

# High Throughput and Sensitivity in Metabolite Profiling of Clozapine Using Predictive MRM and Intelligent Method Building

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## OVERVIEW

- Advanced Predictive MRM methodology.
- Application to *in vivo* metabolic profiling.
- Complete workflow for method construction.
- Data Processing.

## INTRODUCTION

Clozapine, a tricyclic compound, undergoes extensive phase I and phase II metabolism at multiple sites. Metabolic profiling for this class of compound has traditionally employed lower throughput means. *In Vivo* identification of metabolites is particularly challenging due to the complexity of the matrix and the higher sensitivity required. Triple quadrupole scan functions such as precursor ion and neutral loss scanning offer higher selectivity. Predictive multiple reaction monitoring (MRM) is the most sensitive approach. Although most biotransformation reactions are well known, these transformations can occur at multiple sites on the molecule, and primary metabolites often undergo further biotransformation which can complicate metabolite profiling. Here we present the results of a study to evaluate the utility of advanced software tools to automatically construct methods with a large number of MRM transitions which take into account the possibility of multiple transformations occurring on different parts of the molecule. In addition, we present a workflow that allows experimental design, method generation and data analysis using those software tools on a hybrid QqQ/QqLIT system; allowing the highest sensitivity in both detection and MS/MS confirmation of potential metabolites.

## MATERIALS AND METHODS

**In Vivo Administration:** Clozapine (Sigma) was administered by oral gavage at 20 mg/Kg to three Sprague-Dawley rats. Plasma time points were collected at 0, 1, 2, 3, 4 and 6 hours (terminal bleed). Urine was collected over the 0-6 hour interval. Brains were harvested after the 6 hour time point.

**Sample Preparation:** A 400 µL aliquot of plasma was prepared using a 1:1 ACN protein precipitation, followed by evaporation and reconstitution in 200 µL of 20% ACN. Urine was prepared by SPE: A 500 µL aliquot was diluted 1:1 with water and applied to an Oasis HLB SPE cartridge (Waters) following conditioning with methanol and water. Elution was accomplished using 1 mL of methanol followed by evaporation under Nitrogen and reconstitution in 300 µL of 20% ACN.

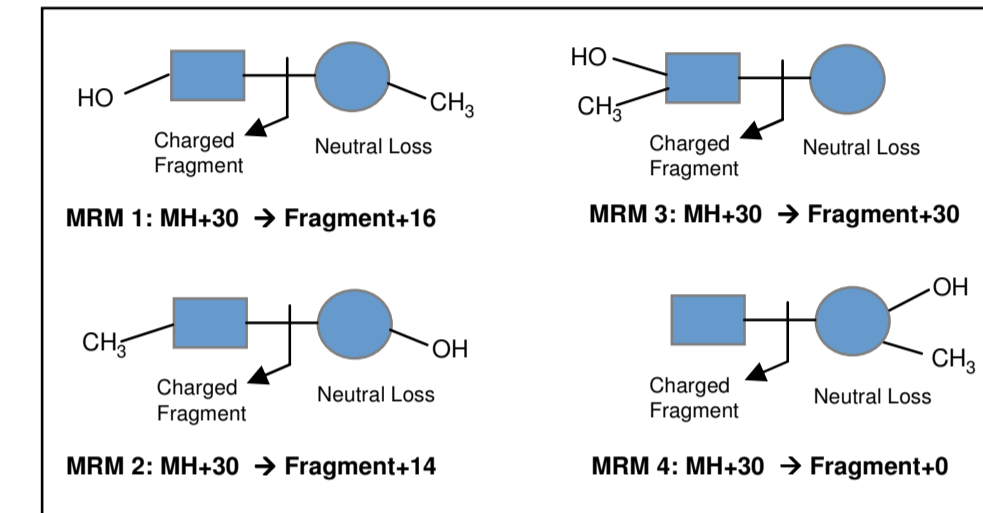
**LC/MS/MS:** A standard ACN/Water/0.1% formic acid gradient was used with a Synergi Polar-RP 4µm, 2x75mm column (Phenomenex). Overall run time was 30 minutes at a flow rate of 200 µL/min. Injection volume was 5-10 µL. The 4000 Q TRAP<sup>®</sup> hybrid QqQ/QqLIT system in ESI mode was used for all analyses. All data was acquired in real time information-dependent acquisition (IDA) mode combined with dynamic background subtraction. No static inclusion or exclusion lists were used in IDA. Dynamic isotopic exclusion was employed to exclude the <sup>37</sup>Cl isotope. For predictive MRM, up to 100 MRM transitions were monitored with a dwell time of 20ms. Full scan MS/MS data was obtained using the Enhanced Product Ion (EPI) mode at a LIT scan speed of 4000 amu/sec with dynamic fill time. The collision energy spread function (45V and a spread of ± 20V) was employed to obtain a full fragmentation spectrum. Overall cycle time (survey + MS/MS) was 3.5 seconds.

