Application Note

Pharma & Biopharma

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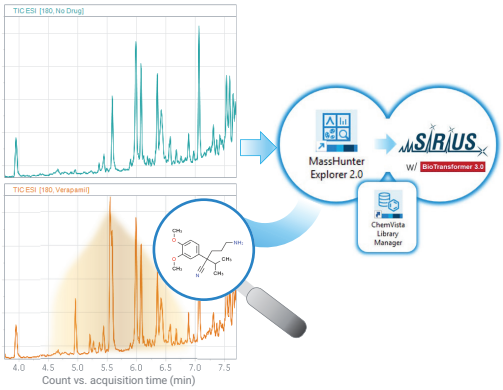
# Drug Metabolite Identification with a Streamlined Software Workflow

## Combining Agilent Revident Q-TOF LC/MS and MassHunter Explorer 2.0





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## Abstract

Drug metabolite identification is a foundational step in understanding the pharmacological and toxicological behavior of therapeutic compounds. However, the absence of biotransformation products from established spectral libraries presents a persistent challenge, particularly in early-stage drug development. This application note describes a streamlined workflow that integrates high-resolution LC/Q-TOF mass spectrometry with chemometric analysis and molecular fingerprint-based structure elucidation. Leveraging the Agilent Revident quadrupole time-of-flight LC/MS system, Agilent MassHunter Explorer 2.0, and SIRIUS with CSI:FingerID software, the workflow enables confident identification of both known and novel drug metabolites. Verapamil, a well-characterized calcium-channel blocker, was selected as a model compound to validate the approach. The workflow demonstrated effective differentiation of drug-related features from complex biological backgrounds and enabled structure-based identification using a custom BioTransformer-generated database. This flexible and scalable strategy supports metabolite profiling in pharmaceutical research and development.

## Introduction

Understanding how drugs are metabolized is essential for predicting their efficacy, safety, and potential interactions in vivo. Drug metabolite identification provides critical insights into pharmacokinetics and pharmacodynamics, yet remains a complex task, partly due to the absence of biotransformation products in public spectral libraries. This limitation is especially pronounced during early drug discovery, where novel compounds and their metabolites lack reference spectra, complicating efforts to characterize low-abundance or structurally diverse transformation products.

To overcome these challenges, we developed an integrated workflow that combines high-resolution mass spectrometry with advanced data analysis and structure prediction tools (Figure 1). The approach uses the Agilent Revident Q-TOF LC/MS system for sensitive and accurate mass detection, Agilent MassHunter Explorer 2.0 for chemometric feature extraction and statistical filtering, and SIRIUS for molecular fingerprint-based structure elucidation. A custom structure database generated using BioTransformer further enhances the workflow's ability to identify metabolites not present in conventional libraries.

Verapamil was selected as a model compound due to its well-documented metabolic profile, making it a reliable benchmark for evaluating the workflow's performance. As a widely used cardiovascular drug, its known biotransformation products support validation of untargeted metabolite identification strategies. In this study, human liver microsomes were used to simulate in vitro metabolism, and differential analysis was applied to isolate statistically significant features associated with drug treatment. The workflow successfully identified known verapamil metabolites, demonstrating its utility for untargeted metabolite discovery and its adaptability for future applications involving new chemical entities.

## Experimental

### Solvents

Ammonium formate (LiChropur) was purchased from MilliporeSigma (St. Louis, MO, USA). Formic acid (Optima LC/MS Grade) was purchased from Fisher Scientific (Waltham, MA, USA). Agilent InfinityLab methanol (part number 5191-5111) and Agilent InfinityLab acetonitrile (part number 5191-5101) were used for both sample preparation and LC/MS mobile phases. Ultrapure water for sample preparation and LC/MS analysis was produced using a Milli-Q Integral system equipped with an LC-Pak Polisher and a 0.22 µm point-of-use membrane filter cartridge (MilliporeSigma).

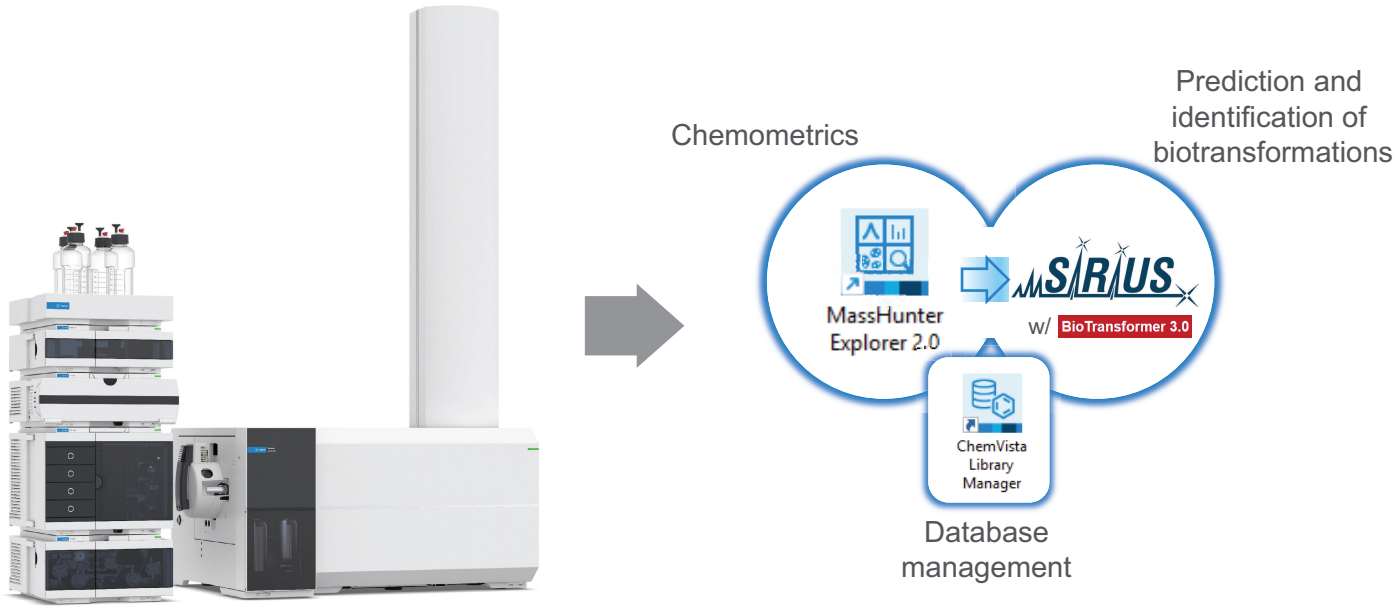



**Figure 1.** Workflow for identification of biotransformations.

## Instrumentation

An Agilent Infinity III LC system was used, consisting of:

- Agilent 1290 Infinity III high-speed pump (product number G7120A)
- Agilent 1260 Infinity III hybrid multisampler with thermostat (product number G7167C)
- Agilent 1290 Infinity III multicolumn thermostat (product number G7116B)

The setup also included the Agilent Revident Q-TOF LC/MS system equipped with an Agilent Dual Jet Stream technology source (product number G6575A).

## Sample preparation

The XenoTech Reaction Phenotyping Kit (BioIVT, Westbury, NY, USA; part number H0500) was employed based on the manufacturer's instructions, with adaptations suited to the experimental design. The (±) Verapamil hydrochloride (MilliporeSigma, St. Louis, MO, USA; part number PHR1131) was prepared as a 1.0 mM stock solution in acetonitrile and added to NADPH reaction buffer; acetonitrile alone served as the "No Drug" control. Reactions were assembled in an Agilent 96-well plate (part number 5043-9310) using pooled human liver microsomes provided in the kit, yielding final concentrations of 5 µM verapamil and 1 mg/mL microsomes. The final acetonitrile concentration was 0.5% in both the verapamil and No Drug control conditions. The plate layout is shown in Figure 2. The plate was incubated at 37 °C with shaking at 500 rpm on an Eppendorf Thermomixer R (Eppendorf North America, Enfield, CT, USA).

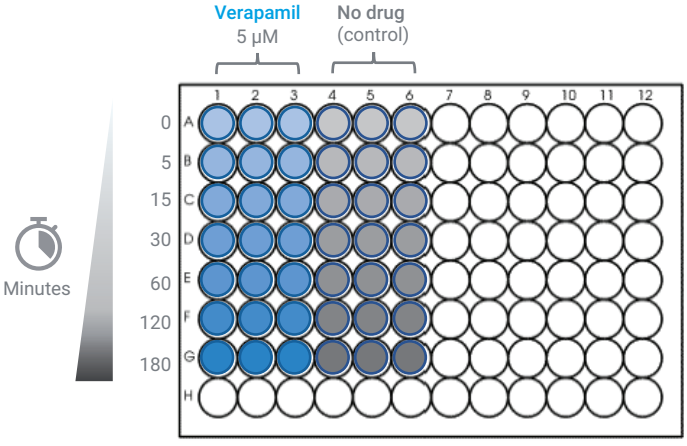



**Figure 2.** Plate layout for microsomal incubations. Seven time points and three replicates (biological replicates) were prepared.

At the designated time points, aliquots were transferred with a multichannel pipettor to a chilled crash plate containing acetonitrile to achieve a 3:1 acetonitrile:aqueous v/v ratio. The aliquots were then mixed by pipetting to quench the reaction and precipitate proteins. After the final time point, the crash plate was further mixed at 500 rpm for 10 minutes at 4 °C and centrifuged at 2,272 × g for 60 minutes at room temperature to pellet protein precipitates. Supernatants were transferred using a multichannel pipettor to a final plate, sealed (Agilent PlateLoc), and placed on the autosampler.

For MS/MS experiments, a five-fold concentrated pooled sample was prepared. This was achieved by combining small aliquots from six wells containing samples for the 120- and 180-minute time points into a separate vial, drying with a vacuum concentrator, and resuspending in one-fifth volume 3:1 (v/v) acetonitrile:water.

## LC/MS methods

Detailed experimental methods for LC conditions, the Q-TOF LC/MS system, and Directed MS/MS parameters are provided in Tables 1 and 2.

