeted Analysis using the SCIEX X500R QTOF System

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Toxicology laboratories worldwide run a variety of assays, ranging from targeted quantitation assays that require good dynamic range to screenings on comprehensive analyte panels where confident detection is critical. Accurate mass platforms can provide this added flexibility as well as providing high quality quantitation.

MRM^{HR} (High Resolution Multiple Reaction Monitoring) is a targeted data acquisition mode for quantification using a high resolution accurate mass instrument. This workflow is similar to monitoring unique fragment ions with MRM on a triple quadrupole instrument, however additional selectivity can be gained by leveraging the high-resolution MS/MS data that is collected for each compound.

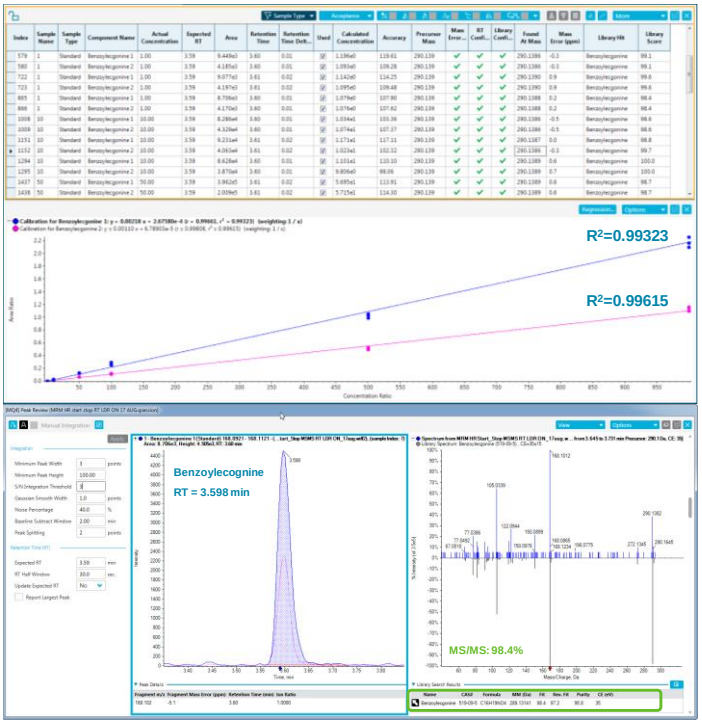

Figure 1. Obtain Both High Quality Quantitation with Confident Identification in a Single Workflow. Quantitation of Benzoylecognine using MRM^{HR} Workflow with the simultaneous generation of full scan MS/MS data provided 4 orders of linear dynamic range (top) for quantitation and a confidence identification using ion ratio computation (bottom left) and automatic library matching (bottom right).

In this technical note, the use of the MRM^{HR} workflow on the SCIEX X500R QTOF was investigated for targeted quantification applications in a forensic toxicological setting. MRM^{HR} allowed for both the quantification and identification of many analytes in biological samples in a single workflow. This high resolution targeted approach enabled sensitive quantitation of low concentration species in urine matrix utilizing the selective high resolution, accurate mass MS/MS information combined with library searching and ion ratio for identification purposes (Figure 1).

The MRM^{HR} Workflow Difference on the X500R QTOF System

- The X500R QTOF System provides high resolution MS/MS at high acquisition rates (up to 100 MS/MS per second) to enable targeted quantitation on high number of analytes
- Easily build and optimize targeted methods on many analytes using streamlined method optimization features in SCIEX OS software 1.3
- Linear dynamic range of quantitation across the 90 targeted analytes averaged ~4 orders
- Confident identification using automatic MS/MS spectra library searching and ion ratios
- Industry leading robustness of Turbo V™ source and Curtain Gas™ interfaces

Methods

Sample Preparation: Calibration curves with a mix of known compound standards (Cerilliant, Round Rock, Texas) were prepared in human drug free urine. Then 10 µL of IS spiking solution was added to 100 µL of urine samples which included both the calibration standards and unknown forensic samples. The mix was then diluted 10-fold with 90:10 (v:v), 0.1% formic acid in water : 0.1% formic acid in methanol followed by ultra-centrifugation.

Internal Standards: 6-MAM-d3, amphetamine-d5, benzoylecgonine-d3, buprenorphine-d4, methamphetamine-d5, codeine-d3 and morphine-d3 were used as internal standards (Cerilliant, Round Rock, Texas). They were mixed and diluted in methanol at concentration of 1000 ng/mL as IS spiking solution.

Liquid Chromatography: HPLC separation was performed at 30 °C on a Phenomenex Kinetex Phenyl-Hexyl column (50 × 2.1 mm, 2.6µm, 00B-4495-E0) on the SCIEX ExionLC™ AC system. Mobile phases used were water and methanol with appropriate additives. The LC flow rate was 0.5 mL/min and the LC runtime was 6.5 minutes. Injection volume was 10 µL.

