# Simultaneous Pharmacokinetic Profiling and Automated Metabolite Identification using the AB SCIEX TripleTOF™ 5600 System and MetabolitePilot™ Software

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## Introduction

Combining quantitative bioanalysis and in vivo metabolite identification (Quant / Qual) has the potential to significantly increase the productivity of pharmaceutical drug discovery and development. Traditionally, quantitative bioanalysis is performed on samples obtained from PK studies while metabolite identification is done in a separate analysis often on two different instruments. Due to sensitivity limitations of traditional LC/MS/MS instruments, studies for metabolite identification are often performed at much higher doses than for PK studies. Therefore, the metabolic profile obtained may not be representative of the profile at therapeutic doses. In addition to the lack of sensitivity, traditional accurate mass instruments used for qualitative studies have suffered from limited speed and linearity required for quantitative bioanalysis.

The AB SCIEX TripleTOF™ 5600 system is a new state of the art high resolution accurate mass instrument with the speed, sensitivity and linearity to deliver triple quadrupole like quantitative performance and accurate mass metabolite identification in the same injection and at the same time.

In this application note, we describe the use of the AB SCIEX TripleTOF™ 5600 system for simultaneous in vivo PK quantification and identification of the major metabolites of buspirone following administration in rat.
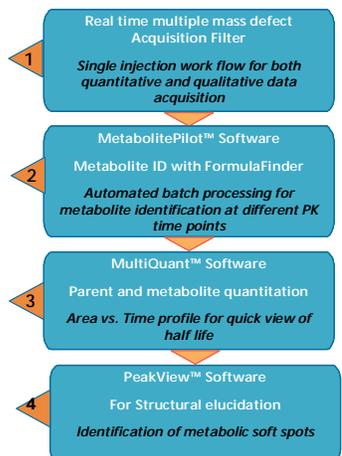


## Key Features of the AB SCIEX TripleTOF 5600 System for Combined Quant and Qual Workflows

- The high speed, accuracy and resolution of the TripleTOF 5600 system in both MS and MS/MS modes enables simultaneous quantification and metabolite identification from in vivo PK studies.

- Using real time multiple mass defect filtering (MDF) for information dependent acquisition (IDA) increases workflow efficiency by acquiring relevant MS/MS data in every run, eliminating the need for re-injection in many cases.

- MetabolitePilot™ Software is a powerful and user friendly tool to quickly identify metabolites.

- The batch processing feature in MetabolitePilot™ Software allows automated data analysis on multiple samples for higher productivity.

- MultiQuant™ software is an efficient tool to quickly perform accurate mass quantitative data processing to generate the parent PK profile as well relative quantification of metabolites.



**Figure 1. Automated PK Profiling and Metabolite Identification on AB SCIEX TripleTOF 5600 System using MetabolitePilot™ and MultiQuant™ Software.**

## Experimental

*In vivo Administration:* Three Sprague-Dawley rats were dosed with buspirone at 10 mg/kg IV. Plasma samples were collected at 0, 15, 30, 60, 120, 240, 360 and 480 minutes.

*Sample Preparation:* Plasma samples were protein precipitated using acetonitrile containing internal standard (buspirone d-8) at 3:1 ratio followed by centrifugation at 10,000 g. The supernatant (100 µL) was mixed with 50 µL water, transferred into autosampler vials and 5 µL was injected.

**Chromatography**: Sample analysis was performed on the AB SCIEX TripleTOF™ 5600 system coupled to an ACQUITY UPLC® system. An acetonitrile / water / 0.1% formic acid gradient was used on a Waters BEH C18, 2.1x100 mm, 1.7 µm column. The flow rate was 0.6 mL/min and the column was heated to 50°C.

**Mass Spectrometry:** System was operated in positive electrospray mode using a DuoSpray™ source. The Information Dependent Acquisition (IDA) method consisted of a TOF MS survey scan (m/z 100 – 1000) followed by 3 TOF MS/MS dependent scans (m/z 50 – 1000). The TOF MS scan data was used for quantification while the MS/MS data was used for accurate mass structure elucidation of potential metabolites detected.

## Real Time Multiple Mass Defect Filter

Mass defect filtering (MDF) has been shown to be a powerful tool in detecting metabolites that are similar in elemental composition to the parent. Traditionally, MDF has been performed as a two step process where full scan data is acquired first, then MDF analysis is performed post processing to identify peaks of interest. A second injection is then performed to acquire the MS/MS spectra of these potential metabolites.

