Investigating the use of the AB SCIEX TripleTOF® 4600 LC/MS/MS System for High Throughput Screening of Synthetic Cannabinoids/Metabolites in Human Urine

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

Xiang He,[1] Xiaohong Chen,[1] Adrian M. Taylor[2] and Hua-fen Liu[1]
[1]AB SCIEX, Redwood City, California, USA; [2]AB SCIEX, Concord, Ontario, Canada

Overview

The AB SCIEX TripleTOF® 4600 liquid chromatography tandem mass spectrometry (LC/MS/MS) system is a hybrid quadrupole/ time-of-flight (QqTOF) instrument with rapid speed in acquisition of highly sensitive full scan MS and MS/MS spectra with high resolution and mass accuracy. An integrated multiplex system [MPX™-2 SP High Throughput System] has been specifically designed to increase the throughput for LC/MS/MS forensic drug screening analysis. The combination of the MPX™-2 SP High Throughput System and TripleTOF® 4600 LC/MS/MS System meets the needs of a high throughput forensic screening workflow. The workflow allows qualitative screening and confirmation in a single automated run with highest confidence based on accurate mass product ion specificity, mass spectral library matching as well as mass error, LC retention time and isotopic ratio pattern. The workflow also automates report generation and facilitates subsequent quantitation when needed.

AB SCIEX TripleTOF® 4600 System

Introduction

Synthetic cannabinoids (K2) are a major health problem and a rapid, sensitive screening test for K2/metabolites is needed. Immunoassays are available, but lack flexibility for adding new analytes. LC/MS/MS offers increased sensitivity and specificity for identifying K2/metabolites and ability to add newly emerging analytes in a forensic screening panel with ease.

TripleTOF® 4600 high resolution accurate mass systems are capable of full MS range data collection and simultaneous Information Dependent Acquisition (IDA) product ion scan confirmation, providing an ideal platform for screening specified targets and also unregulated new analogues. Recent developments in LC multiplexing further improves throughput and makes LC/MS/MS more effective for screening applications. PeakView® software with the MasterView™ add-in was used for targeted data processing. The MasterView™ manages large lists of compounds and performs extracted ion chromatogram (XIC) calculations, both targeted and non-targeted peak finding operations and library searching. It allows the ability to review results based upon retention times, accurate mass, isotopic pattern and MS/MS library searching.

The goal here is to demonstrate a fast and reliable LC/MS/MS based high throughput screening (HTS) method for K2/metabolites on the AB SCIEX TripleTOF® 4600 mass spectrometer and MPX™-2 High Throughput System. Specifically, we aim for optimal sensitivity and specificity, and achieve injection-to-injection cycle time around 2 minutes.

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

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to result in an overall higher throughput system for LC-MS/MS analyses.

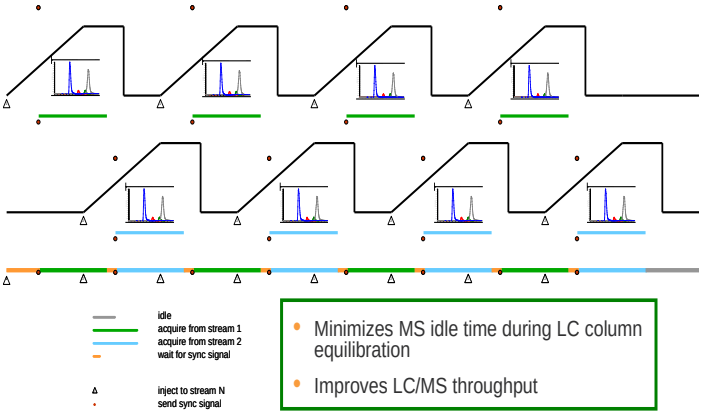

Figure 1. Multiplex LC system Designed to Support Two Parallel LC Streams into a Single MS

Experimental Conditions

Sample Preparation

Urine calibrator samples were prepared by spiking K2/metabolites standards into blank urine at various concentrations. Urine calibrator and urine samples were both spiked with internal standards with optional hydrolysis. Urine samples were diluted 5-fold or 10-fold in 50% MeOH/H2O. The mixtures were 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. MPX™ Driver provides an easy-to-use interface for the user to modify the system configuration in the Settings Pane and to create or update a multiplex LC method in the Method Pane. The information entered in both Settings and Method panes allows the software to calculate the throughput and have it displayed. The interface also allows monitoring the real-time acquisition and system status (including pressure, flow rate, temperature, flow path, and system state for both LC streams) in the Status Pane (Figure 3).