**Software Tools:** LightSight<sup>™</sup> Software V1.1 was used for all data processing as well as construction of biotransformation lists. MetaBuilder prototype software was used to construct predictive MRM data acquisition methods. Transformation lists were constructed based on combinations of the following well known biotransformation reactions: Aromatic oxidation (+16), N-demethylation (-14), oxidative dechlorination (-18), ketone formation (+14) and glucuronidation (+176), without a *priori* consideration of clozapine metabolism.

## RESULTS

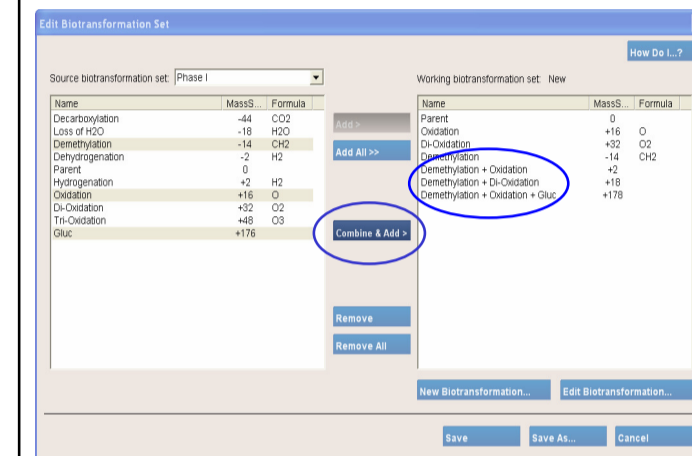
### I. Advanced Construction of Predictive MRM Methods

Figure 1. Predictive MRM for Methylation + Oxidation



A possible metabolite with two transformations requires four MRM's to cover all possible permutations.

Figure 2. Construction of Transformation Lists



Complex metabolism involving multiple transformations is constructed in LightSight<sup>™</sup> software.