Using real time mass defect filtering to trigger IDA, the AB SCIEX TripleTOF™ 5600 system is capable of acquiring both the MS and MS/MS data in the same run. This can eliminate the need for a second injection. Multiple mass defect ranges are often required to detect different metabolite classes. The system is capable of applying multiple filters at the same time (Figure 2). The software automatically calculates the mass defects based on the elemental compositions provided and performs intelligent priority-based MS/MS triggering. This gives priority to peaks matching the mass defect range for MS/MS data acquisition so that both expected and unexpected metabolites can be triggered.
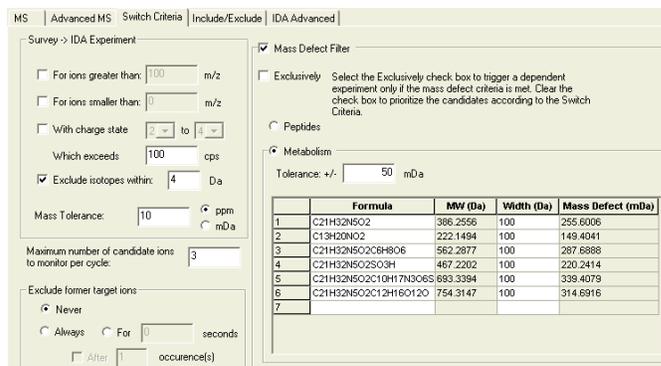


**Figure 2. Automated real time multiple mass defect filter for MS/MS triggering on Phase I, II metabolites and GSH conjugates in IDA mode.**

## Parent Metabolite Quantification

The high speed TOF MS survey scan was used for the dual purpose of quantification of parent and detection of potential metabolites. Using real time MDF to increase IDA efficiency, accurate mass product ion spectra were triggered on potential metabolites and used for structural elucidation. Quantitative PK data for buspirone parent was easily processed in MultiQuant™ software (Figure 3).
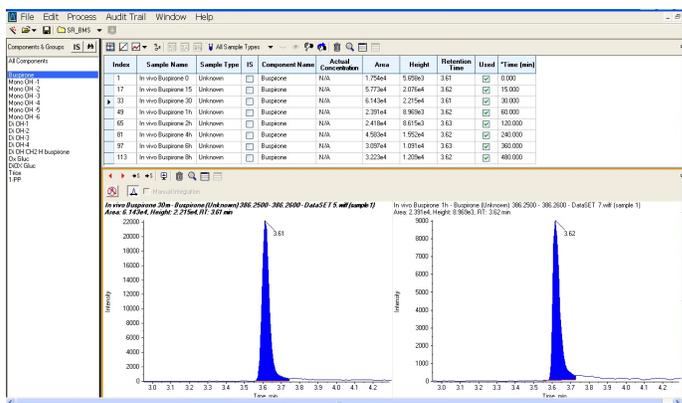


**Figure 3. Quantification of buspirone in rat plasma from TOF MS data using MultiQuant™ software.**

## Metabolite Identification and Confirmation using MetabolitePilot™ Software

Data from multiple PK time points from the buspirone samples were processed in batch mode in MetabolitePilot™ Software (Figure 4). In addition to conventional sample to control comparison, additional detection algorithms such as MDF were applied at the same time. Mass defect filters for multiple phase I and II metabolite classes as well as cleavage metabolites were automatically calculated based on parent compound structure and used for data processing. Other peak finding strategies can also be used such as isotope pattern filtering and searching for common accurate mass product ions or neutral losses.

A confirmation score is automatically calculated for each potential metabolite detected, based on mass accuracy, isotope pattern, mass defect and similarity of the MS/MS spectrum to that of the parent (Figure 5).

