Improved Drug Metabolite Identification with a Streamlined Software Workflow

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Introduction

Drug metabolite identification is the essential first step to understanding drug behavior in humans, including efficacy, toxicity, and drug-drug interactions. However, drug biotransformation products are most often absent from established spectral libraries, presenting a challenge for their identification. Here we describe enhancements to a previously described strategy that combines open-source and vendor software to accurately predict drug metabolite structures, search these structures against MS/MS data through a molecular fingerprinting approach, and map identifications to molecular features resulting from a differential analysis of drug-treated liver microsomes.

Experimental

Samples

Pooled human liver microsomes with buffer (kit, Xenotech) were incubated at 37°C with verapamil (250 nM) or without drug. Reactions were quenched by addition of three volumes of acetonitrile with mixing at time points 0, 5, 10, 15, 30, 45 and 60 minutes followed by centrifugation.

LC/MS and Software

Supernatants were separated with RP-LC and eluents were analyzed with an Agilent Revident LC/Q-TOF in positive ion mode running MassHunter Acquisition 12.1 software. MS and MS/MS datasets were analyzed with MassHunter Explorer 2.0 software. SIRIUS Version 6.2 was used to search the MS/MS spectra against a custom structure database created with verapamil transformation products. Agilent ChemVista 1.0 software was used to manage and merge all exported results into a database.

Workflow

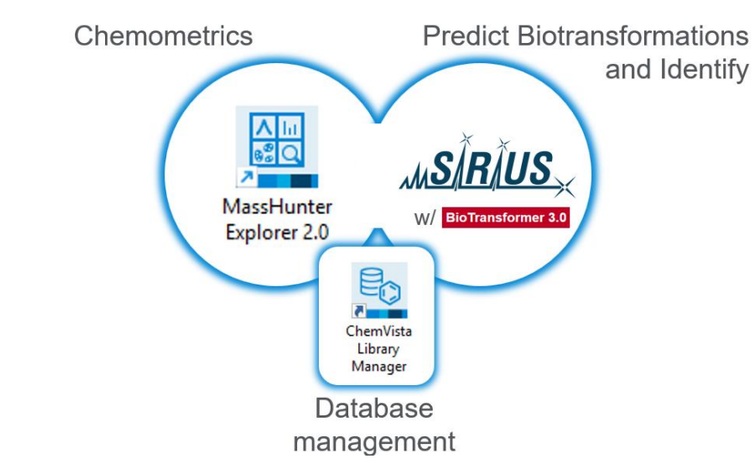

Figure 1. Overview of workflow for identification of biotransformations.

Results and Discussion

Chemometrics

MassHunter Explorer 2.0 is a chemometrics software that enables feature finding, MS/MS data extraction, compound identification, and statistical analyses in a single application. The 42 verapamil and no-drug time course MS1 datafiles were loaded into Explorer along with iterative MS/MS datafiles from a pooled QC. After the Find and Align step, 6,131 compounds groups were extracted.

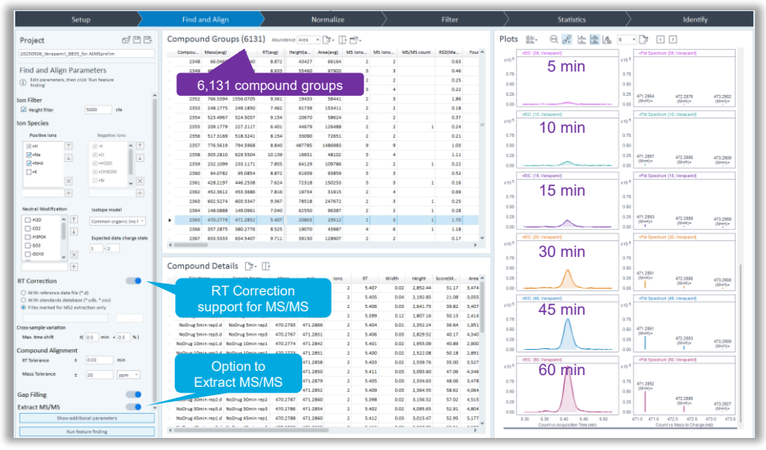

Figure 2. The verapamil Explorer project displaying results after the Find and Align step. Cpd group 2365 is selected, and the EICs show an increase in abundance over time which may be indicative of a biotransformation product.

