Comprehensive Forensic Toxicology Screening in Serum using On-Line SPE LC-MS/MS

SCIEX QTRAP® 4500 LC-MS/MS System and Spark Holland PICO

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Introduction

Comprehensive screening for the detection of drugs and toxic compounds in biological samples is an important function of forensic toxicological analysis. As the demand to monitor the ever increasing number of drugs continues to rise, so too does the need to detect and quantify these compounds in a simple, single and automated run, providing a fast turn-around time for the results.

This application note describes the rapid cleanup of serum samples and detection of over 100 drugs using On-line Solid Phase Extraction (SPE) LC-MS/MS screening and confirmation. The LC-MS/MS was operated in Multiple Reaction Mode (MRM) for detection. Dependent MS/MS spectra were acquired in the Enhanced Product Ion (EPI) mode after being triggered from a Scheduled MRM™ Pro Algorithm Information Dependent Acquisition (IDA) survey scan. Combining MRM and product ion spectral acquisition allows for compound identification with highest confidence based on mass spectral library matching. The automated workflow monitors large panels of analytes, detecting and quantifying these compounds in a single run.

The Scheduled MRM™ Pro algorithm allows for excellent data quality covering the broadest range of drugs possible by only spending time collecting useful data and eliminating triggering on secondary MRMs and product ion spectra if the compound of interest is not present.

Materials and Methods

Materials

130 neat standard solutions of different drug classes were purchased from Cerilliant. An analyte stock solution was prepared by combining all the drugs with methanol, at appropriate concentrations to evaluate the automated serum cleanup method for all the compounds. A detailed list of the drugs used for this study is available upon request. A selected panel of deuterated analogues were purchased from Cerilliant and used for quantification.

Blank serum was spiked with drug mixture stock solutions making concentrations ranging from 0.5 to 2000 ng/mL to prepare the calibrators; 10, 1000 ng/mL for QCs.

Sample pretreatment

Sample Clean-up and LC separation was performed using a Spark Holland Pico System. Figure 2 shows the sample preparation procedure. On-line sample clean-up was achieved using HySphere Resin GP (general purpose) 10; polydivinyl-benzene cartridges.
HPLC Conditions

- Phenomenex Kinetex column (C18, 3 x 50 mm, 2.6 µm, 100 Å); 30°C
- Mobile phase A: Water + 10 mM ammonium formate
- Mobile Phase B: Acetonitrile + methanol (1:1)
- Flow rate: 400 µL/min flow rate.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>1.00</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>10.00</td>
<td>0.4</td>
<td>100</td>
</tr>
<tr>
<td>13.00</td>
<td>0.4</td>
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</tr>
<tr>
<td>13.10</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>15.50</td>
<td>0.4</td>
<td>2</td>
</tr>
</tbody>
</table>

MS/MS Conditions

A SCIEX QTRAP® 4500 LC-MS/MS System operating in Scheduled MRM™ Pro Algorithm mode was used for detection, in positive TurboIonSpray® probe mode. EPI spectra at a scan speed of 10000 Da/s were triggered from the MRM survey scan and acquired using a dynamic fill time and dynamic background subtraction for optimal MS/MS quality. EPI spectra were generated using standardized Collision Energy (CE) of 35 V with Collision Energy Spread (CES) of 15 V to ensure a characteristic MS/MS pattern independently on the compound’s fragmentation efficiency. MS/MS spectra were searched against the iMethod™ Application Meta Library.
Analyst® Software Version 1.6.2 and Scheduled MRM™ Pro Algorithm

SCIEX introduced the Scheduled MRM™ Algorithm in 2008 in Analyst® Software version 1.5. The algorithm adjusts detection windows automatically depending on the retention time of the compound. This means no compromise has to be made between dwell times for each compound or the cycle time. It allows for addition of detecting many more MRM transitions from a single method and gives better signal to noise, accuracy and reproducibility.

The new Scheduled MRM™ Pro algorithm retains all the benefits of Scheduled MRM™ algorithm but also has extended functionality with Grouped MRM-triggered MS/MS, MRM triggered MRM, Dwell Time Weighting, Flexible Window Width and Dynamic Window Extension. All these features allow the mass spectrometer to only spend time collecting useful data.

MRM-Triggered MRM

One or more primary transitions are monitored during the whole acquisition window and only trigger secondary MRMs when the intensity of all primaries exceeds the defined threshold. Secondary MRMs are monitored until primaries drop back below the threshold, thus are monitored across the LC peak. Therefore accurate and quantifiable areas are obtained for all transitions.

Dwell Time Weighting

Higher dwell times improve the signal to noise of MRM transitions. Spending the same dwell time on an internal standard and a difficult to ionize compound is wasteful. However, calculating optimal dwell times for each compound without impacting cycle time during a scheduled run would be difficult. Dwell time weighting allows a desired dwell time to be expressed relatively. Internal standards can be assigned a low weight (<1) while difficult compounds can be assigned a high weight (>1). During the run, the available dwell time will be assigned based on this weighting.

Flexible Window Width

This allows setting acquisition time windows individually for each compound, based on the retention time reproducibility or peak width.

Dynamic Window Extension

MRM detection windows are automatically extended for transitions where the peak has not completely eluted (when the signal is still above the user defined threshold). This functionality allows increasing the robustness to shifts in retention time and ensures the collection of the entire signal of heavily contaminated samples.