Mass Spectrometry: MS and MS/MS data were collected using MRM^{HR} workflow on the benchtop SCIEX X500R QTOF System with SCIEX OS software 1.3, each cycle containing a TOF MS experiment and two MS/MS experiments. *Guided MRM^{HR}* mode in the SCIEX OS software was used to aid in determining the optimal fragment ions for each target compound and to optimize the compound dependent parameters for each transition.

Data Processing: Targeted data processing was performed in the SCIEX OS software 1.3. The target processing method consisting of 90 drugs and internal standards was automatically constructed from the MRM^{HR} acquisition method for post-acquisition data processing.

Linear dynamic range was evaluated through calibration curves with analyte concentrations ranging from 0.1 – 1000 ng/mL. Automated compound library search was performed using the 1700 compound high resolution MS/MS forensic spectral library (Forensic HR-MS/MS Spectral Library version 2.0).

Table 1. List of the 23 Target Compounds Analyzed in a Single Assay using the MRM^{HR} Workflow.

6-MAM	*Fentanyl*	PCP
Alprazolam	*Hydrocodone*	*THC-COOH*
Amphetamine	*Hydromorphone*	*7-Aminoclonazepam*
Benzoylecgonine	*Methamphetamine*	*Amitriptyline*
Buprenorphine	*Morphine*	*Noroxycodone*
Codeine	*Norbuprenorphine*	*Oxazepam*
Diazepam	*Norfentanyl*	*Naltrexone*
EDDP	*Oxymorphone*	

Simplified, Automated Method Development for the MRM^{HR} Workflow

To help transition from the familiarity of MRM performed on a triple quadrupole to MRM^{HR} Workflow performed on the SCIEX X500R QTOF system, the SCIEX OS Software 1.3 offers acquisition method development workflows that enable users to optimize fragments and parameters to build a final optimized acquisition method, in either a fully *Automatic* or a *Guided* workflow (Figure 2a)..

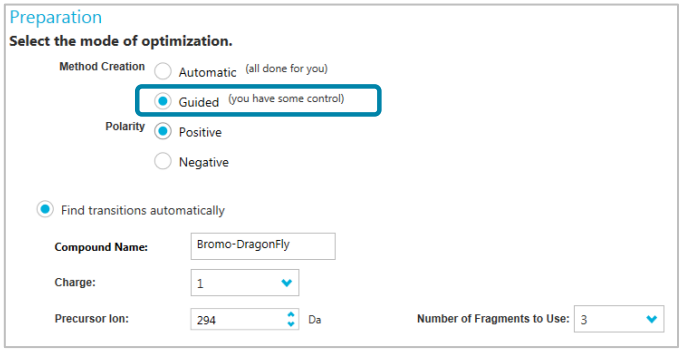

Figure 2a. SCIEX OS Software 1.3 Provides MRM^{HR} Method Optimization Options. Using either the fully automatic or guided workflow for rapid method implementation.

Transitions can be provided or determined automatically and parameters (such as retention time, collision energy (CE) and declustering potential) will be determined for each transition. Methods can also be developed that are fully targeted like an MRM assay or set up to collect full scan MS/MS data (using universal CE and collision energy spread (CES) conditions) that can be used for downstream library searching.

In this case, the *Guided* method development workflow (Figure 2b) was used to automatically create MRM^{HR} acquisition methods containing two looped TOF MS/MS experiments (2 transitions) per compound.

1. **Set Initial Conditions**
   Source Ionization Parameters and TOF MS Scan for precursor ion scan
2. **Optimize declustering potential (DP)**
   Automatic ramp of DP and find most intense value for each MRM
3. **Obtain the Product Ions**
   Software will select most intense fragments from Collision Energy ramp
4. **Optimize Collision Energy (CE)**
   Automatic ramp of DP and find most intense CE value for each MRM
5. **Review Report**
   Software reports optimal MRM parameters for analysis
6. **Send MRM to Method Editor**
   Parameters are automatically imported to MS acquisition method

Figure 2b. SCIEX OS Software 1.3 *Guided* MRM^{HR} Workflow. method parameters (declustering potential and collision energy) can be optimized for every target compound.

Acquiring Full Scan MS/MS for Library Searching

In addition to the fully targeted workflow that mimics the MRM workflow, MRM^{HR} workflow can also be performed using full scan MS/MS acquisition; by defining the m/z range desired using the *Apply TOF start/stop mass* feature. This mode is desirable if the accurate mass of the fragment ions are not known upfront or using library search to confirm identification is required.

This mode allows for acquisition of the full MS/MS fragment *m/z* range and therefore post-acquisition data processing extraction of one or multiple unique accurate mass fragment ions for quantification purposes. This approach also allows for MS/MS spectral library searching after acquisition of looped full scan TOF-MS/MS product ion experiments as well as use of fragment ion ratios.