**Table 1.** LC instrument parameters.

| Parameter | Agilent 1260/1290 Infinity III LC |
|---|---|
| Analytical Column | Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 2.7 µm (p/n 695775-902) |
| Column Temperature | 45 °C |
| Autosampler Temperature | 4 °C |
| Mobile Phase | A) 5 mM ammonium formate and 0.1% formic acid in water<br>B) 5 mM ammonium formate and 0.1% formic acid in methanol |
| Gradient Program | Time (min) %B Flow rate (mL/min)<br>0 3 0.4<br>0.5 3 0.4<br>8.0 97 0.4<br>9.5 97 0.4<br>9.6 3 0.4<br>12.5 3 0.4 |
| Stop Time | 12.5 min |
| Post-Time | None |
| Classical Flow-Through Injection | – Draw speed: 100 µL/min<br>– Injection volume: 10 µL<br>– Inner wash mode: Off<br>– Outer wash mode: Standard, 10 s<br>– Outer wash solvent: 50:50 methanol:water<br>– Injection volume: 10 µL |
| Feed Injection | – Feed speed: 10% of flow (adaptive)<br>– Flush-out volume: 10 µL<br>– Inner wash solvent: 5 mM ammonium formate and 0.1% formic acid in 50:50 acetonitrile:water<br>– Outer wash solvent: 50:50 methanol:water<br>– Inner/outer wash: 150 µL/10 s<br>– Reconditioning: S2 |

**Table 2.** Agilent Revident Q-TOF LC/MS instrument parameters.

| Parameter | Value |
|---|---|
| **Source and Common Parameters** | |
| Instrument Mode | 1700 stable |
| Ion Source | Agilent Dual Jet Stream |
| Polarity | Positive |
| Gas Temperature | 250 °C |
| Drying Gas (Nitrogen) | 11 L/min |
| Nebulizer Gas | 40 psi |
| Sheath Gas Temperature | 350 °C |
| Sheath Gas Flow | 12 L/min |
| Capillary Voltage | 3,500 V |
| Nozzle Voltage | 1,000 V |
| Fragmentor | 125 V |
| Skimmer | 45 V |
| Octopole RF Vpp | 750 V |
| Reference Mass | *m/z* 121.050873, *m/z* 922.009798, 40 µL/min |
| Reference Nebulizer | 8 psi |
| **MS-Only Method Parameters** | |
| MS Range | *m/z* 50–1,700 |
| MS Acquisition Rate | 3 spectra/s |
| **Directed MS/MS Method Parameters** | |
| MS Range | *m/z* 50–1,700 |
| MS/MS Range | *m/z* 20–1,700 |
| MS Acquisition Rate | 8 spectra/s |
| MS/MS Acquisition Rate | 6 spectra/s |
| Isolation Width | Narrow (~ *m/z* 1.3) |
| Delta | 20 ppm |
| RT Window | 0.01 min |
| Collision Energy | Fixed: 20, 40, and 60 eV |
| Maximum Precursors per Cycle | 1 |
| Variable Acquisition Rate | No |
| Precursor Threshold | 3,000 counts and 0.001% |
| Active Exclusion | Disabled |
| Purity | Stringency 100%, cutoff 30% |
| Isotope Model | Common organic molecules |
| Iterative MS/MS Mass Error Tolerance | 20 ppm |
| Iterative MS/MS RT Exclusion Tolerance | ± 0.02 minutes |

## Software

- Agilent MassHunter acquisition software for LC/MS (Q-TOF), version 12.1, was used to operate the Revident Q-TOF LC/MS system.
- Agilent MassHunter Explorer software, version 2.0, was used for compound discovery and differential analysis by Q-TOF LC/MS. The Default.M method was used with the following modifications: Gap Filling: Off; Height filter: 5,000; Use multi-pass exhaustive grouping: selected; Isotope model: Common organic (no halogens); RT Tolerance ± 0.02 minutes; Score (MFE) ≥ 70 in at least two files in at least one sample group. When exporting the Precursor Ion List, a single precursor ion per compound from the most abundant monoisotopic ion was specified. Statistical and Library Identification search parameters are provided in the text and figures. When mapping SIRIUS identifications back to the MassHunter Explorer project with a database search, an RT window of ± 0.05 minutes was specified.
- Agilent ChemVista 1.0 was used for mass spectral database and library management. The Q-TOF LC/MS Applied Markets Personal Compound Database and Library (PCDL) was used to identify known verapamil metabolites. When importing the SIRIUS-exported "chemvista_summary.csv" summary file, the Import File Source type was specified as "PCDL CSV (*.csv)", and the "Classify data" option was cleared in the System Application settings to enable import of multiple compounds with the same identifiers but with different retention times. The 28 substances in the list were selected and exported as PCDL (.cdb) with default settings to be leveraged for a database search in MassHunter Explorer.
- Complementary, direct access from MassHunter Explorer 2.0 allowed convenient access to SIRIUS 6.3.2 and CSI:FingerID (Bright Giant GmbH, Jena, Germany). These tools were used for structure-based identification. A custom database of verapamil biotransformations was built from the integrated BioTransformer 3.0 tool as described in the text. To aid common verapamil metabolite nomenclature, an additional custom database was built from nine known verapamil metabolites. Default computation parameters were used with the following changes: Global Configuration Settings: Search DBs: All clear except the two verapamil databases; SIRIUS Settings: Strategy: Database search; CSI:FingerID Settings: PubChem as fallback: cleared. When exporting identifications, the Top Hits were chosen and the "ChemVista summary" box was selected.
- Agilent MassHunter Qualitative Analysis, version 13.0, was used to create some figures, though it is not required for this workflow.

## Results and discussion

### The 1260 Infinity III hybrid multisampler enables minimal microsomal sample preparation

To generate metabolites, verapamil was incubated with human liver microsomes over a time course using a typical protocol. Following quenching and protein precipitation, the most straightforward approach would be to directly inject the supernatants from the plate onto the Q-TOF LC/MS. However, the high organic content (75% acetonitrile) required for quenching and precipitation results in a strong elution strength for the sample solvent in reversed-phase chromatography. This can lead to poor chromatographic peak shapes and peak breakthrough. Typically, this would necessitate sample concentration, often achieved by drying down and resuspending in a weaker solvent compatible for injection. These additional steps not only increase time and labor but also raise concerns about incomplete resolubilization of certain metabolites.

We evaluated the performance of the 1260 Infinity III hybrid multisampler for direct injection of supernatants from verapamil microsome incubations. The hybrid multisampler operates by mixing the sample with the initial mobile phase at user-defined "feed rates", thereby mitigating solvent effects and effectively trapping and enriching the polar compounds on the column.[1] This autosampler has demonstrated utility in other applications (for example, pesticides) by eliminating peak breakthrough and improving chromatographic peak shapes, thereby enhancing detection, quantification, and automated peak integration.[2] For comparison, this autosampler can also be operated in Classical (flow-through) injection mode.

To assess the impact of injection mode—Feed Injection versus Classical Injection—we injected verapamil microsomal supernatants (prepared in 75% acetonitrile) at varying volumes of 1, 2, 5, 10, and 20 µL. At injection volumes of 10 and 20 µL, Feed Injection produces well-shaped peaks for verapamil, whereas Classical Injection results in pronounced peak splitting (data not shown). Since most biotransformation reactions (for example, hydroxylation, oxidation) increase the polarity of drug metabolites relative to the parent compound, these metabolites often exhibit weaker retention and heightened sensitivity to solvent effects. For closely eluting isobaric metabolites M18, M20, M22, M23, and M26, peak shapes are noticeably worsened with 10 and 20 µL injection volumes using Classical Injection but are fully recovered with Feed Injection (Figure 3). Additionally, peak heights are reduced in Classical mode at larger injection volumes due to peak broadening. Isobaric metabolites M4 and M10, derived from verapamil truncation, exhibit even greater sensitivity, with noticeable peak degradation beginning at 5 µL (Figure 4). Here again, Feed Injection improves peak shapes with consistency across the range of injection volumes. As previously demonstrated, such effects can have quantitative implications, including improvement of relative standard deviation (RSD) values with Feed Injection.[2] In the data analysis strategy described in the current study, poor peak shapes would also pose a challenge for molecular feature extraction algorithms, which would struggle to accurately isolate and extract closely eluting isomeric compounds under suboptimal chromatographic conditions.

Based on these findings, we selected a 10 µL Feed Injection volume for microsomal supernatants extracts, which maintains excellent peak shape while yielding optimal signal quality for metabolite discovery in our LC/MS workflow.

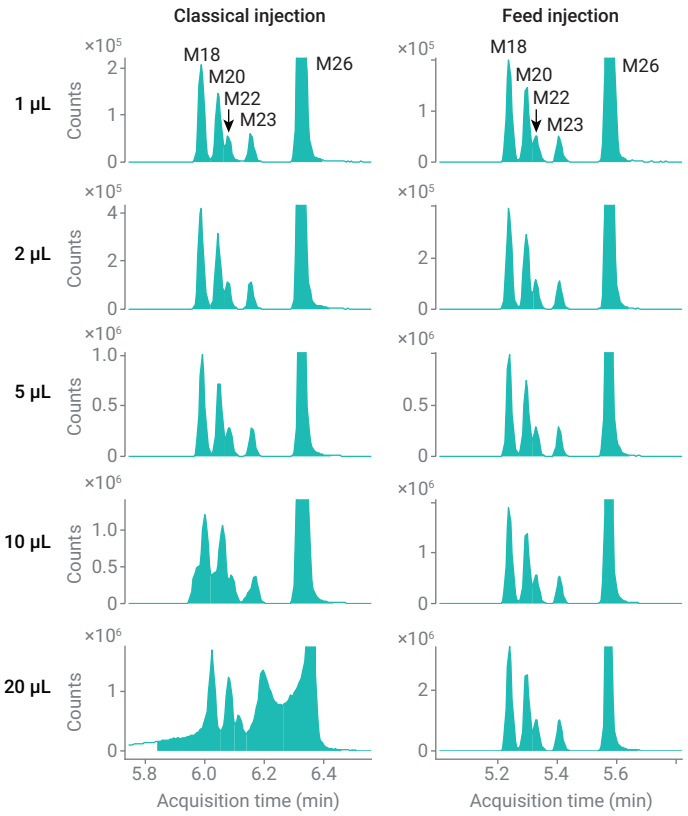



**Figure 3.** Extracted ion chromatogram (EIC; *m/z* 441.2748) comparison of the isobaric verapamil metabolites M18, M20, M22, M23, and M26 in Classical flow-through injection mode and Agilent Feed Injection mode at different injection volumes.