Results and Discussion

Despite the high selectivity that MRM provides, there is always a risk of false positive findings due to endogenous compounds that have the same mass. Typically a second MRM is monitored per analyte and the ratio of quantifier to qualifier transition is calculated for each unknown sample and compared to the MRM ratio of standards for identification. However, targeted compounds with low fragmentation efficiencies (i.e. Low Intensity Ions) have been reported to produce false positive results for compound identification (4-6).
For improved accuracy, compound identification was performed using full scan MS/MS experiments in Enhanced Product Ion (EPI) mode with automated library searching capabilities to compare the unknown with a standard spectrum. The dependent MS/MS spectra were acquired using the EPI mode of the QTRAP® system after being triggered from a Scheduled MRM™ Pro Algorithm IDA survey scan and searched against mass spectral libraries for compound identification. The information of the complete molecular fingerprint saved into EPI spectra significantly reduces the risk of false positive and negative results (7-10). In this method, a large library of MS/MS spectra (SCIEX iMethod™ Meta MS/MS Spectral Library) containing more than 2400 compounds including pesticides, mycotoxins, veterinary drugs, pharmaceuticals and drugs of abuse compounds was used to search against in order to confirm compound identifications. Quantitative analysis was performed in the same run allowing for both quantification and qualitative data to be collected simultaneously.

Figure 4 shows representative Scheduled MRM™ chromatograms for over 100 different drugs. Figure 5 shows the signal-to-noise levels for selected compounds obtained from 1 ng/mL spiked serum. Figure 6 shows example calibration curves (0.5-1000 ng/mL) created for compounds identified from the library matching from the same run.

![Figure 4](image1.png)

**Figure 4.** Extracted ion chromatograms for 130 drugs from a 10 ng/mL extracted serum calibrator

![Figure 5](image2.png)

**Figure 5.** Chromatogram of select analytes in spiked serum at 1 ng/mL. The signal to noise was calculated by dividing the average background signal intensity from the peak by a 1 times the standard deviation of the noise region

![Figure 6](image3.png)

**Figure 6.** Selected example calibration curves for spiked serum calibrators, generated from the same run in which qualitative information was obtained for confident identification through library searching.
Regression analysis for the analyzed samples within this method resulted in $R^2$ values of 0.99 or greater. Typical recoveries were greater than 80% (Table 1). Matrix effects were evaluated at 10 ng/mL concentrations, using one lot of serum, and % accuracy differences were typically less than 20%. Figures 7 and 8 show specific examples with % CV and % Accuracy values across the calibration curve concentrations.

<table>
<thead>
<tr>
<th>Drug</th>
<th>% CV 1ng/mL</th>
<th>% Accuracy</th>
<th>Matrix Effect (% accuracy difference)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>0.9</td>
<td>102</td>
<td>15</td>
<td>98</td>
</tr>
<tr>
<td>Beclometasone</td>
<td>8.5</td>
<td>109</td>
<td>6</td>
<td>61</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>20</td>
<td>80</td>
<td>23</td>
<td>55</td>
</tr>
<tr>
<td>Clonazepam</td>
<td>20</td>
<td>112</td>
<td>4</td>
<td>112</td>
</tr>
<tr>
<td>Nalorphine</td>
<td>9</td>
<td>87</td>
<td>1</td>
<td>81</td>
</tr>
<tr>
<td>Norfentanyl</td>
<td>5</td>
<td>99</td>
<td>3</td>
<td>14</td>
</tr>
<tr>
<td>Oxycodone</td>
<td>3</td>
<td>97</td>
<td>11</td>
<td>86</td>
</tr>
<tr>
<td>Propanolol</td>
<td>6</td>
<td>90</td>
<td>49</td>
<td>61</td>
</tr>
<tr>
<td>Sotalol</td>
<td>2</td>
<td>103</td>
<td>15</td>
<td>97</td>
</tr>
<tr>
<td>Terfenadine</td>
<td>5</td>
<td>87</td>
<td>16</td>
<td>76</td>
</tr>
</tbody>
</table>

Table 1. Typical recoveries and matrix contribution values as represented by these selected examples

Extracted spectra and library search Purity Score values using an MS/MS library search algorithm are shown in Figure 9 for extracted serum samples with low analyte concentrations.

The developed method was quickly imported into the Cliquid® Software as a pre-configured method. The Cliquid® Software allows the simple selection to run the method and after sample batch set up the software performs automated acquisition, data processing and reporting of the results. Figure 10 shows an example report that is generated by the Cliquid® Software.
Figure 8. %CV and %Accuracy values obtained for selected drugs, across the calibration curve concentrations.

Figure 9. Example of extracted spectra and library search purity score values using an MS/MS library search algorithm.
Figure 10. The Cliquid® Software was used to automatically generate a report after automated acquisition, data processing and library searching. An extract from the report is shown, showing the integrated MRM signal of Salbutamol and the comparison of the acquired EPI spectrum and the spectrum from the library.

Conclusions

As a result of this study, we were able to show:

- The Spark Holland PICO system in combination with the SCIEX QTRAP® 4500 LC-MS/MS System, as part of an On-Line SPE LC-MS/MS workflow, allowed the automated injection and cleanup of serum samples for screening and confirmation for over 100 analytes in a single run.
- The QTRAP® allowed high accuracy compound identification by performing full scan MS/MS experiments using the Enhanced Product Ion mode after being triggered from a Scheduled MRM™ Pro Algorithm IDA survey scan with automated library searching capabilities to compare the unknown with a standard library spectrum.
- Quantitative analysis was performed in the same run allowing for both quantification and qualitative data to be collected simultaneously. Linear calibration curves resulting in R2 values 0.99 or greater were achieved for the samples analyzed.
- Analysis for large panel of drugs in serum with automated sample preparation, detection and confident identification in a single injection was achieved.

References