A diverse range of buspirone metabolites was successfully detected (Table 1). Due to the high mass accuracy, an unambiguous assignment of elemental composition was achieved. High resolution, high mass accuracy product ion spectra were obtained without the need for a second injection due to the high IDA triggering efficiency of the system.
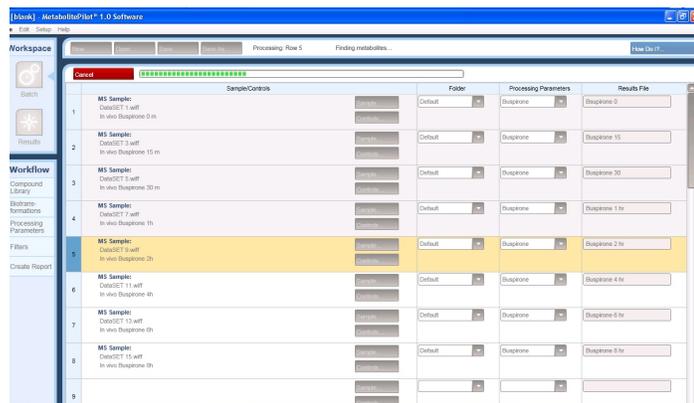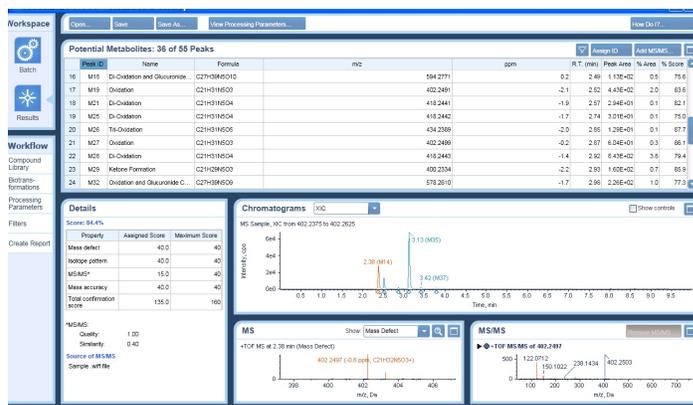


Figure 5. MetabolitePilot™ software results window showing phase I and II metabolites with proposed elemental composition, mass accuracy, % area, XIC of metabolites, TOF MS and MS/MS spectra, with scoring information, all in one window for easy visualization.

## Structural Elucidation with PeakView™ Software

High mass accuracy for the fragments ions in the product ion spectra is particularly valuable to aid in identification of the site of metabolism, since elemental compositions can be unambiguously assigned to the product ions. The FormulaFinder in PeakView™ Software quickly performs this task. In addition, structure based fragment interpretation can be performed (Figure 6) to elucidate the fragments based on theoretical fragment prediction for the proposed metabolite structure.
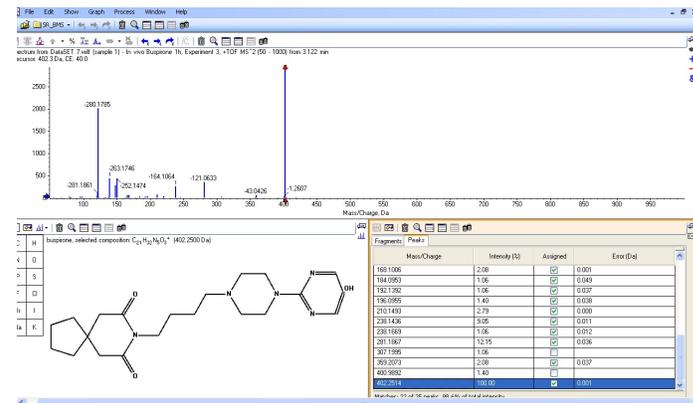


Figure 4. Automated batch processing of multiple samples from the PK time course for metabolite identification in MetabolitePilot software.



Figure 6. Structural elucidation of a mono-hydroxy buspirone metabolite using PeakView software.

