Application Note #LC-MS 80

Simultaneous Quantitative and Qualitative Measurements in a Single Workflow to Increase Productivity in Primary Drug Metabolism Investigations

At-a-Glance

- Increased productivity in drug metabolism studies via simultaneous quantitative and qualitative measurements from a single workflow
- Easily obtained accurate mass drug profile and clearance data comparable to triple quad
- SmartFormula 3D and Metabolite Detection software allows metabolites to be rapidly identified and profiled
- Accurate mass, auto MSMS data from the same data set used to elucidate the structure of identified metabolites
- Metabolite identification, profiling, and structure elucidation without the need for repeat analyses

Bruker’s impact™ Quadrupole-Time-of-Flight mass spectrometer technology combines high mass accuracy with sensitivity that approaches a triple quadrupole MS, enabling accurate quantitation at 1µM levels with linearity over 3 decades of concentration. Complemented by Compass and SmartFormula 3D software, the impact uniquely combines high mass accuracy at fast scan speeds with high sensitivity quantitation to enable a simultaneous quantitative/qualitative workflow for metabolism studies.

The ability to simultaneously collect quantitative and qualitative information from a DMPK analysis has the potential to significantly increase productivity in pharmaceutical drug discovery and development. Due to limitations in traditional LC-MS technology, quantitative and qualitative information is traditionally collected from 2 parallel sample lines at differing concentrations, and often on separate LC/MS/MS instruments. Most current LC/MS/MS systems that have the high mass accuracy required for metabolite identification lack the sensitivity and linear range to perform quantitative bioanalysis at therapeutic dosing levels.

This work describes an investigation using the impact Quadrupole-Time-of-Flight mass spectrometer to obtain clearance data, metabolite identification, structure elucidation and metabolite profiles from P450 microsomal incubations at a drug concentration of 1 µM from a single sample set.

P450 microsomal incubations of commercially available drug substances including Pindolol, Verapamil, Haloperidol, Dichlofenac, Dapsone and Fluconazole were prepared at 1 µM concentration. The incubations were sampled and quenched at intervals to provide a time course over a period of 60 minutes. The analysis of the samples was carried out by LC-MS using an impact Quadrupole-Time-of-Flight mass spectrometer to obtain data suitable for measuring clearance and plotting metabolic profiles. Data dependent MSMS spectra were collected in order to identify and elucidate the structures of the observed metabolites. The clearance results are compared to those obtained using a triple quadrupole mass spectrometer (ABSciex, API 5000™) to analyse the same sample set.

Verapamil  $C_{27}H_{38}N_2O_4$  $M+H^+ = 455.2904$
**Workflow**

In a single workflow, data dependent MSMS spectra identify and elucidate metabolite structures and drug clearance is measured.

First the metabolism profile of the drug is determined using the theoretical m/z of the drug ion and internal standard to create high resolution mass chromatograms. In the case of Verapamil this would be 455.2904 +/- 0.005. The drug profile is plotted and the pseudo first order rate constant determined. From this the half life and clearance value are calculated.

Metabolite detection is carried out using Bruker Metabolite Detect software to produce BPI chromatograms of the difference between a selected time point and the respective control sample. The measured m/z values for the metabolites are obtained from their spectra. At this point Smartformula may be used to predict their formulae.

The measured m/z can then be used to create high resolution mass chromatograms for the detected metabolites. This allows profiling to be done without metabolite identification (even formulae) or structure elucidation.

Finally the metabolite formula determination and structure elucidation can be carried out on the metabolites of interest using the accurate mass msms data and Smartformula3D.

In some cases when the drug is not cleared by the P450 enzymes, there may be no interest in the metabolites. The workflow is flexible, allowing only the steps of interest to be followed, creating greater efficiency and avoiding the creation of redundant information. The full data set along with its processing is saved for further examination in the future should the need arise.

**Results**

Feasibility of the workflow was confirmed by experiments to measure Verapamil clearance and half life, identify metabolites, determine linear dynamic range for quantitation, profiling metabolites, and comparing clearance results from the impact system to results from a triple quadrupole LC/MS/MS. All results demonstrate that the Bruker impact enables accurate quantitative and qualitative analysis in a single workflow.
Protocol
1. 50 μl sampled at each time point
2. Quenched by adding to 550 μl CH₃CN containing IS (30 nM)
3. Samples left on ice for 1 hour
4. Centrifuged at 2000g for 10 min @ 4°C
5. Remove 200 μl supernatent
6. Reduce to dryness under N₂ @ 37°C
7. Stored at 4°C
8. Reconstituted with 20 μl CH₃CN and 80 μl H₂O

Incubation
Drug Concentration: 1 μM
Protein Concentration: 0.5 mg / ml
NADPH Concentration: 2 mM
MgCl₂ Concentration: 1 mM
ICA Isocitric dehydrogenase 1 unit / ml
Total volume 600 μl
Sampled at 3, 7, 13, 20, 28, 38, 50, 60 mins
NCF preparation and sampled at 0, 60 mins

Chromatography
Column: Kinetex, 2.6 μm, C18, 2.10 x 100mm
Column Temp: 30°C
MPA: 0.1% Formic Acid in H₂O
MPB: 0.1% Formic Acid in CH₃CN
Gradient: 0.0 0.5 10.0 12.5 15.0 min
MP %: 95 95 5 5 95 95 %
Flow Rate: 300 μl/min
Injection vol: 5 μl
On column: 0.8 pmoles drug and 0.3 pmoles IS = 72 pg

Metabolite Detection
Metabolite detection software compares the data file for the drug (in this case t₆₀) with the corresponding control sample. A base peak chromatogram of the difference is created allowing the metabolites to be easily observed and their mass determined to 4 decimal places.

