Application Note

Small Molecule Pharmaceuticals

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Benefits of 2D-LC/MS/MS in Pharmaceutical Bioanalytics

Avoiding Matrix Effects—Increasing Detection Sensitivity

Suitable for Agilent
1290 Infinity III LC

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Abstract

This Application Note demonstrates the use of the Agilent InfinityLab 2D-LC Solution coupled with the Agilent 6495 Triple Quadrupole LC/MS for the analysis of drugs and their metabolites in pharmacokinetic studies. 2D-LC can eliminate coelution, thereby reducing signal suppression and the risks of cross-talk, which can occur in one-dimensional analysis of these complex samples. The second dimension of a 2D-LC/MS/MS analytical method can be used to enhance the signal intensity of mass spectrometric detection by introducing the analytes to the MS source in a more suitable eluent.

Introduction

In the past decade, LC/MS/MS has become a well established technology for the analysis of drugs and their metabolites in pharmacokinetic studies. In addition to the clear benefits of this technology, such as high selectivity and analytical sensitivity, peak assignment, or molecular weight information, there are some well-known challenges, for example when analyzing biological samples with complex matrices, such as plasma or cell-culture media. Some of the challenges are, cross-talk due to coelution of isobars or in-source fragmenting metabolites as well as signal suppression due to coeluting matrix components. Signal suppression might be especially problematic in the quantification of analytes with very low concentrations, and can result in insufficient quantitation limits.

This Application Note details two examples of how the Agilent InfinityLab 2D-LC Solution coupled with the Agilent 6495 Triple Quadrupole LC/MS can be used in a bio-analytical research environment to overcome the previously mentioned challenges:

- How two-dimensional separation can eliminate coelution and the risks of cross-talk or signal suppression when dealing with complex analyte mixtures.
- How a second dimension can be used to enhance the signal intensity of mass spectrometric detection by changing the eluent conditions, thereby introducing the analytes in a more suitable eluent to the MS source, improving quantitation limits significantly.

Experimental

Equipment

The Agilent 1290 Infinity II 2D-LC System comprised the following modules:

- Two Agilent 1290 Infinity II High Speed Pumps (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler (option #100)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/4-port-duo valve (2D-LC valve 1300 bar: part number 5067-4244)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with Multiple heart-cutting valves (G4242-64000) equipped with 40 µL loops
- Mass spectrometric (MS) detection was performed using an Agilent 6495 Triple Quadrupole LC/MS equipped with an Agilent Jet Stream ESI source (G1958-65538).

Software

- Agilent OpenLAB CDS ChemStation Edition Rev. C.01.07 SR2 [263] with 2D-LC Software version A.01.03 [025].
- Agilent MassHunter Workstation Software LC/MS Data Acquisition, Version B.08.00, Build 8.0.8023.5 SP1
- Agilent MassHunter Workstation Software Quantitative Analysis, Version B.07.01, Build 7.1.524.0

Chemicals

LC/MS grade acetonitrile and methanol were purchased from TH Geyer (Renningen, Germany). Formic acid and ammonium acetate for LC/MS analysis were obtained from Sigma-Aldrich (Steinheim, Germany).

Fresh ultrapure water was obtained from an ELGA Pureflex 2 water purification system equipped with a 0.2-µm membrane point-of-use cartridge (Veolia Water Technologies Deutschland GmbH, Celle, Germany).

Samples and Methods

2D-LC as a Tool to Avoid Signal Suppression and Cross-Talk

An *in vitro* cell suspension-based assay was performed to identify the key enzymes responsible for metabolism of a drug candidate. The drug candidate (a small molecule) and multiple enzyme-specific probe-substrates with corresponding enzyme inhibitors were incubated in a cell suspension. After a defined time period, the samples were collected, and the formation of metabolites was analyzed. To avoid signal suppression or cross-talk, the high-abundant substrates and inhibitors were chromatographically separated. The injected solution contained a mixture of the cell supernatant and a solution of stable-isotope-labeled internal standard. See Table 1 for method parameters.

2D-LC to Increase MS Detection Sensitivity by Solvent Switching

An *in vitro* cell culture-based assay was performed to evaluate the concentration of parent compound (drug candidate) and its three major metabolites in culture medium and cells. After a predefined incubation time, samples were collected and, before injection, a non-stable-isotope-labeled internal standard was added. See Table 2 for method parameters.

