Investigating the High Throughput Capability of a High Resolution, Accurate Mass Method using the AB SCIEX TripleTOF® 4600 LC/MS/MS System for Toxicology Urine Screening

AB SCIEX MPX™-2 SP High Throughput and TripleTOF® 4600 LC/MS/MS System

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Overview
Sample preparation and Liquid Chromatography (LC) separation are usually the most time consuming steps in a typical drug screening analysis. LC separation is an effective way of separating out the compounds of interest from interfering components from the sample matrix and therefore reducing ion suppression effects as well as separating out isobaric compounds. LC therefore still remains a valuable tool in ensuring accurate and precise data quality for a broad compound analysis for both targeted and non-targeted screening. At the same time retention time can also be used as part of multiple points of selectivity to ensure proper identification of drugs in samples. High throughput requirements however necessitate fast chromatography and short run times. An integrated multiplex system [MPX™-2 High Throughput System] has been specifically designed to increase the throughput of Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) analysis. Combining high resolution MS and MS/MS acquisition with LC separation, allows for retrospective compound identification with highest confidence based on accurate mass product ion specificity, mass spectral library matching as well as mass error, isotopic profile and molecular formula finding.

The AB SCIEX TripleTOF® 4600 LC/MS/MS system is a hybrid quadrupole/ time-of-flight (QqTOF) instrument allowing acquisition of highly sensitive full scan MS and MS/MS spectra with high resolution and mass accuracy. The combination of the MPX™-2 High Throughput System and TripleTOF® 4600 LC/MS/MS System meets the needs of a high throughput screening, less than 50 seconds injection to injection. The workflow allows qualitative screening and confirmation in a single automated run, for generating and reporting results, while maintaining good precision and accuracy.

Introduction
Laboratories performing drug screening analyses on samples often wish to identify as many compounds as possible from a single experiment. Furthermore, laboratories may also desire the capability and flexibility to perform retrospective data analysis, for the purpose of identifying compounds that were not included in the initial targeted screening experiments.

Recently, interest in high-resolution/accurate-mass MS systems as a potential tool for drug screening has grown, largely due to the ability to run simple and generic methods, and the possibility of performing retrospective data analysis.
The aim of this work was to investigate the throughput capabilities of the AB SCIEX TripleTOF® 4600 system as a potential alternative to traditional methods used to screen for drugs in urine. This method employs both (i) a TOF-MS scan, along with (ii) multiple product ion scans, during each cycle. The non-targeted TOF-MS full-scan is acquired during every cycle throughout the chromatographic run, and enables the identification of compounds based on accurate mass measurements of the parent ions. As with any non-targeted data acquisition, this approach has the advantage that retrospective data analysis may be performed at a later date, to identify any compound—whether known or unknown—at any mass. The inclusion of product ion scans for the list of target compounds ensures that MS/MS data is acquired for these compounds and enables highly selective extracted ion chromatograms (XICs) of the characteristic fragment ions for each compound. This approach, known as MRMHR, is analogous to the MRM scan mode on a triple quadrupole, with the added benefit of high-resolution/accurate-mass measurements and has the advantage of providing more criteria for compound identification through the use of MS/MS library matching. Combining all available MS and MS/MS data allows more comprehensive empirical calculations for potential molecular formula determinations.

Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) is a widely used analytical tool for the screening of drugs. LC separation is an effective way of separating out the compounds of interest from interfering components from the sample matrix and therefore reducing ion suppression effects as well as separating out isobaric compounds. LC therefore still remains a valuable tool in ensuring accurate and precise data quality for a broad compound analysis for both targeted and non-targeted screening. A comprehensive drug screening approach however often requires long LC run times. These times can be reduced by multiplexing LC systems, and synchronizing to a single MS, generating the high throughput needed by maximizing the efficiency of the MS. An integrated multiplex system has been specifically designed to synchronize two LC systems and a mass spectrometer, allowing injection of samples into two LC streams in parallel (Figure 1). The overlapping LC runs and efficient use of MS detection, achievable with the MPX™-2 High Throughput System, is shown to result in an overall higher throughput system for common routine screening analyses. The example presented in this paper compromised on the LC separation over the need for a fast screening using the MPX™-2 High Throughput System and used a C18 guard column (20 x 2.1 mm, 5 mm) to impart a small separation but the specificity was gained by the use of highly selective extracted ion chromatograms (XICs) of the characteristic fragment ions for each targeted compound.

The potential of the high resolution and accurate mass AB SCIEX TripleTOF® 4600 LC/MS/MS system to effectively screen for and identify drugs from urine that are present at or above a cutoff concentration was investigated. PeakView® Software with the MasterView™ add-in was used for targeted data processing.

MasterView™ manages large lists of compounds and performs extracted ion chromatogram (XIC) calculations, both targeted and non-targeted peak finding operations and library searching. MasterView™ allows the ability to review results based upon retention times, accurate mass, isotopic pattern, MS/MS library searching and formula finding. The MasterView™ Software can further be used to aid in the structural elucidation, elemental composition determination and fragmentation interpretation to confidently identify unknowns using acquired MS/MS data.