Figure 3. Software Workflow

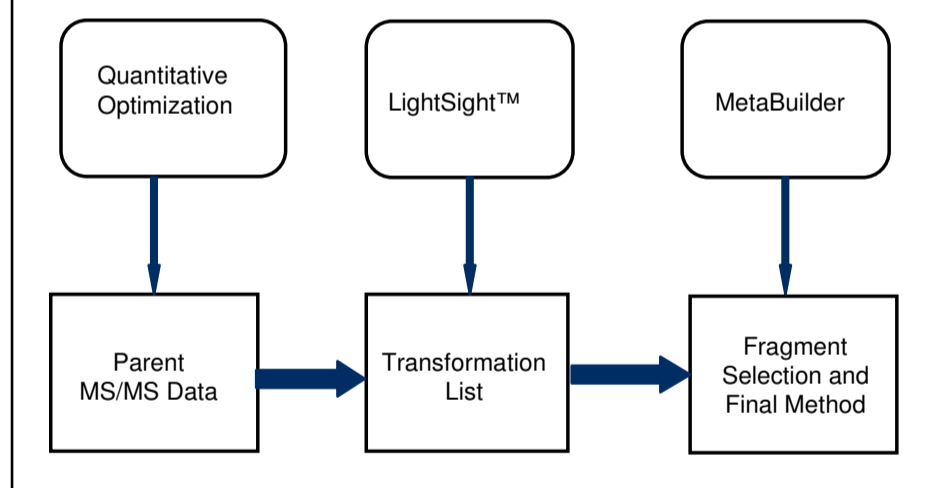
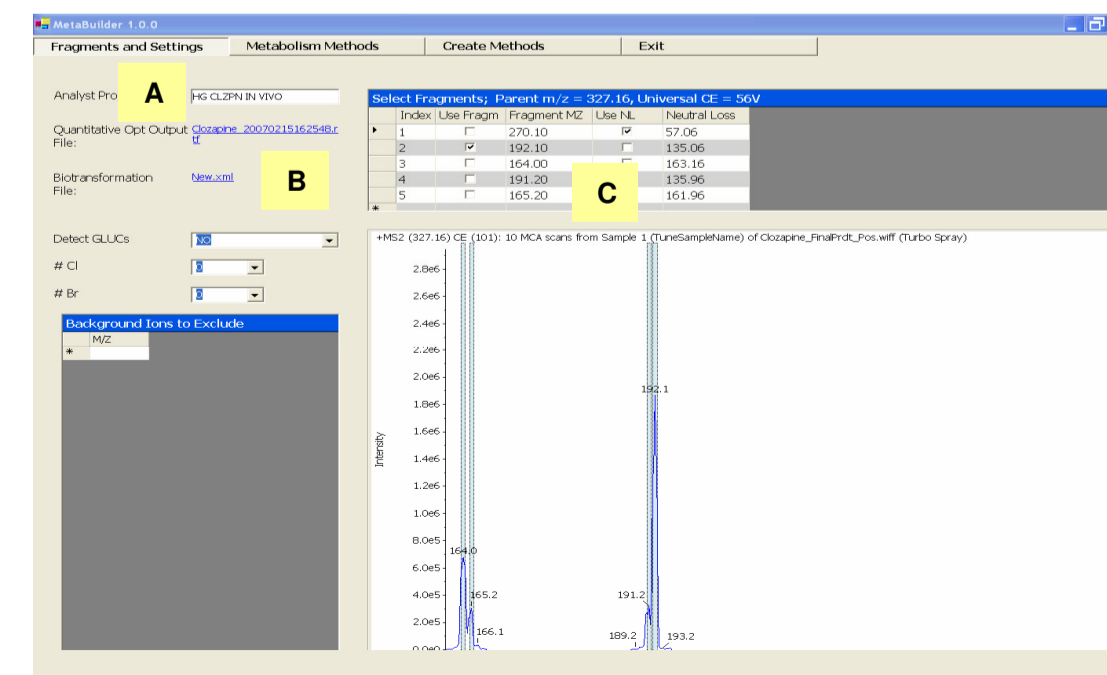


Figure 4. MetaBuilder Prototype Software

- (A) Selection of MS/MS quantitative optimization results with fragmentation and tuning information.
- (B) Selection of biotransformation list produced by LightSight<sup>™</sup> software.
- (C) Fragment selection for method generation.