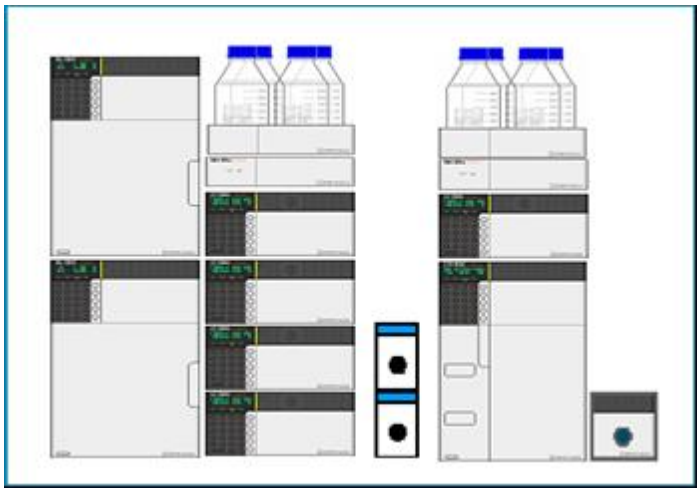

Figure 2. MPX™-2 High Throughput System Configuration and Module Arrangement Used in this Application.

Figure 3. MPX® TF Driver 1.0 interface with exemplary LC configuration and method.

For this fast screening method a Restek Ultra Biphenyl (50 x 2.1 mm, 5 µm) was used, with a 3.5 minute gradient of water and methanol with 5 mM NH_4FA and a 1.5 minute acquisition window (Figure 3).

Multiplex data acquisition for a batch of samples is automated by the MPX™ TF Driver 1.0. For the particular LC method used in multiplexed mode, compared to single-stream data acquisition, two-fold increase in throughput was achieved and injection-to-injection cycle time was 2.09 min (Figure 4). In comparison, current sample throughput of regular immunoassay screening for synthetic cannabinoids was roughly 2 minutes.

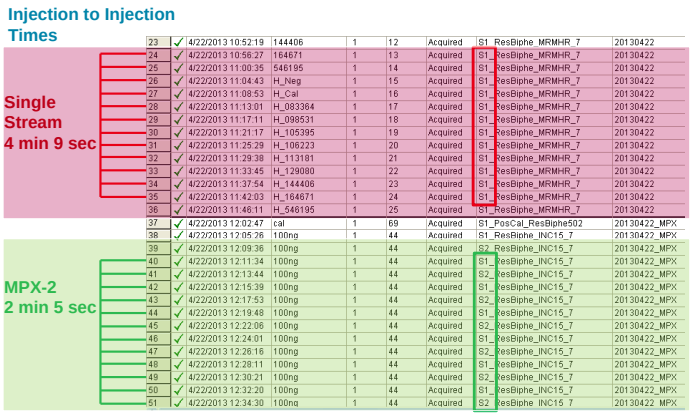

Figure 4. Increasing throughput by 2 times. Demonstration of injection-to-injection cycle time reduction (from 4.15 to 2.09 min) using both LC streams in an interleaved fashion with MPX® TF Driver 1.0 interface.

Mass Spectrometric Conditions

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. This automatic calibration workflow was based on a multiple-mass mixture and specific retention time was assigned to each mass covering a wide mass range for both TOF-MS and TOF-MS/MS modes calibration. The duration of each calibration was 3 min and the frequency of this calibration can be user-defined (e.g. once in several hours or longer).

The AB SCIEX TripleTOF® 4600 system was operated with DuoSpray™ source and Electrospray (ESI) probe. The data acquisition method consisted of a TOF-MS full scan and several IDA-TOF-MS/MS product ion scans using 35 V Collision Energy (CE) with 15 V (Collision Energy Spread) CES used for each candidate ion in each data acquisition cycle. An inclusion list consisting of >40 pairs of m/z and retention times was used for IDA-TOF-MS/MS product ion scans. 150 msec TOF-MS and 35 msec TOF-MS/MS accumulation time were used. This workflow allows quantitation (using TOF-MS primarily) and confirmation (using IDA-TOF-MS/MS) in the single run. Further, the TOF-MS data allows for retrospective data analysis on a later date, making it possible for data interrogation on the compounds that were not targeted in the first round. The flexibility in acquiring IDA-TOF-MS/MS product ion scan without an inclusion list makes it possible to acquire complementary confirmative MS/MS data in a non-targeted fashion, ideal for added analytes at later times.