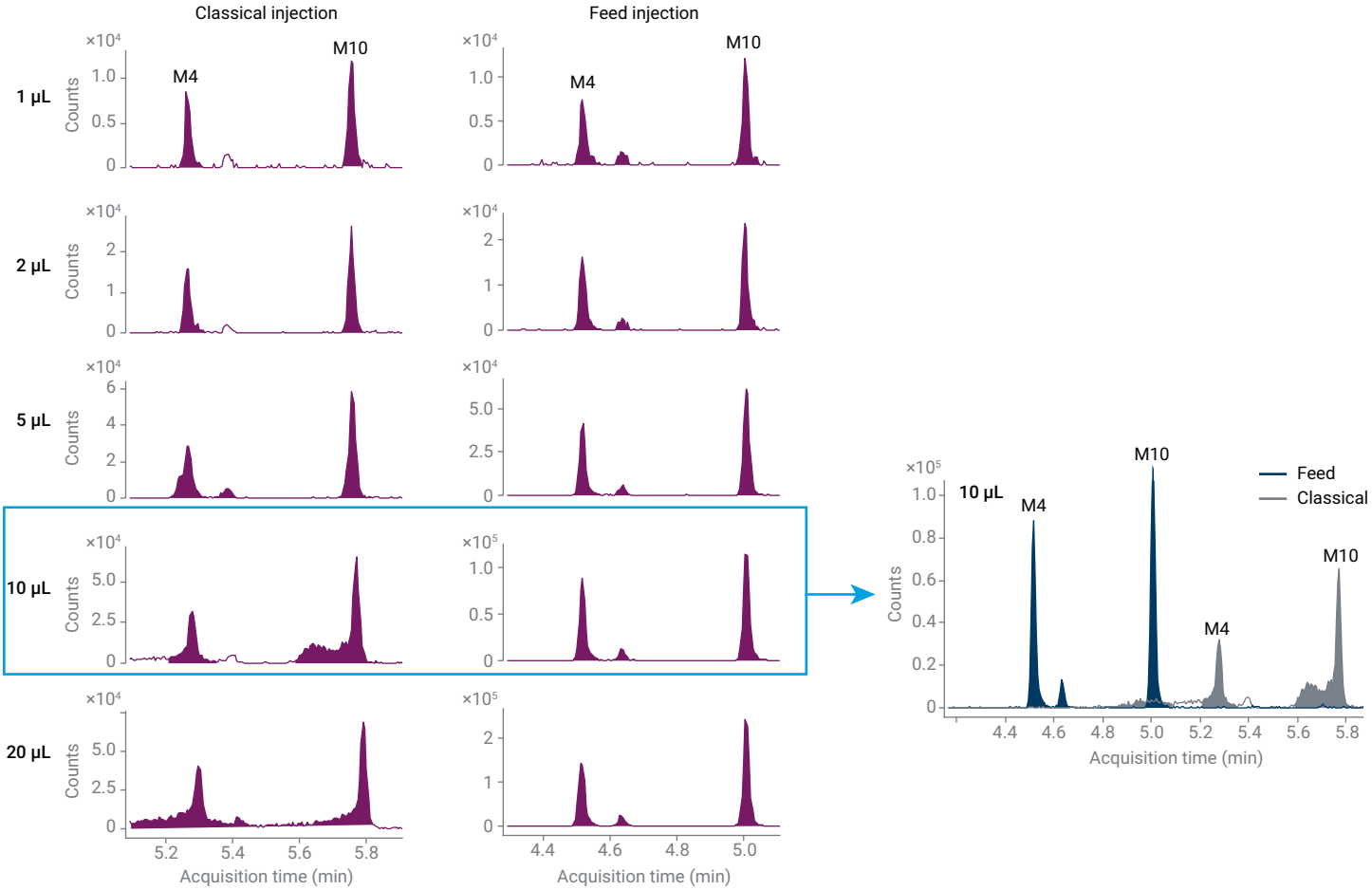



**Figure 4.** EIC (*m/z* 277.1911) comparison of the isobaric verapamil metabolites M4 and M10 in Classical flow-through injection mode and Agilent Feed Injection mode at different injection volumes. Overlaid EICs are also shown for the 10 µL injection.

## Chemometrics selects potential metabolites from Q-TOF LC/MS datasets

The 42 microsomal supernatants were each injected and separated with a 12.5-minute reversed-phase method generally well suited for separation of moderately polar, small-molecule drug metabolites. LC eluents were analyzed with a Revident Q-TOF LC/MS, an instrument that delivers improved spectral quality resulting in increased mass resolution, dynamic range, and isotopic fidelity.

To find and identify the verapamil biotransformation products, the complex dataset was analyzed with MassHunter Explorer 2.0, a chemometrics software that enables feature finding, MS/MS data extraction, compound identification, and statistical analyses in a single application. In the Find and Align step, the 42 verapamil and no-drug time course MS1 data files were added to an Explorer project along with iterative MS/MS data files from a pooled QC. Two sample groups were created: 1: Time (7 time points) and 2: Treatment (Verapamil and No Drug). After processing, 7,501 compounds groups were extracted.

In the Statistics tab of MassHunter Explorer, a strategy was taken to select for potential drug metabolites using sequential volcano plot analyses. First, to select compounds that change in abundance over time, verapamil-treated samples at 180 minutes were compared to verapamil samples at time point 0 with a volcano plot, resulting in 808 significant compounds that were saved as a compound list (fold change > 5, p-value < 0.01, Benjamini-Hochberg correction, data not shown). However, this compound list not only contained drug biotransformation products, but also endogenous metabolites that naturally change over time from the metabolically active microsomal extracts. To remove the latter, a second volcano plot paired the 180-minute samples with and without verapamil (No Drug), narrowing the list to 132 significant compounds, 88 of which were elevated in the verapamil-treated samples and should include the drug biotransformation products (Figure 5A).

For drug metabolite identification, high-quality MS/MS spectra were essential. To achieve this, a targeted acquisition strategy was applied, focusing exclusively on compounds of interest. Using the "Export Precursor Ion List" feature in MassHunter Explorer, a precursor ion list was generated from the 88 target compounds, including their observed retention times, for setup of a Directed MS/MS acquisition method (Figure 5D).

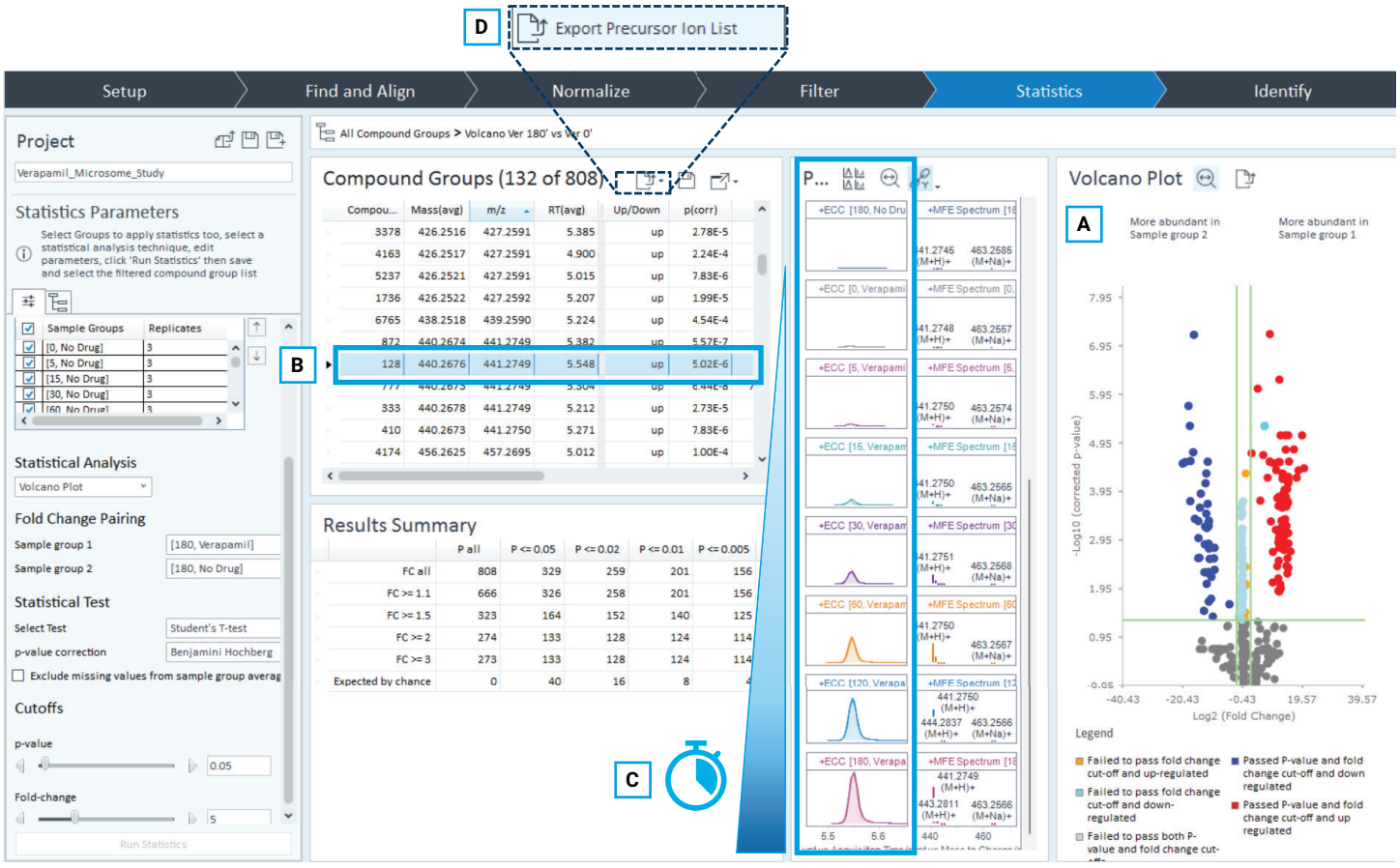



**Figure 5.** Agilent MassHunter Explorer software user interface displaying results from the "Statistics" procedure. (A) The second volcano plot, generated from the 808-compound subset identified in the initial analysis, comparing verapamil-treated samples to untreated controls at the 180-minute time point. (B) Compound group 128 is selected, and (C) the corresponding EICs exhibit a time-dependent increase in abundance, consistent with the expected profile of a biotransformation product. Fold-change > 5, p-value < 0.01, Benjamini-Hochberg correction. (D) The 88/132 significant compounds that were "up" were exported as a precursor ion list to set up a Directed MS/MS method for further Q-TOF LC/MS data acquisition.