Figure 1. Multiplex LC system Designed to Support Two Parallel LC Streams into a Single MS

Figure 2. MPX™-2 High Throughput System Configuration and Module Arrangement Used in this Application.
**Experimental Conditions**

**Sample Preparation**

Urine samples were prepared by spiking analytical standards (Cerilliant) into blank urine (Lot 1) to create 100, 30, 10, 4, 1, 0.5 and 0.25 ng/mL concentration calibrants. 4 ng/mL and 10 ng/mL urine samples were also prepared in urine Lot 2 and 3 respectively. 50 µL of each urine sample was transferred to a microcentrifuge tube and 20 µL of internal standard mix added and vortexed. The samples were then diluted with 300 µL 10% MeOH in water with 0.1% formic, vortexed and centrifuged before being transferred to autosampler vials for LC-MS/MS injection.

**Multiplex Software Configuration and Operation**

An integrated multiplex LC-MS/MS System was used, consisting of an AB SCIEX TripleTOF® 4600 LC/MS/MS system, two Shimadzu Prominence XR LC systems, a pump containing a four solvent selection valve for sample loading and 3 switching valves for flow path control (Figure 2).

The two chromatographic channels were not independent as they share a single high pressure loading pump which provides additional flexibility for injection and wash solvent composition. All hardware modules are controlled by Analyst® TF 1.6 software with MPX™ TF Driver 1.0 add-on.

Precise timing of the switching valves allowed each LC stream to perform interleaved injection and LC gradient elution. Targeted MS data acquisition was enabled by selecting a retention time window around the peaks of interest in the MPX™ TF Driver 1.0 Method Editor. Figure 3 shows common flow paths for a typical LC-MS analysis.

After running the multiplex software installer, the user may easily configure and activate the multiplex option from the Hardware Profile dialog within the Analyst® program. MPX™ Driver provides an easy-to-use interface for the user to modify the system configuration in the Settings Pane (Figure 4) and to create or update a multiplex LC method in the Method Pane (Figure 5).
An example chromatogram can simply be loaded into the software and the region that should be directed to the mass spectrometer for data acquisition is highlighted. The timing is automatically calculated and throughput increased displayed in the gauge.

Automated multiplex data acquisition for a batch of samples is very similar to performing a regular LC-MS data acquisition by creating and submitting a batch with Batch Editor in Analyst® software. During a multiplex run a staggered injection timing schedule is calculated in real-time by the MPX™ TF Driver 1.0. All switching valves and synchronization between LC components and the mass spectrometer are also controlled by the MPX™ TF Driver 1.0.

Mass Spectrometric Conditions

The AB SCIEX TripleTOF® 4600 system was operated with DuoSpray™ source and Electrospray (ESI) probe. The method consisted of a TOF-MS full scan and several looped TOF-MS/MS product ion scans for the 41 target compounds with an optimized Collision Energy (CE) used for each compound. 50 msec TOF-MS and 10 msec TOF-MS/MS accumulation time were used.

For mass calibration of the AB SCIEX TripleTOF® 4600 system, an on-column injection of an AB SCIEX APCI calibration solution via the LC autosampler was performed.

Acquired MS/MS spectra were searched against an accurate mass drug LC/MS/MS library.

Results and Discussion

Enabling Rapid, High Resolution Screening with the MPX™-2 High Throughput TripleTOF® 4600 System with High Confidence Identifications

The effectiveness of the MPX™-2 High Throughput System with MPX™ TF Driver 1.0 software in combination with the TripleTOF® 4600 System, as a potential rapid drug screening solution, was investigated. An LC-MS/MS method targeting 41 drug compounds has been developed that has an injection to injection time of less than 50 seconds. The method uses full advantage of the high resolution, accurate mass capabilities of the TripleTOF® system using multiple points of selectivity for identification based on mass error, isotope matching, retention time, MS/MS library searching and formula finding. Relying solely on accurate mass and isotopic matching can potentially lead to false negative conclusions as illustrated in Figures 7 and 8. Figures 7 and 8 show examples where, due to a co-eluting unknown compound, the isotopic ratio for the targeted compound was off. This may cause a false negative conclusion but because MS/MS data was collected for the compound and a good library match was generated, the compound was successfully identified.
This potential interference is increased when using fast screening methods with limited separations leading to higher instances of co-eluting compounds. False negative rates can therefore be reduced by collection of MS/MS data during these fast screening workflows.

Figure 8 Interference caused isotopic ratio change, but MS/MS confirmed identity and eliminated possible false negative

Defining an XIC List and Processing Options in the XIC Manager for Targeted Qualitative Screening and Identification

After opening a data file in PeakView® software the MasterView™ add-in can be launched from the ‘XIC Manager’ menu in the PeakView® toolbar.

The table contains a number of columns with values that can be edited, including name, formula, adduct/modification, retention time, width and more. To define an XIC, a mass must be entered. This can be done by directly editing the cell, having the software calculate the value based on formula, isotope, and adduct provided, or by pasting values from a spreadsheet. The generated XIC list can be saved for future processing (Figure 9).