### II. In Vivo Metabolites Detected in Rat Urine:

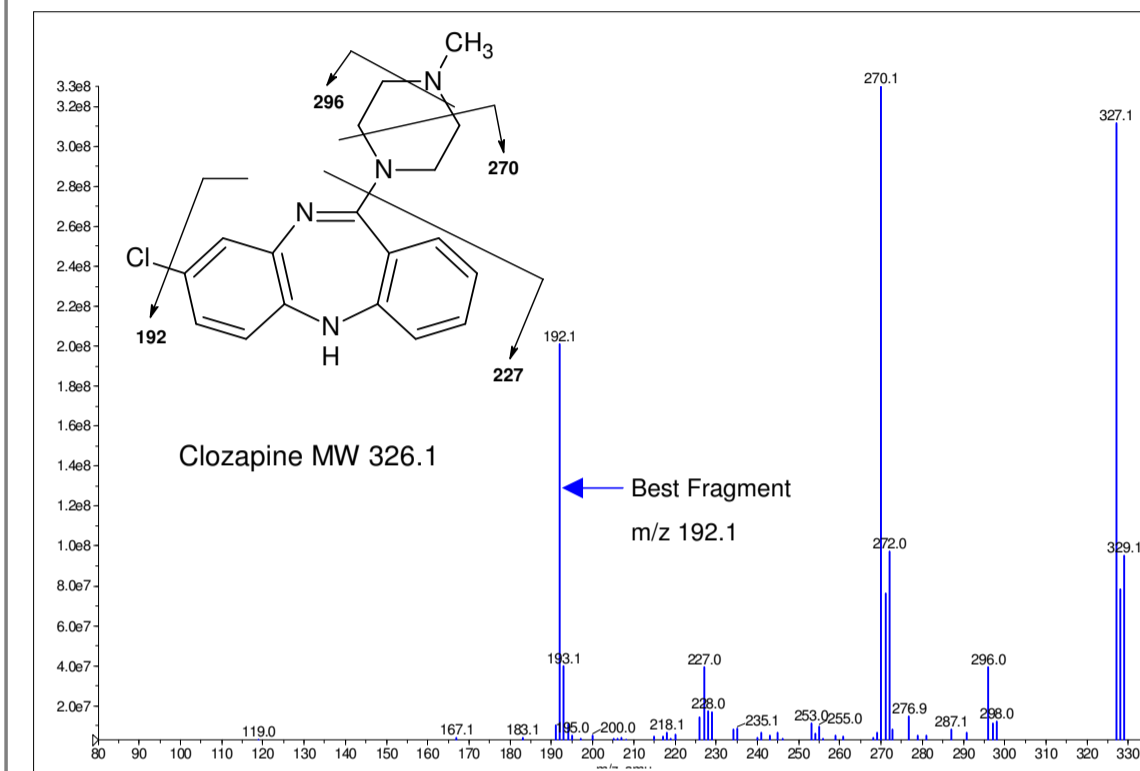


Figure 5. Fragment Selection

Enhanced product ion spectrum of clozapine parent with proposed fragment assignments. The m/z 192 fragment represents the best candidate for predictive MRM due to its structure and low mass relative to the parent.

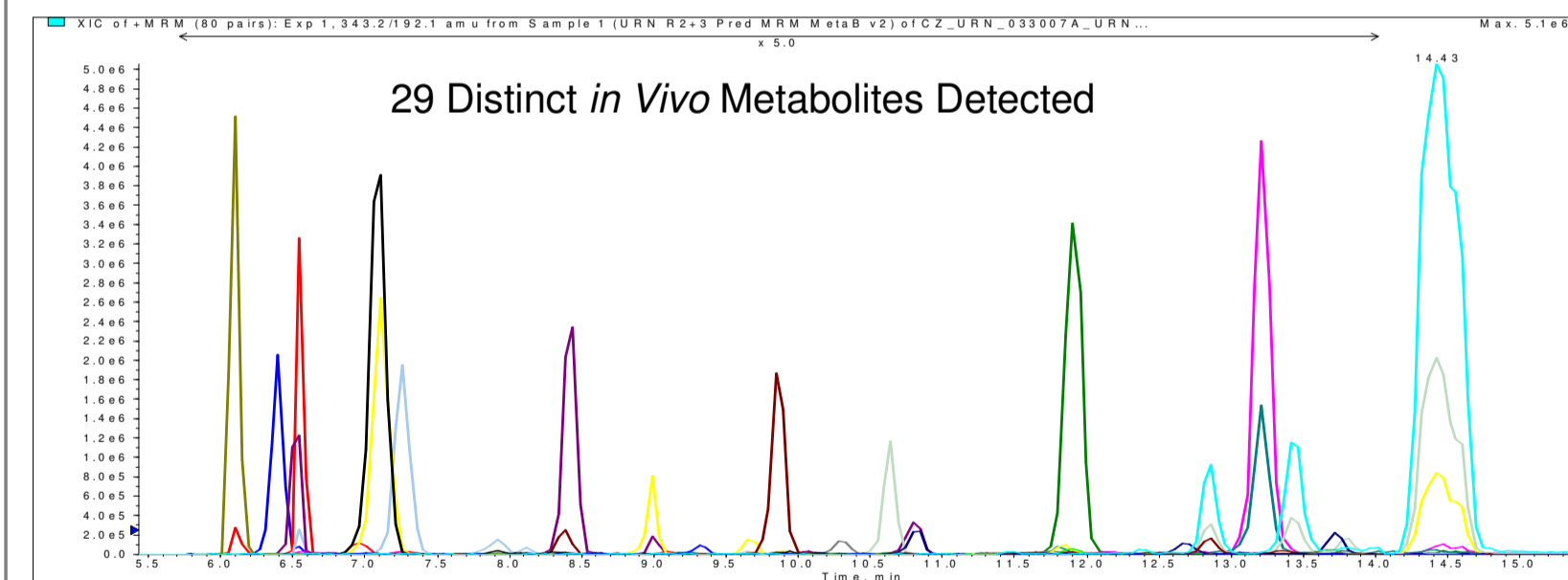


Figure 6. Overlaid MRM XIC's of 29 Metabolites Detected in Rat Urine Extract using Predictive MRM