A narrow retention time window (0.01 minutes) was specified in the acquisition parameters to capture spectra near the apex of each chromatographic peak, thereby reducing interference from closely eluting isomers and enabling collection of relatively pure MS/MS spectra (Figure 6). The Directed MS/MS method was operated in Iterative mode, and two injections were needed to cover the compounds in the precursor ion list. The two MS/MS data files were added to the Explorer project, and the MS/MS spectra from these new files were extracted with the Find and Align step (not shown).

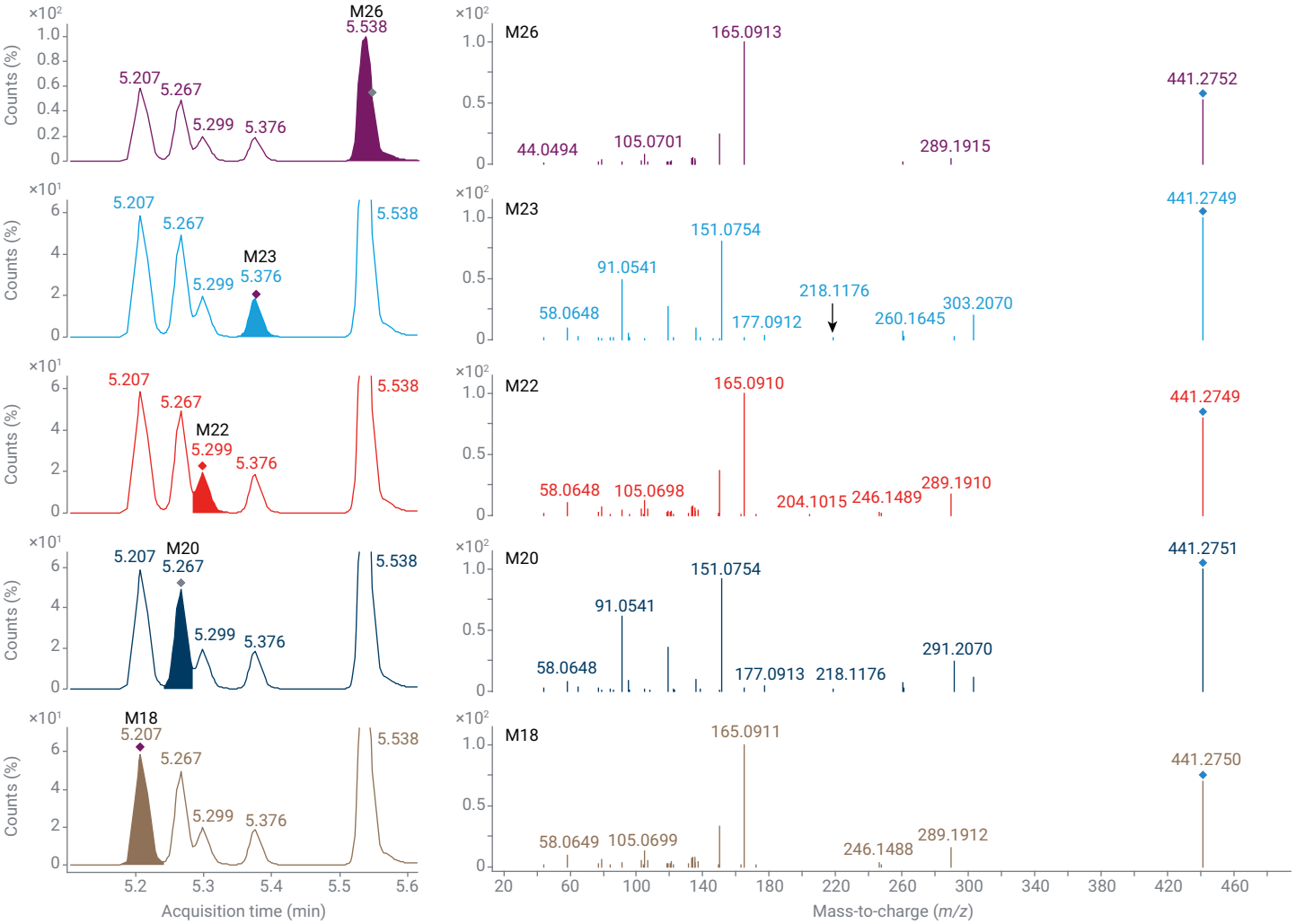



**Figure 6.** Directed MS/MS data acquisition of significant compounds. EICs for five isomers (M26, M23, M22, M20, and M18; *m/z* 441.2748) are shown on the left, with trigger points for each acquisition cycle indicated. On the right, the corresponding MS/MS spectra are displayed, averaged across three collision energies (20, 40, and 60 eV).

## Identification of verapamil metabolites

As verapamil is a well-characterized drug, its parent compound and several metabolites are likely present in established MS/MS libraries. MassHunter Explorer supports spectral library matching, and to demonstrate this capability, a library search was performed against the Agilent Applied Markets PCDL, which contains numerous known drug compounds and metabolites (Figure 7A). Verapamil and a few of its known metabolites, norverapamil, D-617, and D-620, were confidently identified using MS1 scoring and MS/MS reference library matching. Furthermore, these metabolites were included among the 88 significant compounds, reinforcing the effectiveness of the statistical filtering strategy for prioritizing compounds of interest.

However, in typical drug discovery pipelines, resulting novel drugs and their metabolites would not be found in public spectral libraries, necessitating an alternative identification approach. For this purpose, the 88 compounds and their extracted MS and MS/MS spectra were exported to SIRIUS software through the single-click option in MassHunter Explorer (Figure 7B). The extracted compound corresponding to verapamil was additionally exported and added to the same project within SIRIUS.

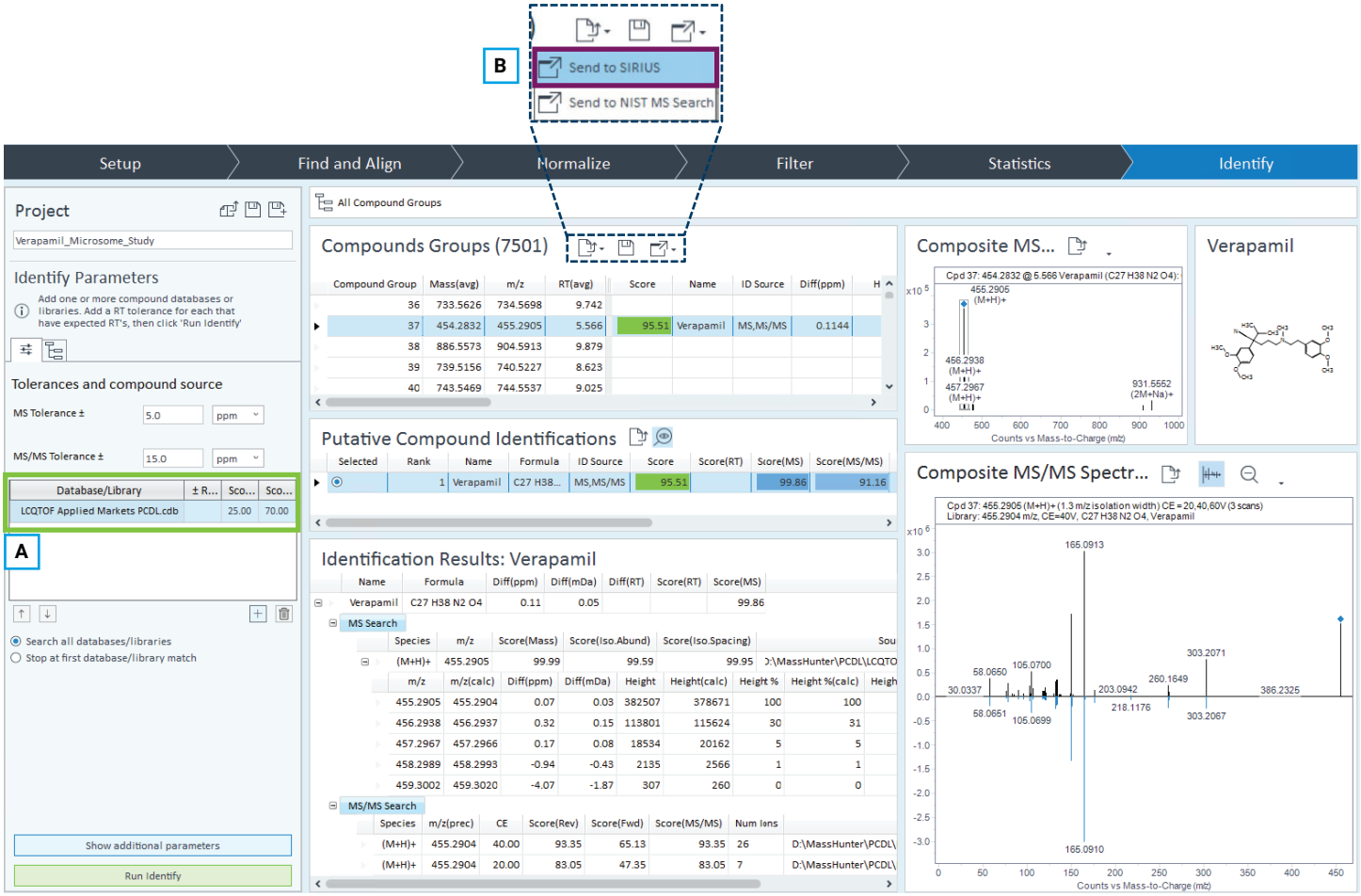



**Figure 7.** Agilent MassHunter Explorer software user interface displaying results from the "Identify" procedure. (A) A search of the Applied Markets PCDL (green box) was performed to identify compounds. Detailed results for compound 37 are shown with a single high-scoring library hit (Score = 95.51) for "Verapamil". (B) Single-click options for exporting compound information to SIRIUS and NIST MS Search (purple box) are shown.