| Peak ID | Name | Formula | Theoretical (M+H)⁺ | Experimental (M+H)⁺ | Mass Accuracy (ppm) | R.T. (min) | Peak Area | % Score |
|---------|------|---------|---------------------|----------------------|----------------------|------------|-----------|---------|
| | Parent | C21H31N5O2 | 386.2556 | 386.255 | -1.1 | 3.62 | 4.24E+02 | 93.6 |
| M1 | Oxidation | C21H31N5O3 | 402.2505 | 402.249 | -2.2 | 3.41 | 8.16E+01 | 88.8 |
| M2 | Oxidation | C21H31N5O3 | 402.2505 | 402.251 | 1.3 | 3.13 | 2.31E+03 | 83.2 |
| M3 | Oxidation | C21H31N5O3 | 402.2505 | 402.25 | -0.2 | 2.87 | 6.04E+01 | 83.3 |
| M4 | Oxidation | C21H31N5O3 | 402.2505 | 402.249 | -2.1 | 2.52 | 4.29E+02 | 82.3 |
| M5 | Oxidation | C21H31N5O3 | 402.2505 | 402.25 | -0.6 | 2.38 | 9.53E+02 | 83.2 |
| M6 | Ketone Formation | C21H29N5O3 | 400.2349 | 400.233 | -2.2 | 2.93 | 1.60E+02 | 83.9 |
| M7 | Ketone Formation | C21H29N5O3 | 400.2349 | 400.235 | 0.8 | 2.32 | 8.39E+02 | 86.1 |
| M8 | Di-Oxidation | C21H31N5O4 | 418.2454 | 418.245 | -0.1 | 3.31 | 6.09E+01 | 79.5 |
| M9 | Di-Oxidation | C21H31N5O4 | 418.2454 | 418.244 | -1.4 | 2.92 | 8.35E+02 | 78.8 |
| M10 | Di-Oxidation | C21H31N5O4 | 418.2454 | 418.243 | -3.8 | 2.4 | 7.19E+01 | 76.2 |
| M11 | Di-Oxidation | C21H31N5O4 | 418.2454 | 418.244 | -2.4 | 2.18 | 3.42E+02 | 84.3 |
| M12 | Di-Oxidation | C21H31N5O4 | 418.2454 | 418.244 | -1.6 | 2.04 | 6.79E+02 | 82.7 |
| M13 | Demethylation to Carboxylic Acid | C21H29N5O4 | 416.2298 | 416.229 | -0.9 | 2.14 | 2.45E+02 | 85 |
| M14 | Demethylation to Carboxylic Acid | C21H29N5O4 | 416.2298 | 416.228 | -2.1 | 2 | 3.59E+01 | 77.3 |
| M15 | Oxidation and Glucuronide Conjugation | C27H39N5O9 | 578.2826 | 578.281 | -1.7 | 2.98 | 2.20E+02 | 83.4 |
| M16 | Di-Oxidation and Glucuronide Conjugation | C27H39N5O10 | 594.2775 | 594.278 | 0.8 | 2.49 | 1.12E+02 | 83.8 |
| M17 | Dealkylation +Hydrogenation | C9H15NO2 | 170.1181 | 170.117 | -3.9 | 2.47 | 5.27E+01 | 57.8 |

**Table 1. Buspirone Metabolites Detected in the 1 Hour Sample.** Detailed list automatically generated with MetabolitePilot™ Software showing proposed biotransformation and elemental composition, experimental m/z, mass accuracy, retention time, peak area and score.
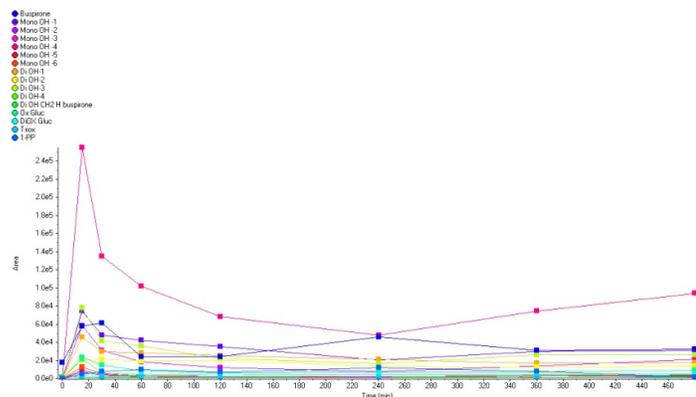


**Figure 7. Relative Quantification for Parent and Metabolites.** Peak area vs. time profiles (0-8hr) for buspirone parent and metabolites in rat plasma, generated using MultiQuant™ Software.

## Relative Quantification of Metabolite Levels

Once metabolites are identified, it is often very important to estimate their concentrations and kinetic profile relative to the parent. This was easily performed by exporting the accurate masses of the metabolites to MultiQuant™ Software and plotting the areas vs. time (Figure 7). Since the TOF MS scan is completely non-targeted, metabolite information can be queried without the need to reacquire data. This would also aid in identifying metabolites that are disproportionate across different species to address MIST questions.

## Conclusions

- High throughput in vivo Quant / Qual was successfully performed using the AB SCIEX TripleTOF™ 5600 system combined with MetabolitePilot™ and MultiQuant™ software.

- The high speed and sensitivity of the system enabled metabolite detection and identification on standard PK plasma samples.

- Efficient data processing software and the successful application of multiple peak finding strategies resulted in comprehensive metabolite coverage for Phase I and II as well as unexpected cleavage metabolites for buspirone.

- Obtaining high mass accuracy product ion spectra without compromising speed is invaluable for structure elucidation of metabolites and identification of the site of metabolism for soft spot optimization.

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## Acknowledgements

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