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

Results and Discussion

This workflow adopts a simple dilute-and-shoot sample preparation procedure. It is known that some K2 metabolites can be converted to their phase-2 metabolites such as glucuronide or sulfate conjugates, therefore, enzyme hydrolysis can be optionally performed to release the parent analytes/phase-1 metabolites. With hydrolysis, sample preparation will be extended but data processing will be simplified as fewer analytes need to be monitored. Further, after hydrolysis, all unconjugated analytes share similar hydrophobicity which shortens analytes elution window and improves the throughput of the workflow. Figure 5 shows the extracted ion chromatograms of all analytes that elute during a very short 1.5 minute data window.

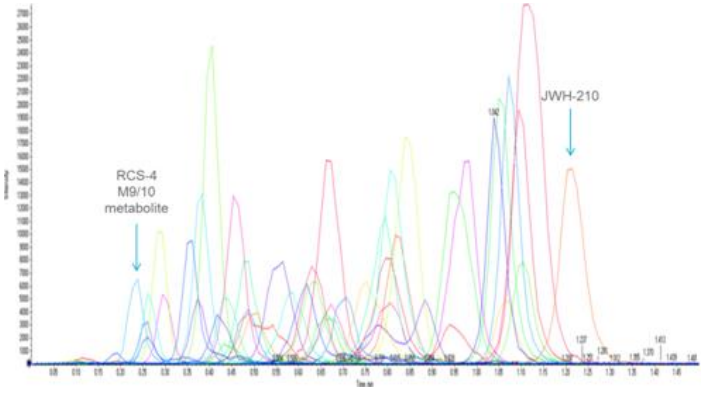

Figure 5. Extracted ion chromatograms of all analytes that elute during a 1.5-min data window.

Using MasterView™ for Targeted Qualitative Screening and Identification

MasterView™ add-in is an integral part of PeakView®. It is the main software for viewing, analyzing, processing, and finally reporting the screening results. An XIC list needs to be constructed to extract ions from the data, and subsequently data is analyzed using multiple processing settings such intensity and signal-to-noise threshold; and confidence settings such as mass error, retention time, isotope matching, library searching and formula finding (Figure 6).

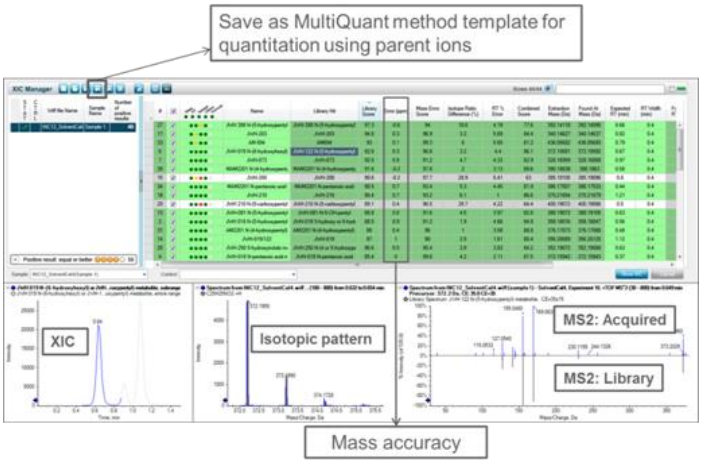

Figure 6. PeakView® software with MasterView™; the top pane shows an XIC table loaded for target drug screening and identification

Figure 6 shows all targeted analytes that were identified from a spiked urine sample (25 ng/mL) using information of the isotopic pattern of the detected molecular ion, retention time, accurate mass and MS/MS spectral searching against an accurate mass forensic toxicology LC/MS/MS 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 5 shows an example of the spectral comparison of library versus urine sample (isobaric mixture of two JWH-019 and 120 metabolites). The sample spectrum is the top blue trace and the library is the grey trace.

In screening applications, both false positive and false negative hits need to be kept to a minimum so it is critical to utilize all possible screening criteria for data review. In terms of false positives, for instance, when only mass accuracy and retention time were used for data review and report generation, it was observed that the sheer file size doubled the report generated with all criteria enforced (mass accuracy, retention time, isotope ration difference and library score) for a 10-sample batch (Figure 7).

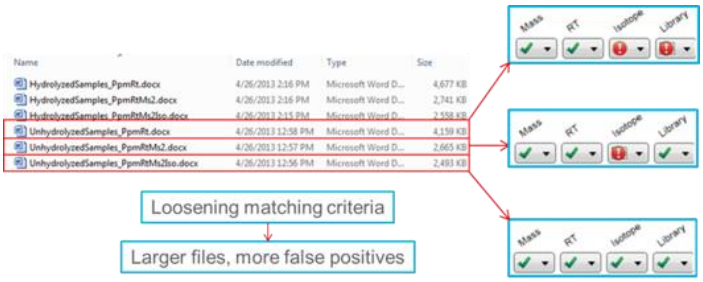

Figure 7. Report file size comparison between reports generated using various number of confidence points.