Figure 7. Data Processing in LightSight<sup>™</sup> Software

- (A) Table of metabolites detected following sample / control comparison of MRM data.
- (B) Overlaid XIC of sample and control for currently selected metabolite.
- (C) IDA MS/MS data for selected peak along with parent MS/MS data.

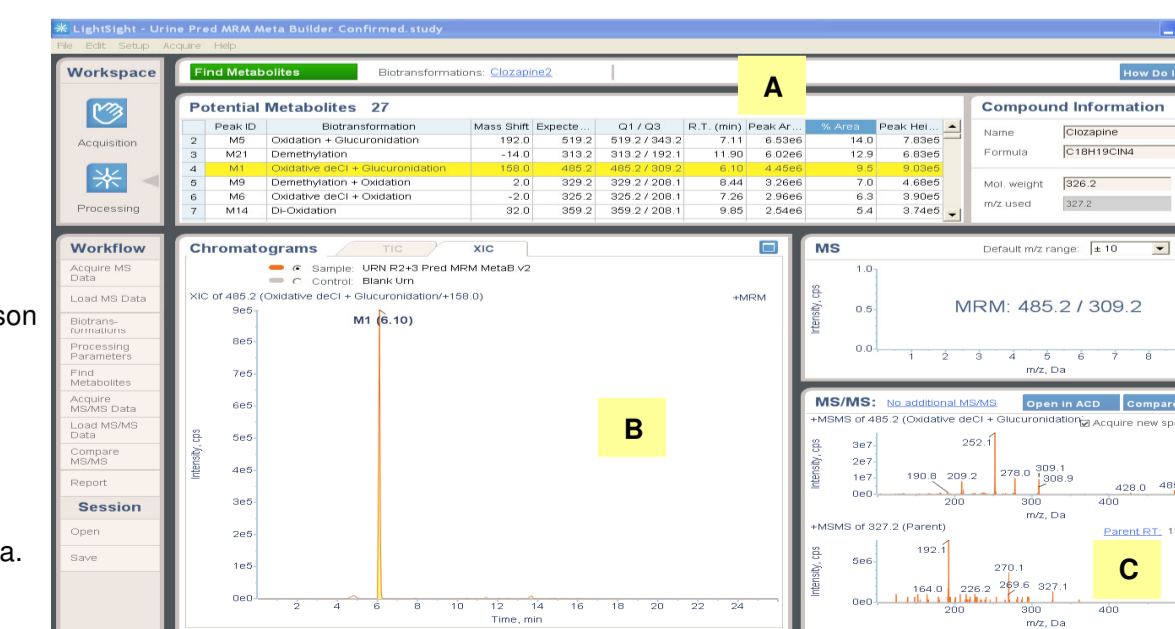


Figure 8. Detection of a complex metabolite involving a double transformation on two different sites of the molecule: (a) MRM detection, (b) IDA MS/MS spectrum.