SIRIUS with CSI:FingerID analyzes the MS/MS spectra of a compound to predict its molecular formula, which is then used to search for candidate structures within a molecular structure database. The software uses fragmentation trees, molecular fingerprinting, and machine learning to rank possible structures, enabling identification even when the compound is not present in spectral libraries.[3,4] Typically, large structure databases are searched, but even the largest database PubChem, with > 100 million structures, would most likely not contain a novel drug nor its metabolites. To address this challenge, SIRIUS provides the ability to create a custom database of structures and has integrated BioTransformer, a computational tool designed to predict the metabolism of small molecules.[5,6] BioTransformer uses both a knowledge-based approach and a machine learning-based approach to simulate how compounds are metabolized in various environments or how drugs are metabolized in the human body.

To create a custom database using the BioTransformer tool, users provide the molecular structure of the parent compound(s) as input—either as .sdf files or Simplified Molecular Input Line Entry System (SMILES) identifiers. Although MS/MS spectra are not required, including them adds value by enabling analog spectral library searches within SIRIUS. In most research settings, MS/MS spectra for the drug of interest can be readily acquired. In this study, we curated verapamil's MS/MS spectra using Agilent ChemVista, a library management software, and exported the compound information as an .sdf file. This file was then imported into a custom database creation tool within SIRIUS, where the BioTransformer 3 option was selected (Figure 8A). Given that liver microsomes primarily support Phase I (CYP450) activity and limited Phase II metabolism, both phases were included with multiple iterations. The computation yielded 1,946 predicted biotransformation products, representing 164 unique molecular formulas and highlighting a substantial number of structural isomers (Figure 8B).

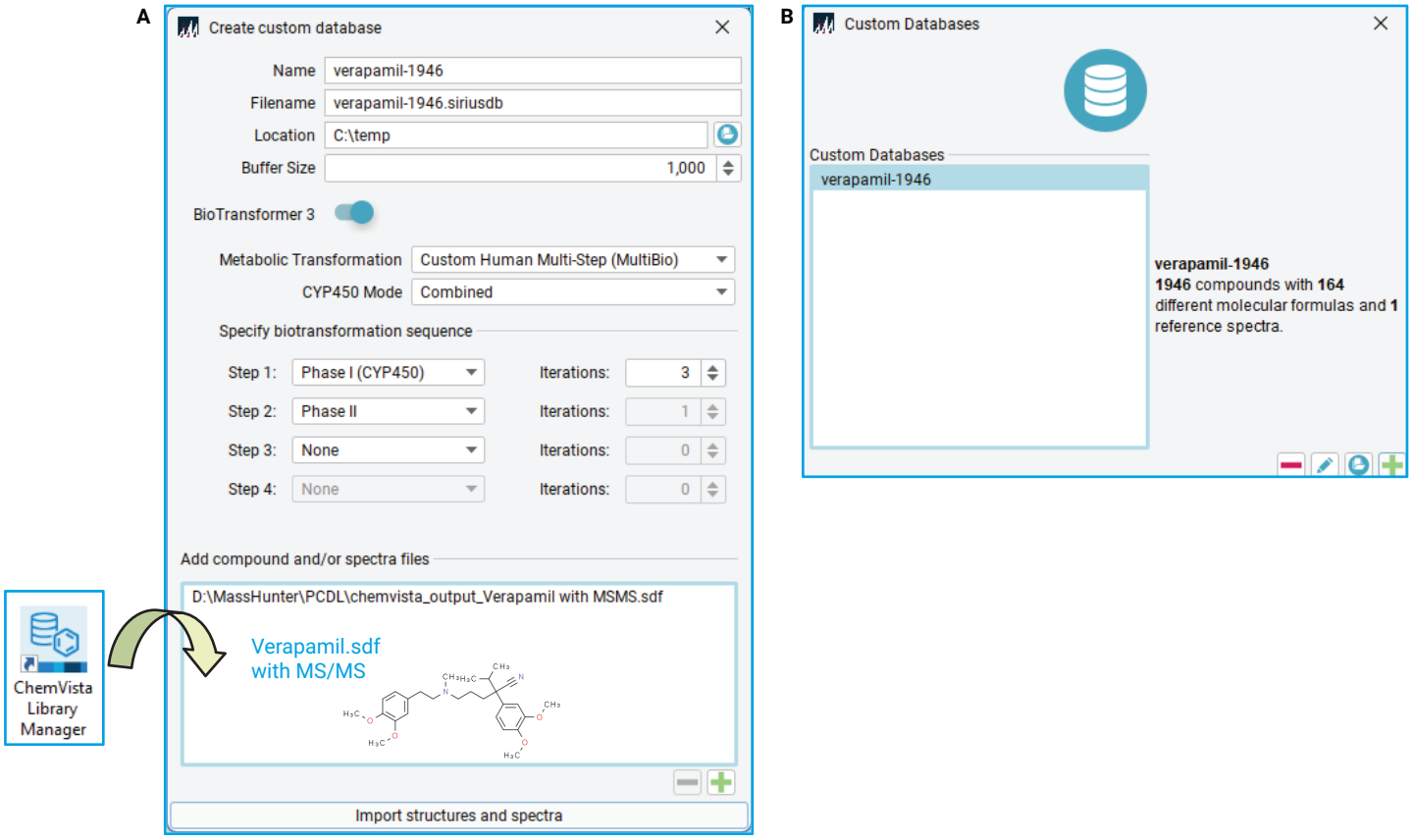



**Figure 8.** Creation of a custom verapamil biotransformation database within SIRIUS. (A) The verapamil.sdf containing MS/MS spectra was exported from Agilent ChemVista and dragged and dropped into the "Create custom database" dialog box. The BioTransformer 3 option was selected and the parameters chosen are shown. (B) Results from the custom database creation yielding 1,946 biotransformation products.

After creating the custom database, we selected computation parameters within SIRIUS. The "Database search" strategy was chosen to restrict both molecular formula identification (SIRIUS) and structure database searching (CSI:FingerID) to the custom databases only. Additionally, with ChemVista software, we created a small compound database of several known verapamil metabolites using structural information from the literature. This was used solely to map common verapamil metabolite names to their corresponding biotransformations for interpretive clarity and did not influence identification rankings.

Upon computation, 28 compounds representing 16 unique molecular structures—including verapamil—returned hits with a confidence score greater than 0.01. To evaluate how BioTransformer settings affected identification outcomes, we compared results across different combinations of Phase I and Phase II iterations (Table 3). The analysis revealed that three iterations of Phase I metabolism yielded the highest number of identifiable compounds, while Phase II iterations did not contribute additional identifications. Therefore, a custom database incorporating only three Phase I iterations was selected for all subsequent analyses. For our purposes, we refer to the 27 identified compounds as M1 through M27, ordered by retention time.

**Table 3.** Results of custom database creation with BioTransformer using different combinations of Phase I and Phase II iterations.

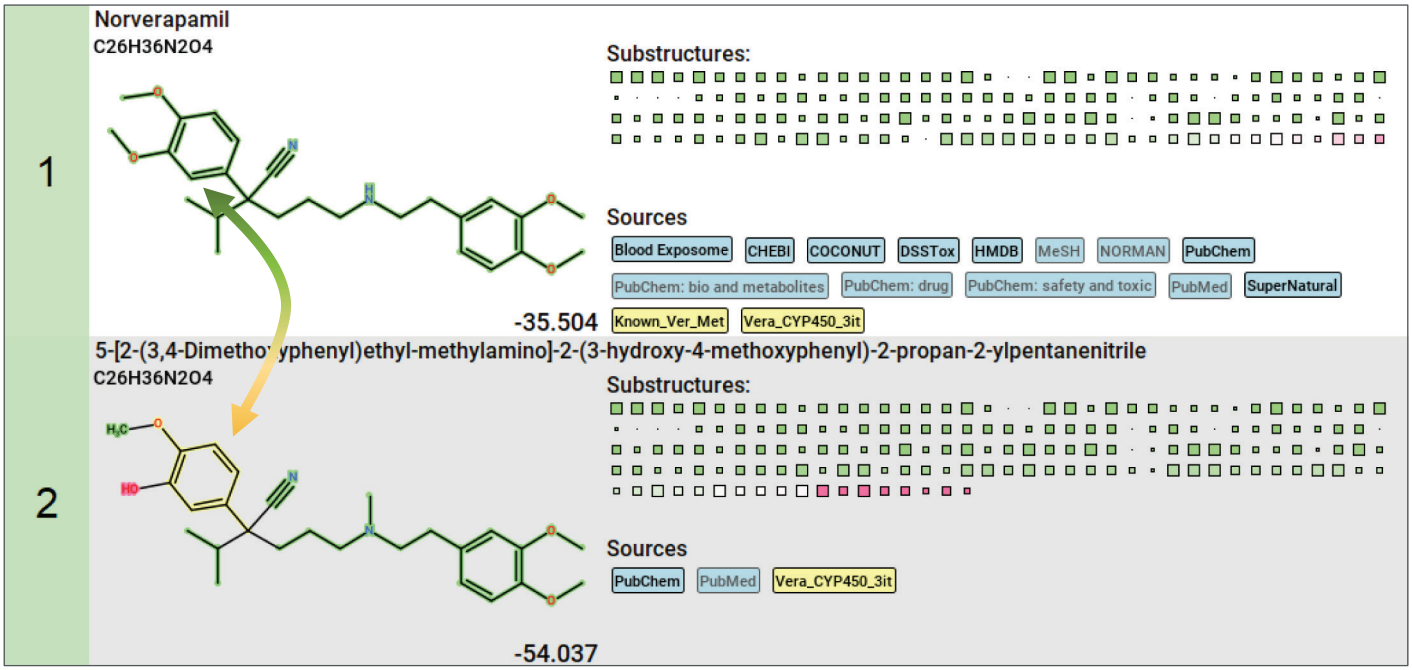
| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Number of Iterations | Phase I (CYP450) | 1 | 2 | 3 | 1 | 2 | 3 |
| | Phase II | 0 | 0 | 0 | 1 | 1 | 1 |
| Number of Biotransformations | | 17 | 117 | 496 | 43 | 377 | 1946 |
| Unique Formulas | | 8 | 26 | 56 | 15 | 66 | 164 |
| Number of Compound Hits in SIRIUS (confidence > 0.01)* | | 10 | 25 | 27 | 10 | 25 | 27 |