On the other hand, when all criteria were strictly in place, it was inevitable that some false negatives were created. Figure 7 shows a situation in which one spiked K2 analyte JWH-200 was categorized as “negative” based on its elevated isotope ratio difference due to a co-eluting substance that shares similar molecular weight as JWH-200’s second isotopic peak. However, with >90% library matching score, finding of JWH-200 in this particular sample was shown to be a true positive. This example highlighted the importance of using MS/MS library matching in data review. In our testing, it was frequently observed that the isotope ratio patterns of many analytes were altered in real urine samples and having MS/MS data proved to be critical.

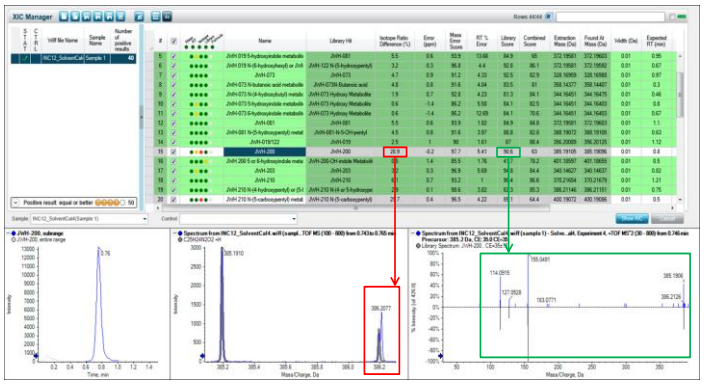

Figure 8. Interference caused isotopic ratio change, but MS/MS confirmed identity and eliminated possible false negative caused by elevated isotope ratio difference for JWH-200 in neat solution.

From Screening to Quantitation

After sample data was processed in MasterView™, the *m/z*s and retention times were determined for all target analytes, along with the related extraction windows for both *m/z* and retention time. Such results can be linked with MultiQuant® software and used for quantitation. Figure 9 shows a calibration curve for JWH-018 that was generated in MultiQuant™ software. The process was fast, reliable and flexible.

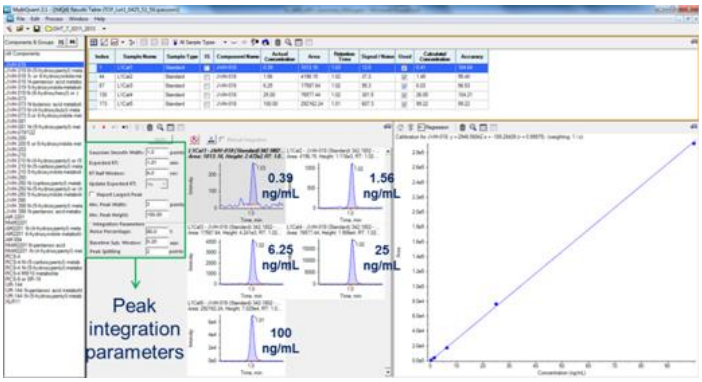

Figure 9. Calibration curve of JWH-018 in human urine.

Reporting

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 10).

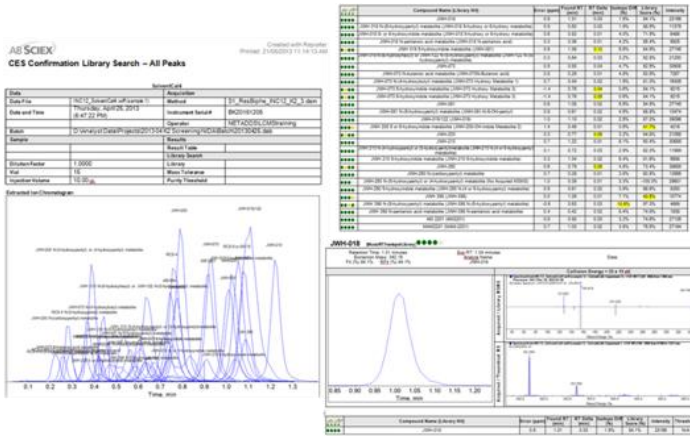

Figure 10. Flexible Reporting Features in MasterView™

Conclusion

The MPX-2 High Throughput system with the AB SCIEX TripleTOF® 4600 high resolution accurate mass system has enabled a fast forensic screening, around 2-min injection to injection time, to screen for synthetic cannabinoids/metabolites utilizing multiple points of selectivity to ensure confident ID of components in urine samples. The workflow is simple, highly flexible in adding new analytes, and has superior sensitivity and specificity than immunoassay-based screening approaches.

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AB SCIEX

Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
Phone 508-383-7700
www.absciex.com

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