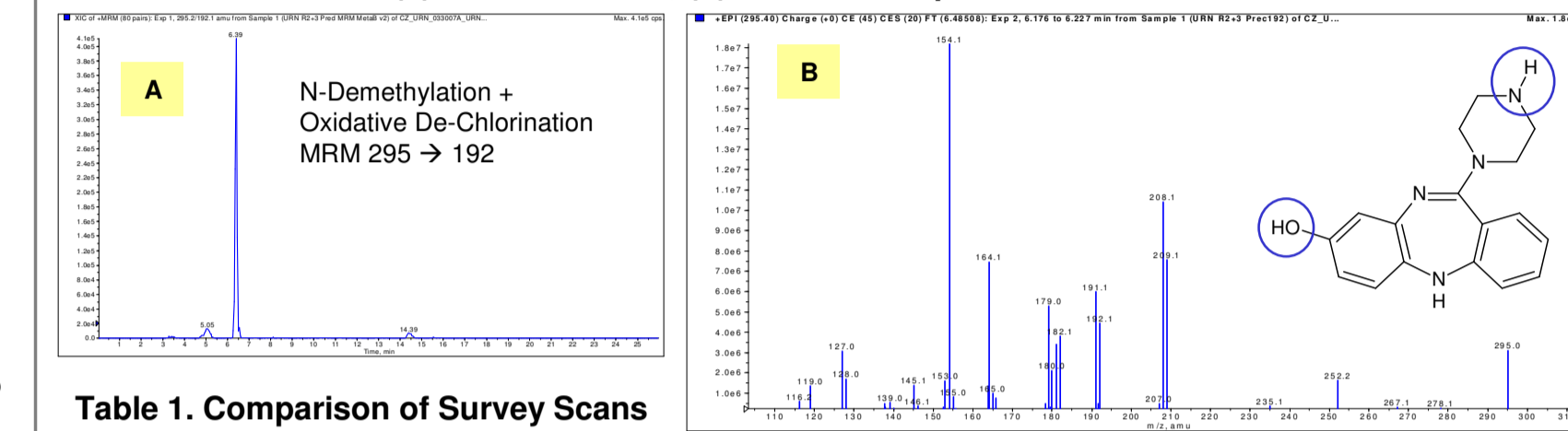


Table 1. Comparison of Survey Scans

Scan Type	No. of Metabolites Detected
Neutral Loss of 57	7
Precursors of 192	16
Full Scan EMS	13
Advanced Predictive MRM	29

Predictive MRM detected the highest number of *in vivo* metabolites of clozapine in urine when compared with other survey scan types.

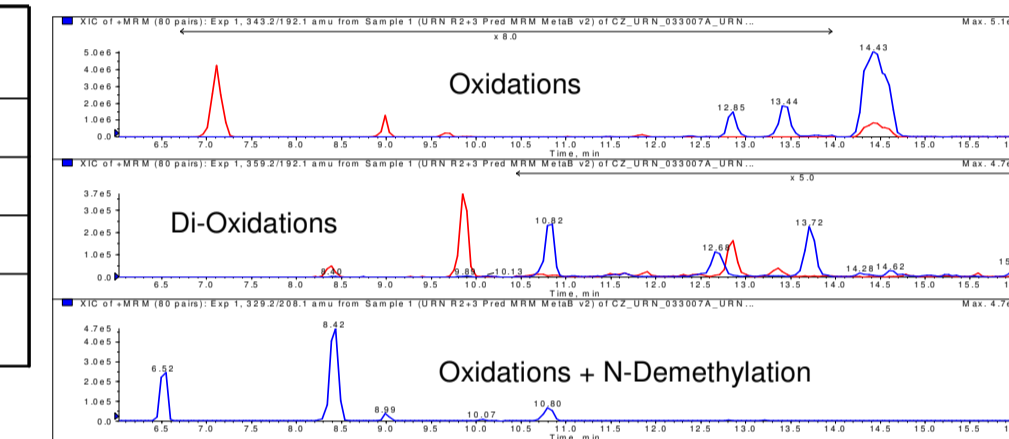


Figure 9. Predictive MRM Provides Broad Coverage of Phase I Metabolism.

## CONCLUSIONS

Predictive MRM is capable of detecting diverse metabolites with the highest sensitivity in a single experiment.

Predictive MRM is a viable first line option for complex *in vivo* metabolic profiling.

Advanced method building logic is essential for ensuring maximum metabolite coverage.

LightSight<sup>™</sup> Software, combined with MetaBuilder prototype software, represents a powerful and versatile workflow for metabolic profiling.

## REFERENCES

- J.G. Dain, J. Nicoletti, F. Ballard, Biotransformation of clozapine in humans, *Drug Metab Dispos.* **25**, 603-609 (1997).
- C.J. Bramwell-German et al, Development of automated software for creation of metabolite LC/MS methods for a QQQ/LIT hybrid mass spectrometer, ASMS TP06 (2006).

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