* The Metabolomics Transformation was set as "Custom Human Multi-Step (MultiBio)" and the CYP450 Mode was set as "Combined" for all analyses.

SIRIUS with CSI:FingerID results for compounds with *m/z* values corresponding to known verapamil metabolites were examined in detail. Compound 128 (M26, *m/z* 441.2750) was identified with norverapamil as the top-ranked structure (top hit). Among five database hits for this compound, norverapamil has the best CSI:FingerID score. Using the "highlight matching substructures" tool, we confirmed that the full structure of norverapamil aligns well with the molecular fingerprint, whereas the second-best hit shows weaker evidence in key structural regions (Figure 9A). To further support the identification, we compared the MS/MS spectra of compound 128 with reference spectra of verapamil using SIRIUS's analog spectral library search (Figure 9B). Compound 128 (M26) was one of five chromatographically resolved isobaric compounds, all assigned to the same molecular formula C26H36N2O4 by SIRIUS; however, only M26 is uniquely identified as norverapamil. To confirm this identification, we analyzed an authentic norverapamil standard using the same LC/MS method. The standard produced a single chromatographic peak matching the retention time of M26 (Figure 9C).

A

| Cpd Grp 128, M = 440.2676, Verapamil_Micr | |
|---|---|
| Det. Adduct | [M + H]+ |
| Precursor | 441.275 m/z |
| RT | 5.55 min |
| Confidence (A) | 0.350 |

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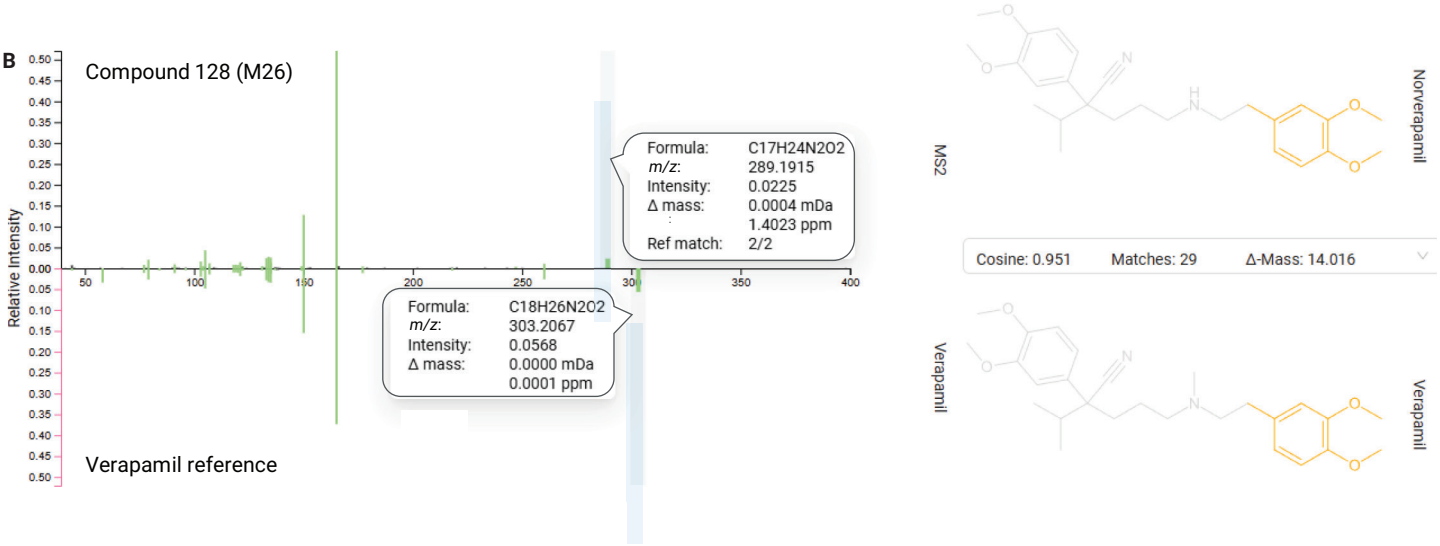

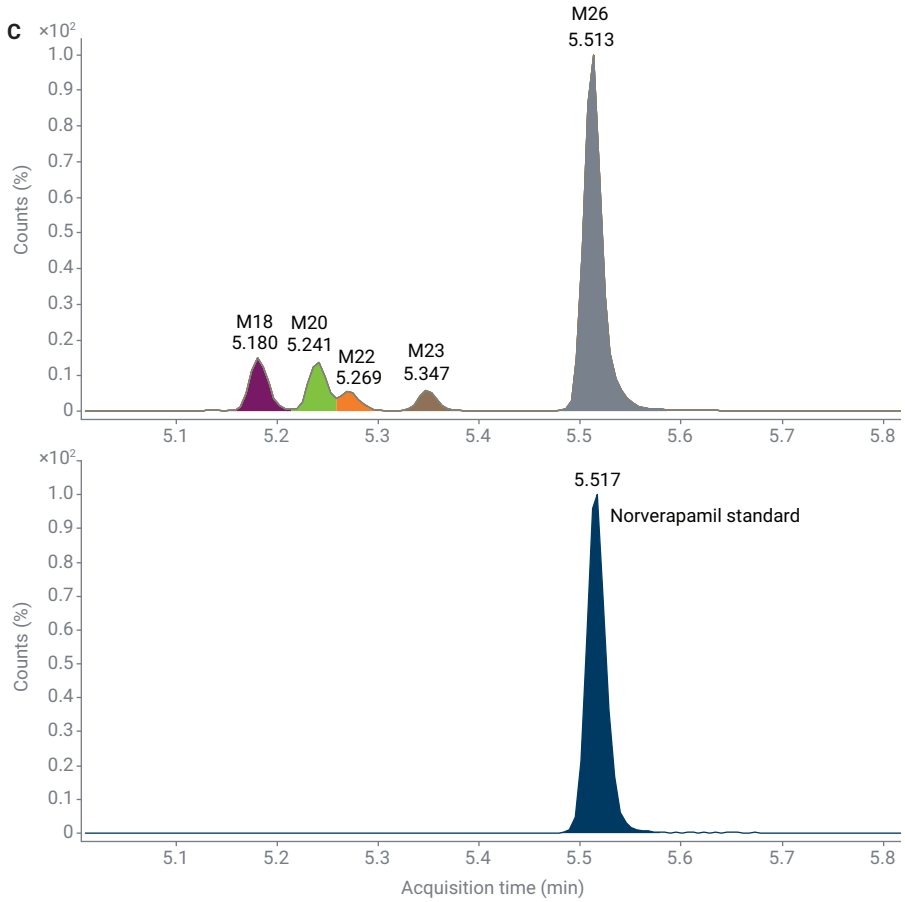


**Figure 9.** SIRIUS with CSI:FingerID results and further evidence for the identification of compound 128 (M26) as norverapamil. (A) SIRIUS with CSI:FingerID structure database search results from compound 128 (M26) showing norverapamil as the top hit. For comparison, the second hit is also shown. The arrow points to differences in the structural regions informed by the molecular fingerprints: green indicates strong support, while yellow and red reflect weaker or poor support. (B) Confirmation with spectral library analog search comparing norverapamil to verapamil. Peaks highlighted in green are explained by a substructure. Clicking on a peak reveals its corresponding matched peak in the mirror spectrum by grey background highlighting. For both spectra, the respective substructure is highlighted in the structure to the right. For an analog match, some peak matches will represent shared losses instead of shared fragments. Loss substructures are highlighted in yellow in the structure to the right. (C) LC/MS EICs (*m/z* 441.2750) of pure norverapamil standard (bottom) compared to isobaric metabolites M18, M20, M22, M23, and M26 (top).

SIRIUS with CSI:FingerID results were examined for the neighboring isomer M23 (compound 872). In this case, the known metabolite p-O-desmethylverapamil was identified as the top hit, with a significantly improved CSI:FingerID score compared to the fifth hit, norverapamil. A key structural difference between these molecules is the presence of a tertiary amine in p-O-desmethylverapamil versus a secondary amine in norverapamil. Support for the tertiary amine in p-O-desmethylverapamil with the molecular fingerprint is highlighted in SIRIUS (Figure 10).