Results and Discussion

To select for potential drug metabolites, sequential Volcano plot analyses were performed in Explorer. First, verapamil-treated samples at 45 minutes were compared to time 0, revealing 274 significantly elevated compounds. PCA showed separation by time (PC1) and treatment (PC2). To exclude endogenous metabolites that naturally increase over time, a second Volcano plot compared 45-minute samples with and without verapamil (not shown), narrowing the list to 52 candidate compounds for further analysis.

Figure 3. (A) Volcano plot of Verapamil-45min vs Verapamil-0min samples. Fold-change > 3, p-value <0.01, Benjamini-Hochberg correction. (B) PCA based on 274 significant compounds.

From the 52 significant compounds, EICs were examined for characteristic biotransformation profiles over time, resulting in 8 compounds. From these an interesting set of 5 isobaric compounds were resolved chromatographically, all with associated MS/MS spectra extracted using Explorer 2.0.

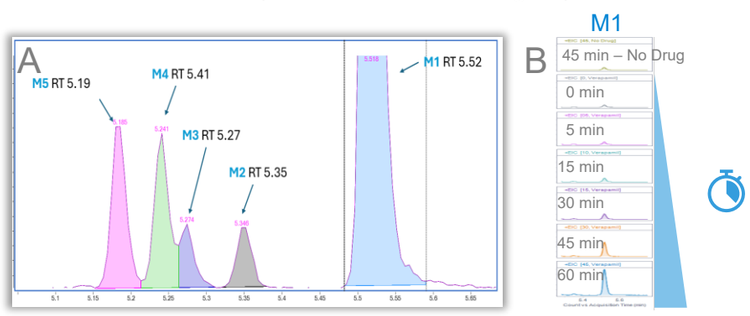

Figure 4. (A) Representative chromatogram of a set of 5 putative metabolites (M1 through M5) with mass 440.2746 ± 0.5 ppm. (B) Characteristic biotransformation profile for M1.

Identification

In the Identify step, all compound groups were subjected to library searching. Many endogenous metabolites were identified with the Agilent METLIN PCDL, and the parent drug verapamil was identified with Agilent Applied Markets PCDL.

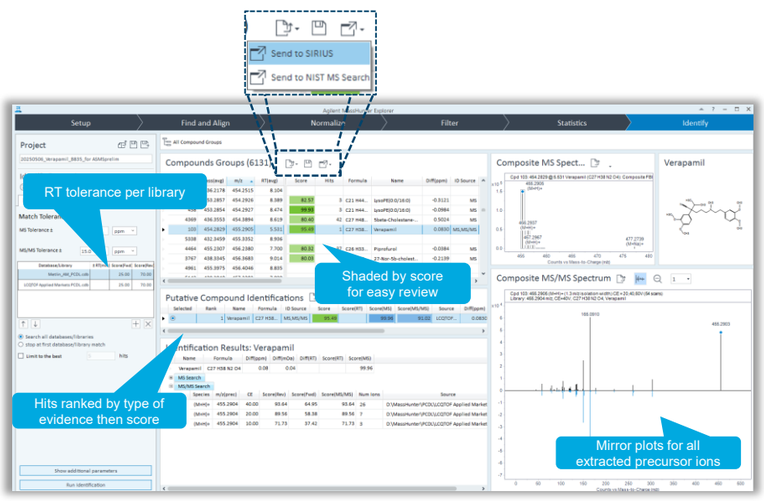

Figure 5. Explorer project displaying results after the Identify step, showing spectral library matching for verapamil. The "Send to SIRIUS" and "Sent to NIST" functions are highlighted.

Creation of Custom Biotransformation Database

SIRIUS 6.2 allows for creation and searching of custom structure databases of transformation products via integration of Biotransformer 3 software[1], a capability critical for this workflow, because metabolites from novel drugs are most likely to be absent from public structure databases.