MS/MS spectral library searching can be performed by using the SCIEX Forensic HR-MS/MS Spectral Library version 2.0 for added confidence in identification in addition to ion ratios. Figure 3 highlights another positive spectral match from a library search for PCP as shown by the mirror image plot and the ion ratio XIC plot (bottom).

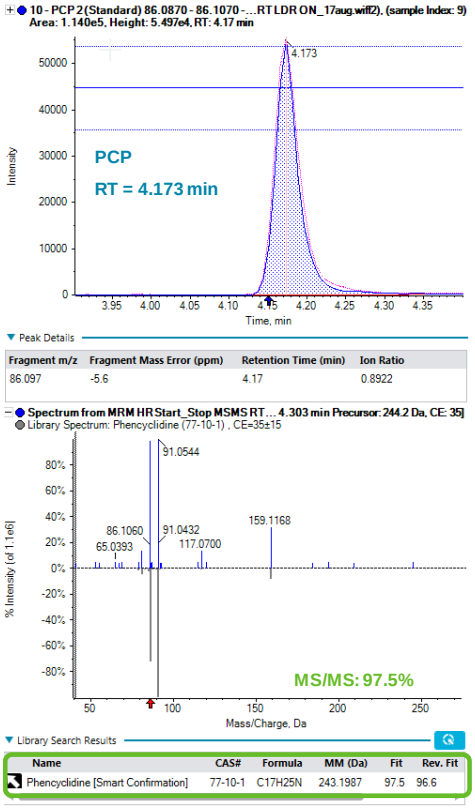

Figure 3. Automatic MS/MS Library Search. XIC (top) of a PCP calibration standard in urine (10 ng/mL) with MS/MS spectrum and library match score (bottom).

Dynamic Range

The MRM^{HR} targeted acquisition method involving the full MS/MS *m/z* range still allows the preservation of the desired linear dynamic range plus the identification through ion ratios; in this study a similar dynamic range was observed for most compounds between both targeted methods. Figure 4 shows calibration curves and XICs obtained for EDDP using both approaches.

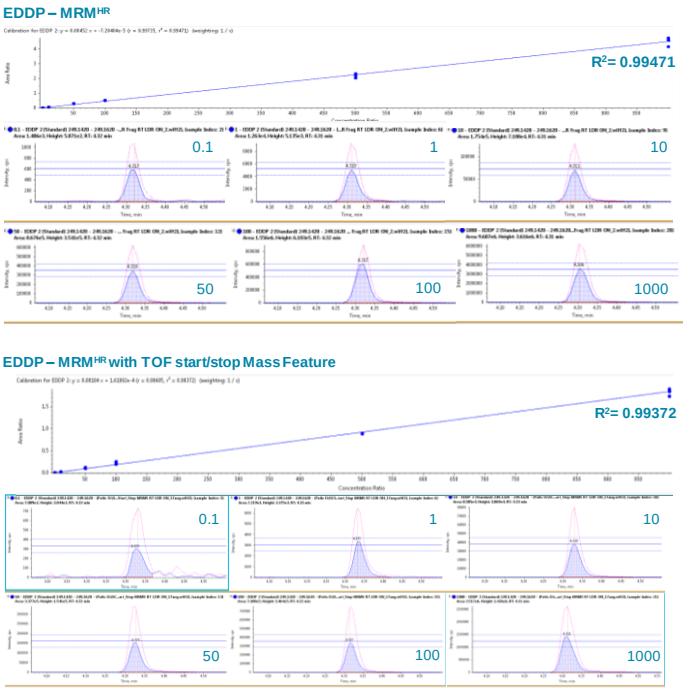

Figure 4. Linear Dynamic Range. Good Linear Dynamic Range was achieved across the targeted compounds using the both MRM^{HR} and MRM^{HR} with full scan MS/MS. Calibration curves for EDDP are shown as well a few representative XIC traces to demonstrate consistency in ion ratio. Linear dynamic range averaged ~4 orders with concentrations ranging from 0.1 to 1000 ng/mL.

Conclusions

The SCIEX X500R QTOF System combined with the intuitive SCIEX OS software 1.3 has made the MRM^{HR} workflow very straightforward to incorporate into the toolkit of the forensic toxicology lab.

- Automated method development simplifies building and optimizing acquisition methods.
- Fully targeted MRM^{HR} workflow can be used to transition methods from a triple quadrupole assay or develop new highly multiplexed assays.
- MRM^{HR} workflow with full scan MS/MS can also be used when library search confirmation is required or when best fragment ions for quantitation are not yet known.
- Both methods can be combined with time scheduling, monitoring each analyte only across its expected elution time, for highest compound multiplexing. In this example, 23 compounds were quantified in a 6.5 min method.
- Typical linear dynamic range for quantitation averaged ~4 orders of magnitude across the compounds monitored in this study.

MRM^{HR} workflow is a targeted data acquisition strategy enabling sensitive quantitation of low concentration species in complex matrices utilizing the selective high resolution, accurate mass MS/MS information as well as using ion ratio and MS/MS spectral library matching for identification purposes.

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