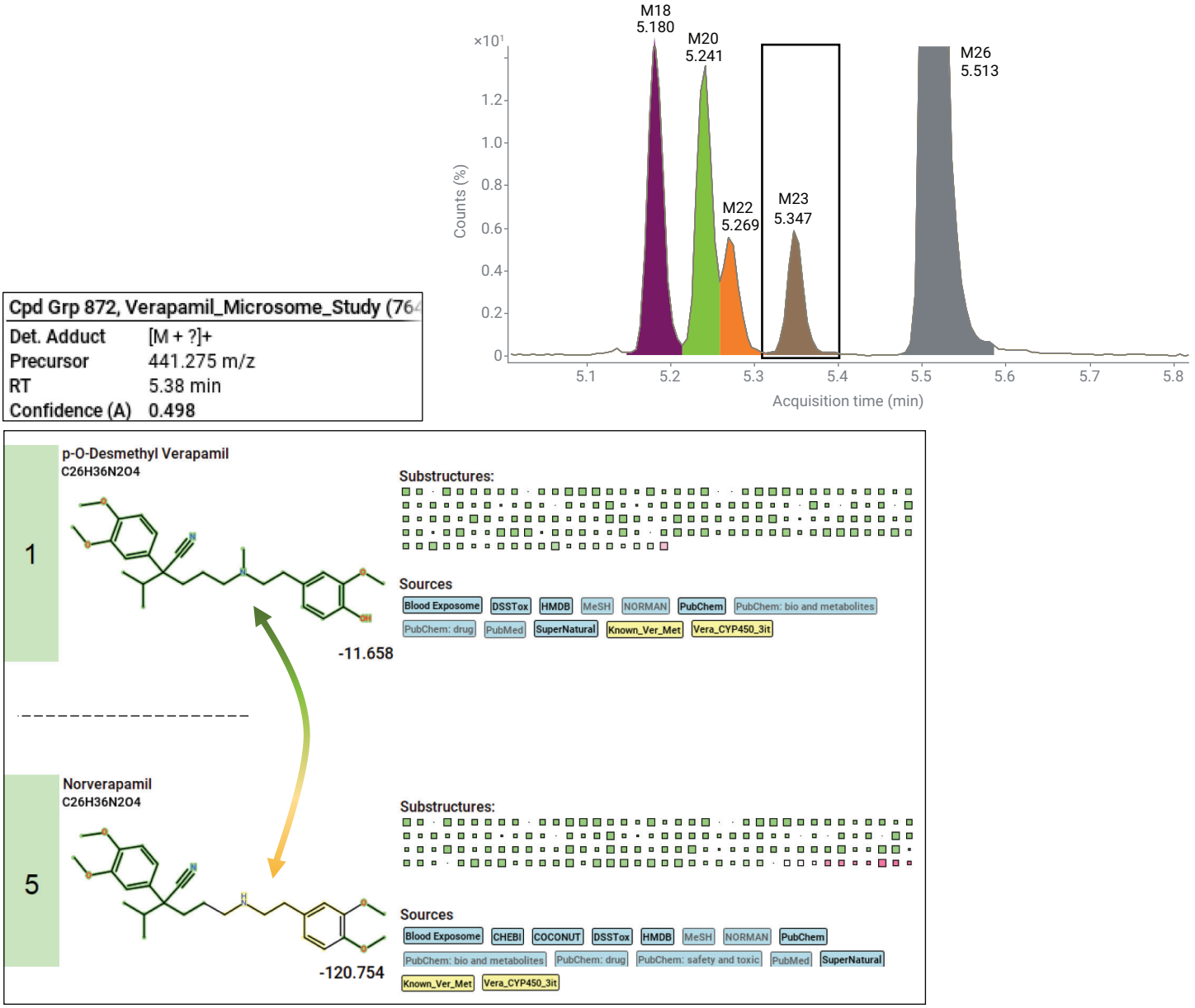


**Figure 10.** SIRIUS with CSI:FingerID structure database search results for compound 872 (M23) showing p-O-desmethyl verapamil as the top hit. For comparison, the fifth hit (norverapamil) is also shown. The arrow points to differences in the structural regions informed by the molecular fingerprints: green indicates strong support, while yellow and red reflect weaker or poor support.

Additional SIRIUS with CSI:FingerID results were reviewed for known verapamil metabolites. Two significant isobaric compounds—M4 (compound 2542) and M10 (compound 2362)—were chromatographically resolved and both empirically assigned the molecular formula $C_{16}H_{24}N_2O_2$ (Figure 11A). Notably, PR-25 and D-620 are the only well-characterized verapamil metabolites known to share this molecular formula, so it is fitting that the untargeted approach identified exactly two compounds with this formula.

SIRIUS with CSI:FingerID identified PR-25 as the top hit for M4 (Figure 11B) and D-620 for M10 (Figure 11C), each with a substantially higher CSI:FingerID score than alternative candidates, supporting confident identification.

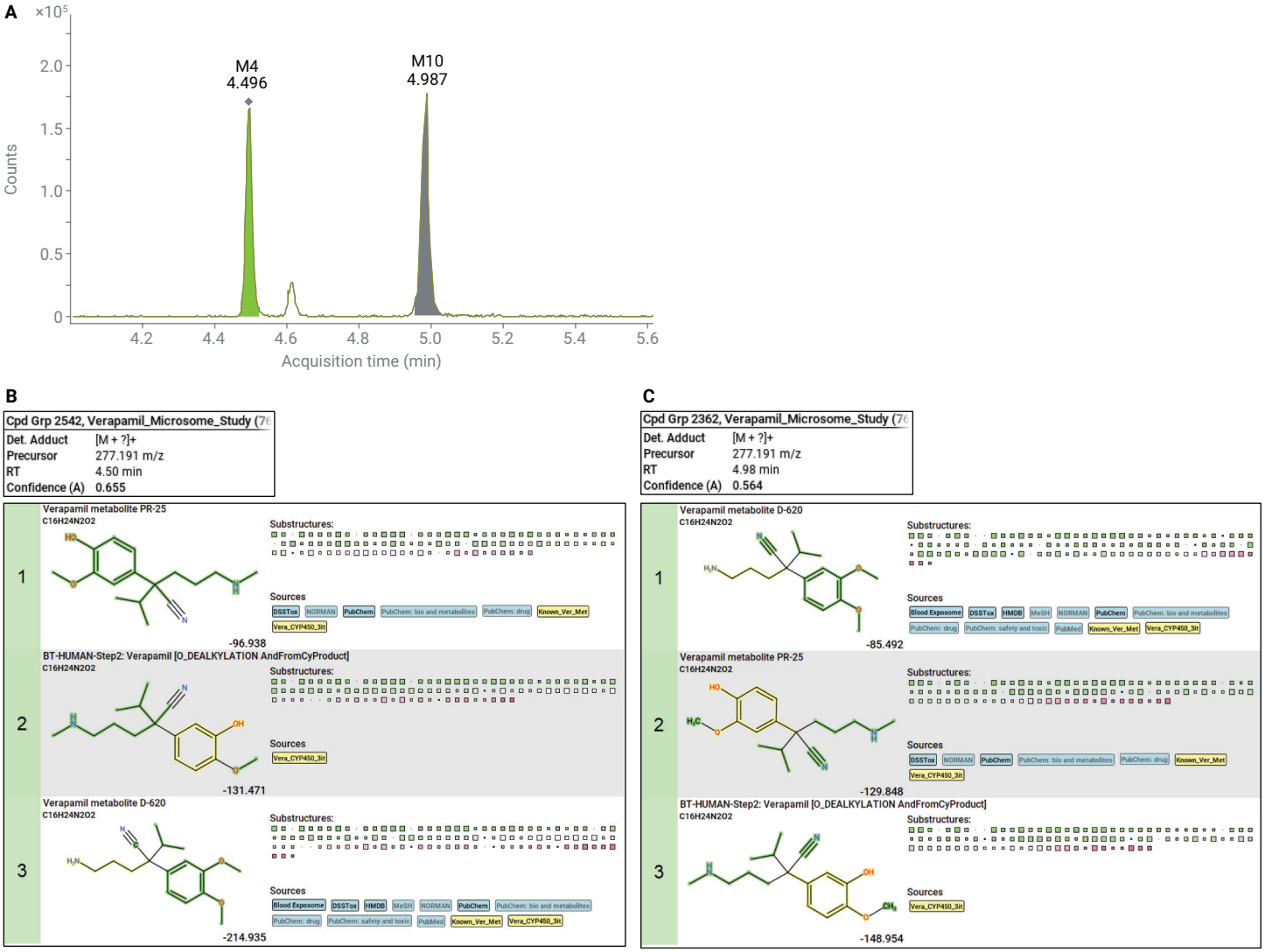


**Figure 11.** SIRIUS with CSI:FingerID identification results for metabolites M4 and M10. (A) LC/MS EICs (*m/z* 277.1910) of isobaric metabolites M4 and M10. (B) SIRIUS with CSI:FingerID structure database search results for compound 2542 (M4) showing verapamil metabolite PR-25 as the top hit. For comparison, hits 2 and 3 are shown. (C) SIRIUS with CSI:FingerID structure database search results for compound 2362 (M10) showing verapamil metabolite D-620 as the top hit. For comparison, hits 2 and 3 are shown.

In some cases, users may wish to incorporate SIRIUS with CSI:FingerID identifications into the MassHunter Explorer project to facilitate downstream analysis. This integration enables easier review of compound behavior in statistical tests and visualization of abundance changes across time points and treatment conditions. Figure 12 outlines a streamlined workflow for this process. Key features include SIRIUS's ability to export a ChemVista-compatible summary of top-ranked identifications and MassHunter Explorer support for compound matching based on accurate mass and retention time, ensuring precise alignment between structural identifications and extracted compounds. Using this approach, all 27 compounds identified by SIRIUS with CSI:FingerID were successfully mapped back to their corresponding compounds in MassHunter Explorer.