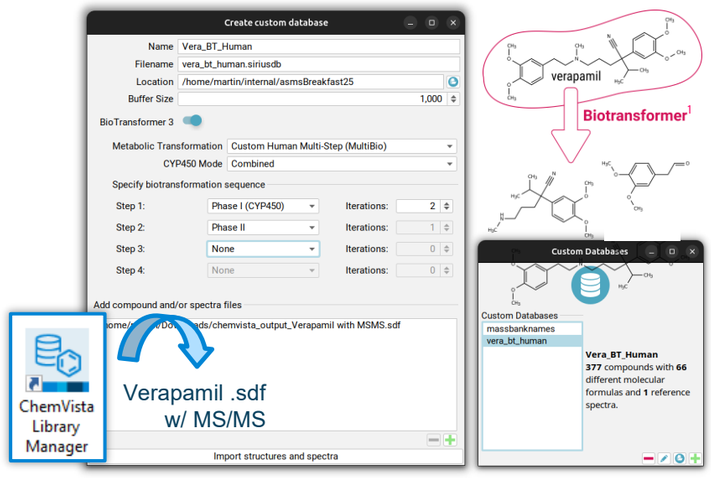

Figure 6. Creation of a custom verapamil biotransformation database within SIRIUS 6.2. A combination of phase I and phase II biotransformations was specified, resulting in 377 compounds.

Results and Discussion

Structure Elucidation

The MS/MS spectra for the 8 putative metabolites were sent from Explorer to SIRIUS using the "Send to SIRIUS" function, and the custom database was leveraged for both formula generation[2] (fragmentation trees) and database searching with molecular fingerprinting[3] using CSI:FingerID. The putative metabolite M1 was identified as "norverapamil" as the top hit. This well-characterized metabolite was confirmed by retention time and MS/MS with an authentic standard (data not shown).

Figure 7. SIRIUS with CSI:FingerID Structure DB Search results showing norverapamil as the top hit for metabolite M1. (B) Confirmation with spectral library analog search comparing norverapamil to verapamil, highlighting key fragments in peak-to-substructure assignments resulting from neutral losses.

The putative metabolite M2 was identified from the custom database as a dealkylation product as the top hit, which corresponds to the well-described metabolite O-Desmethyl Verapamil. The molecular fingerprints suggested differences with other possible isobaric biotransformation products.

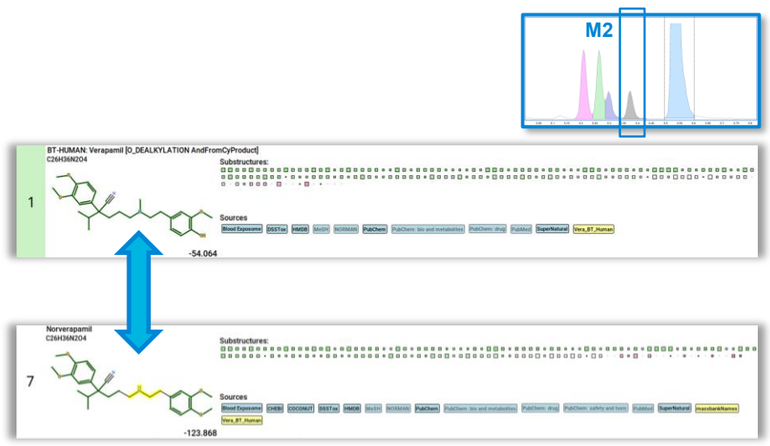

Figure 8. Structure DB Search results comparing molecular fingerprints for the top hit (O-Desmethyl Verapamil) and the 7th hit (norverapamil). Key differences in matching substructures are highlighted with the software.

Conclusions

A streamlined and novel drug metabolite ID workflow was demonstrated:

- Applies chemometrics to profile and reveal putative drug metabolites from liver microsomes, with simultaneous MS/MS spectral extraction.
- Identifies resulting structures through molecular fingerprinting from a custom biotransformation database
- Opportunities for extension to xenobiotic applications

References

1 Wishart DS et al. Biotransformer 3.0—A Web Server for Accurately Predicting Metabolic Transformation Products. *Nucleic Acids Res.* 2022 May 10;50(W1):W115-W123. PMID: 35536252

2 Kai Dührkop, Markus Fleischauer, Marcus Ludwig, Alexander A. Aksenov, Alexey V. Melnik, Marvin Meusel, Pieter C. Dorrestein, Juho Rousu and Sebastian Böcker. SIRIUS4: a rapid tool for turning tandem mass spectra into metabolite structure information. *Nat Methods*, 16, 2019.

3 Kai Dührkop, Huibin Shen, Marvin Meusel, Juho Rousu and Sebastian Böcker. Searching molecular structure databases with tandem mass spectra using CSI:FingerID. *Proc Natl Acad Sci* U S A, 112, 2015.