A number of processing and display settings can be adjusted in the ‘Options’ dialog. This includes intensity, signal-to-noise threshold, and confidence settings for mass error, retention time, isotope matching, library searching and formula finding (Figure 10).

To start data processing, simply click the button in the lower right of the table. MasterView™ will automatically calculate XICs, perform compound identification and display results.

Figure 10. Confidence settings for compound identification

Results Display in MasterView™

After processing, the results are displayed to show the mass error (ppm or mDa), found at retention time and library search results as well as formula findings. XICs above a defined intensity threshold are highlighted in green and confidence data for compound identification is visualized using traffic lights (Figure 14). The extracted compounds that are displayed can be customized based on the strength of the hit (Figure 15). For example the software can be set up to only display compounds with 5 green traffic lights.

The example data presented in Figures 11 and 13 shows the automatically generated XICs for each targeted analyte and compared against the user defined threshold. High resolution and accurate mass LC-MS/MS chromatograms contain comprehensive information of all molecules present in the sample that are amenable to the ionization techniques and polarity used. Narrow XICs can be generated to selectively screen for targeted compounds. The AB SCIEX TripleTOF® 4600 system provides high resolution of up to 35,000 dependent on the mass detected (Figure 11) and stable mass accuracy of ~2 ppm at fast acquisition speed in MS and MS/MS mode.
This allows the generation of narrow XICs to achieve both selectivity and increased S/N when screening for a large set of targeted drug compounds in complex samples (Figure 12).

Figure 13 and Table 1 show 41 drug compounds that were identified from a spiked urine sample using information of the isotopic pattern of the detected molecular ion, retention time accurate mass and MS/MS spectral searching against an accurate mass MS/MS drug library. The accurate mass TOF-MS/MS product ion scan of a drug compound is shown as a mirror view with the matched library spectrum for easy comparison. The bottom right panel of Figure 13 shows an example of the spectral comparison of library versus urine sample of meperidine. The sample spectrum is the top blue trace and the library is the grey trace.

Figure 16 shows the details of the library search and formula finder results that are automatically performed during the extraction of the ion chromatograms. Both results are based on MS and MS/MS results. Formula Finder uses high resolution accurate mass information of the molecular ion, adducts, isotopic pattern and fragment ion information to empirically calculate potential molecular formula which can only be achieved by combining all available MS and MS/MS data.
Figure 12. Increasing selectivity and S/N using narrow extracted ion chromatograms (XICs)

Figure 13. 41 drugs were detected and identified using retention time, mass error, isotope pattern and confirmed with MS/MS library matches and formula finder results in a urine sample at 10 ng/mL.

Data Review Functionality

- Confidence in identification is visualized using five ‘traffic lights’ (including RT error, mass error, isotope matching, library search, and formula finding)

Figure 14. Data Review

- XIC highlighted in green:
  XIC above a user defined intensity threshold will be highlighted in green as specified by the criteria chosen in ‘Select combination for positive result (equal or better)’ box (Figure 15). The identification criteria above defined thresholds can be displayed.

Positive ID with high confidence

Identified compounds to review

Figure 15. Identifying which components to review

- Display highlighted XIC only
- Show MS and MS/MS spectra
- Show details of library searching and formula finding

Figure 16. Library Search and Formula Finder Results are automatically generated using both MS and MS/MS information
MasterView™ has flexible reporting features showing the compound identifications using both high resolution and accurate mass MS and MS/MS information. Customizable report templates can be used to generate word, csv text or PDF reports (Figure 17).

How much is in my sample?

MasterView™ can also quantitatively compare samples to highlight identified compounds at or above a target concentration.

The unknown sample is selected using the drop-down menu below the XIC table. A control is then selected. This can be a solvent standard or matrix sample spiked with all targeted compounds at relevant concentration (i.e. cutoff levels). A threshold is then defined for comparative screening (ratio sample and control). A threshold of 1 will highlight all compounds in the XIC table which are present in unknown samples at a concentration higher than in the control sample, a threshold of 0.5 will highlight all compounds having at least 50% of the signal in comparison to the control sample. Figure 18 shows how samples containing drugs above a cutoff concentration can be quickly identified by comparison to a standard at the cutoff concentration and the extracted drug concentrations in the sample is automatically calculated.

Table 1: Results table generated by MasterView™, detailing the compounds identified from this 100 ng/mL urine sample based on both MS and MS/MS data generated in a fast 48 second injection to injection screening method.
Conclusions

We have shown that Multiplexing LC systems, and synchronizing to a single MS, can potentially generate the high throughput needed by modern day laboratories to analyze increasing numbers of samples but maintain or improve turn-around times.

The above features are achieved by the ability to automatically adjust program overlapping runs. By simply inputting the times or highlighting the analyte retention area of a sample chromatogram the mass spectrometer acquisition period is automatically updated.

The MPX™-2 High Throughput System has enabled a fast screening, less than 50 second injection to injection time, to screen for targeted compounds utilizing multiple points of selectivity to ensure confident identification of components in samples.