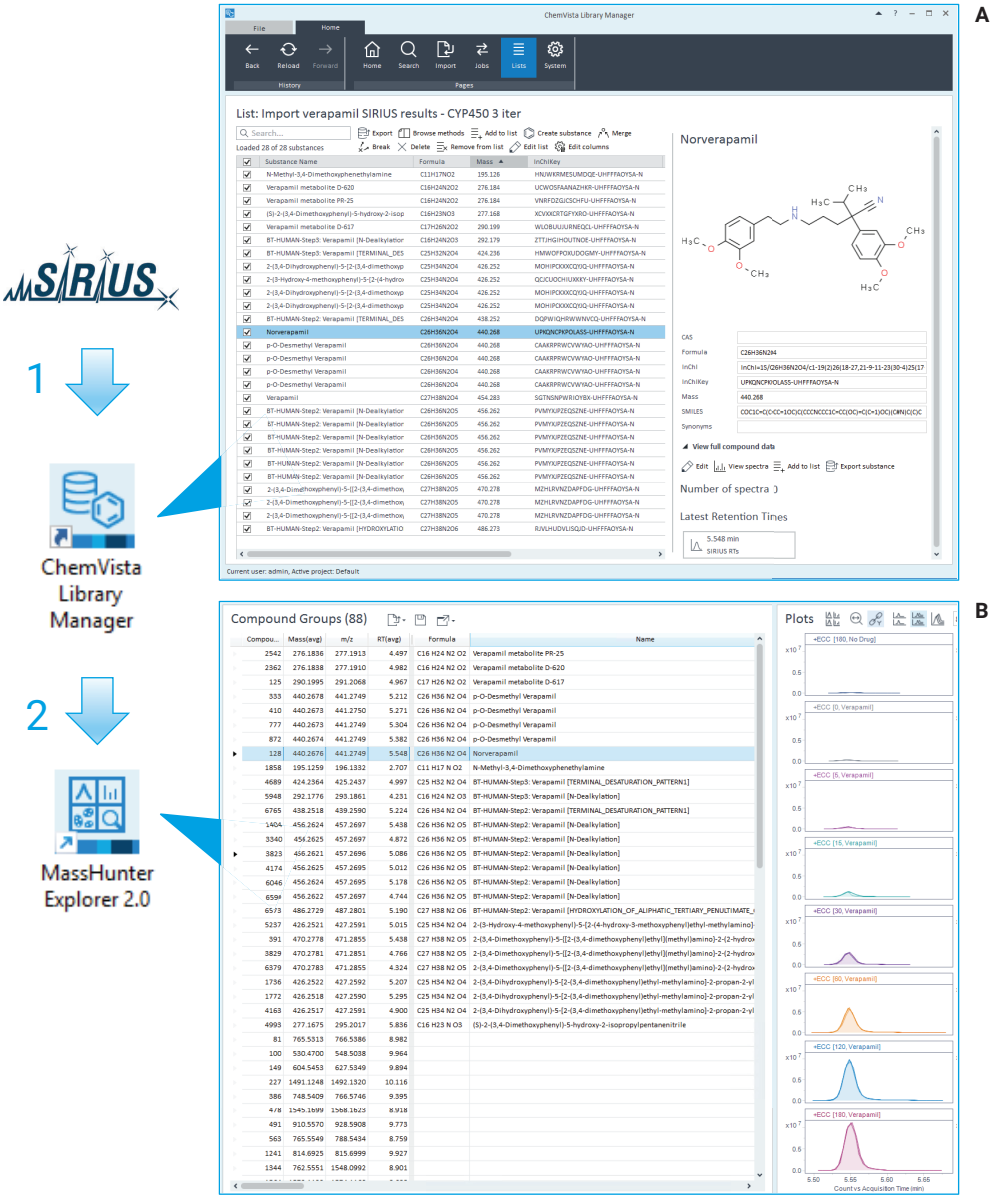


**Figure 12.** Workflow for mapping SIRIUS with CSI:FingerID identifications to Agilent MassHunter Explorer project. In step 1, the top hits for structure identifications and observed retention times (RTs) are exported from SIRIUS and imported into Agilent ChemVista library management software. (A) The 27 identified metabolites imported and the parent drug verapamil in ChemVista are shown. The metabolite norverapamil is selected, and its compound details are shown. In step 2, the compound database is exported from ChemVista and leveraged in MassHunter Explorer software to match compounds based on both accurate mass and RT. (B) The 27 compounds identified in the list of 88 significant compounds in MassHunter Explorer are shown, and compound norverapamil is highlighted. EIC plots across the time series are displayed.

To illustrate time-dependent trends, we exported selected verapamil metabolite abundances from MassHunter Explorer and plotted them on the established verapamil metabolic pathway (Figure 13). Comparing their metabolite formation profiles strengthened confidence in the identifications predicted by SIRIUS. Notably, metabolites D-620 and PR-25, predicted secondary biotransformation products, show a delayed rise in abundance relative to their predicted precursors, norverapamil and D-617 (primary biotransformation products).

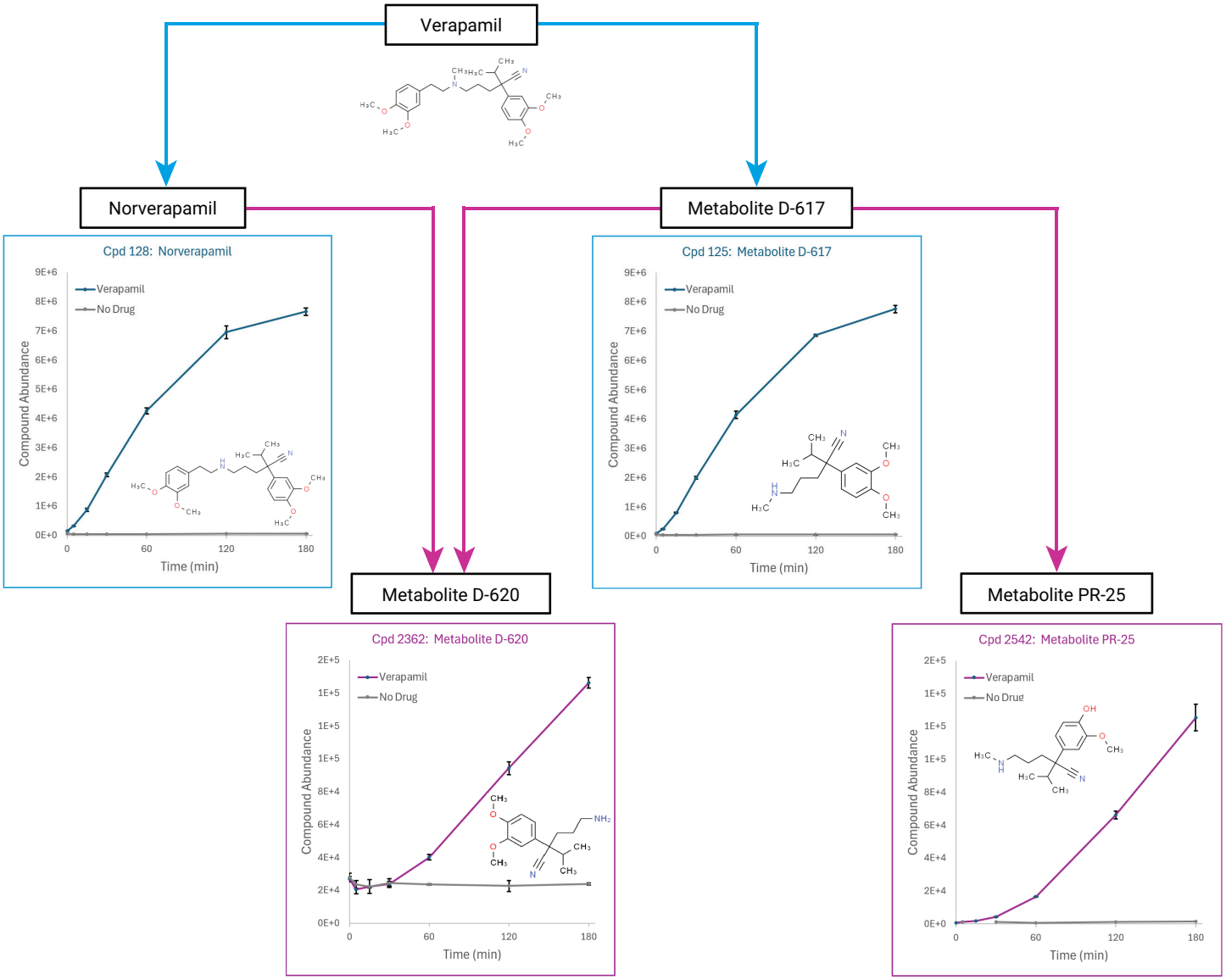


**Figure 13.** Verapamil metabolism pathway (partial) with observed metabolite formation profiles in human liver microsomes. Selected compound abundance values from Agilent MassHunter Explorer in control (No Drug) and verapamil-treated microsomes were plotted against treatment time. Blue arrows/boxes = 1 iteration Phase I metabolism, purple arrows/boxes = 2 iterations Phase I metabolism. Error bars indicate ± 1 standard deviation.

## Conclusion

This application note demonstrates a streamlined workflow for drug metabolite identification, integrating the Agilent Revident Q-TOF LC/MS system with Agilent MassHunter Explorer 2.0 and SIRIUS software. Using verapamil as a model compound, the workflow successfully identified both known and novel biotransformation products through a combination of chemometric filtering, MS/MS acquisition, and molecular fingerprint-based structure elucidation.

Key innovations include the use of the Agilent 1260 Infinity III hybrid multisampler with Feed Injection, which enabled direct injection of minimally prepared microsomal supernatants while preserving chromatographic integrity and peak shape—critical for accurate feature extraction and quantification. The differential analysis strategy in MassHunter Explorer 2.0 effectively prioritized drug-related features from complex datasets, while the coordination with SIRIUS software and its integration of a custom BioTransformer-generated database allowed confident identification of metabolites not present in traditional spectral libraries.

Although verapamil is a well-characterized drug, the workflow was designed with flexibility to accommodate novel compounds, making it particularly valuable in early-stage drug discovery where reference spectra are unavailable. The approach is additionally applicable to other xenobiotics and other types of transformations, offering a powerful solution for metabolite identification in broad research and development settings.

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RA260121.603





Printed in the USA, February 2, 2026
5994-8867EN


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