Comparision of drug with control sample

Metabolite detection software compares the data file for the drug (in this case t₆₀) with the corresponding control sample. A base peak chromatogram of the difference is created allowing the metabolites to be easily observed and their mass determined to 4 decimal places.

EIC chromatogram
XIC Chromatograms +/- 0.005 Da for Verapamil and the observed metabolites at t₂₈, (1)Internal standard, (2)Metabolite 277, (3) Metabolite 291, (4) Metabolite 441, (5) Verapamil
Metabolite Profiles

Using the accurate mass measurement, integration is carried out on the XIC for the measured m/z of each metabolite +/- 0.005 Da. Plotting the ratio of metabolite to internal standard (M/IS) with respect to time produces the metabolite profiles. The rapid decay of Verapamil is observed in the profile above along with the rapid increase of metabolite 291 (referring to its observed nominal mass) which remains at a constant concentration. The concentration of metabolite 441 increases, reaching a maximum at about 15 minutes and then decreases. This suggests that 441 may itself be metabolised by P450. The concentration of metabolite 277 increases slowly but steadily throughout the incubation.

SmartFormula combines accurate mass information and True Isotopic Pattern data to identify a compound’s molecular formula. SmartFormula is more powerful and accurate than simple algorithms that just rely on exact mass and isotopic pattern of the precursor ion and fragment ions to determine molecular formula. SmartFormula 3D extends the capabilities of the basic SmartFormula package by incorporating the isotopic pattern from fragment ions into molecular formula determinations. This additional information results in a dramatic reduction in potential candidate molecular formulae and often produces only a single molecular identification – the correct choice.

Linear calibration of 50 pg/ml to 50 ng/ml (3 decades) was achieved using the XIC for the measured m/z of each metabolite +/- 0.005 Da. R² = 0.9987.

SmartFormula provides 4 predictions for the m/z = 441 metabolite formula with a clear favourite but cannot identify the position of demethylation.

SmartFormula3D predicts a single (correct) formula for m/z = 441 and rapidly enables the identification of the site of demethylation.

Metabolite ID

SmartFormula: predicting potential metabolities

SmartFormula: identifying the correct metabolite
Verapamil Profile and Clearance (t1/2)

Integration is carried out on the XIC for the theoretical m/z of the Drug and internal standard +/- 0.005 Da. Plotting the ratio of drug to internal standard (V/IS) with respect to time produces the clearance profile.

The half-life of the drug under these incubation conditions and the clearance value is determined from the $\ln V/IS$ versus time plot assuming pseudo first order kinetics.

$t_{1/2} = 0.693/k$
$t_{1/2} = 0.693/0.0771 = 9.0\text{ min}$

Clearance rate = $k$/enzyme concentration.

Clearance = 0.0771/0.5 ml/min/mg, or 0.0771/0.0005 ul/min/mg = 154 ul/min/mg
Comparison of Clearance data from 3Q and impact

The linearity and gradients of the ln V/IS vs time plots are nearly identical and result in identical values for $t_{1/2}$. The difference in y intercept is due to a difference in relative response of the internal standard and has no influence on the clearance results.

Clearance comparison ($t_{1/2}$ minutes)

<table>
<thead>
<tr>
<th>Compound</th>
<th>3Q</th>
<th>impact</th>
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</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Dichlofenac</td>
<td>15</td>
<td>16</td>
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<tr>
<td>Haloperidol</td>
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<td>47</td>
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<tr>
<td>Pindolol</td>
<td>540</td>
<td>770</td>
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<tr>
<td>Dapsone</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>NC</td>
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</tbody>
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Conclusions

- The high sensitivity and linear range of the Bruker impact enables simultaneous quantitative and qualitative measurements from a single workflow to increase productivity in drug metabolism studies.
- Comparable drug profile and clearance data can be obtained using impact and a 3Q (API5000) mass spectrometers at 1 μm drug concentrations.
- SmartFormula 3D metabolite detection software allows metabolites to be rapidly identified and profiled without prior knowledge.
- Accurate mass, auto MSMS data from the same data set can be used to elucidate the structure of the identified metabolites.
- The Quan–Qual workflow allows metabolite identification, profiling, and structure elucidation to be carried out without the need for repeat analyses or further incubations at higher concentration.

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Keywords

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Quantitation

Instrumentation & Software

impact
SmartFormula 3D

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