Table 1. Method parameters for 2D-LC to avoid signal suppression.

One-dimensional analysis and first dimension of 2D-LC analysis	
Column	C18 column, 2.1 × 150 mm, sub-2 µm
Solvent	A) Water + 0.1 % formic acid B) Methanol
Gradient	0 minutes – 15 %B 2 minutes – 30 %B 20 minutes – 70 %B 23 minutes – 95 %B 27 minutes – 95 %B 27.5 minutes – 15 %B
Flow rate	0.300 mL/min
Temperature	40 °C
Detection	^{1}D Analysis: Agilent 6495 Triple Quadrupole LC/MS 2D-LC Analysis: no detection after ^{1}D separation
Injection	Injection volume: 2 µL Sample temperature: 10 °C Needle wash: 3 seconds in water/methanol (50/50, v/v)
Second dimension	
Column	PFP column, 2.1 × 50 mm, sub-2 µm
Solvent	A) Water + 0.2 % formic acid B) Methanol + 0.2 % formic acid
Gradient	0.00 minutes – 40 %B 0.70 minutes – 90 %B ^{2}D gradient stop time: 0.90 minutes ^{2}D cycle time: 1.35 minutes
Flow rate	0.600 mL/min
Temperature	40 °C
Detection	Agilent 6495 Triple Quadrupole LC/MS
2D-LC	
2D-LC mode	Heart-cutting Time-based multiple heart-cutting was performed with the heart-cuts (sampling table) set according to the first-dimension retention times.
Agilent 6495 Triple Quadrupole LC/MS parameters	
	Polarity: Positive and negative Scan type: Scheduled MRM Drying gas temperature: 140 °C Drying gas flow: 15 L/min Nebulizer pressure: 40 psig Sheath gas temperature: 385 °C Sheath gas flow: 12 L/min Source voltages and iFunnel RF were optimized individually for each compound using Agilent MassHunter Source Optimizer.

Table 2. Method parameters for 2D-LC for increased MS detection sensitivity.

One-dimensional analysis	
Column	Hybrid silica C18 column, 2.1 × 100 mm, sub-2 µm
Solvent	A) 0.1 % Formic acid in water B) 0.1 % Formic acid in acetonitrile
Gradient	0.0 minutes – 5 %B 0.5 minutes – 5 %B 4.5 minutes – 40 %B 6.5 minutes – 90 %B Stop time: 8.0 minutes Post-time: 1.5 minutes
Flow rate	0.500 mL/min
Temperature	40 °C
Detection	Agilent 6495 Triple Quadrupole LC/MS
Injection	Injection volume: 5 µL Sample temperature: 10 °C Needle wash: 10 seconds in water/methanol (50/50, v/v)
First dimension of 2D-LC analysis	
Column	Hybrid silica C18 column, 2.1 × 50 mm, sub-2 µm
Solvent	A) 0.1 % Formic acid in water B) 0.1 % Formic acid in acetonitrile
Gradient	0.00 min – 15 %B 1.50 min – 40 %B 2.10 min – 90 %B 2.50 min – 90 %B 2.53 min – 15 %B
Flow rate	0.900 mL/min
Temperature	40 °C
Detection	No detection after ^{1}D separation
Injection	Injection volume: 1 µL Sample temperature: 10 °C Needle wash: 10 seconds in water/methanol (50/50, v/v)
Second dimension	
Column	Non-endcapped silica C18 column, 2.1 × 50 mm, sub-2 µm
Solvent	A) 5 mM ammonium acetate and 5 % methanol in water B) Methanol
Gradient	0.00 minutes – 40 %B 0.50 minutes – 95 %B ^{2}D gradient stop time: 0.60 minutes ^{2}D cycle time: 1.00 minute
Flow rate	0.600 mL/min
Temperature	40 °C
Detection	Agilent 6495 Triple Quadrupole LC/MS
2D-LC	
2D-LC mode	Heart-cutting Time-based multiple heart-cutting was performed with the heart-cuts (sampling table) set according to the first-dimension retention times. The smart peak parking functionality was used.
Agilent 6495 Triple Quadrupole LC/MS	
MS Parameters	Polarity: positive Scan type: MRM Drying gas temperature: 200 °C Drying gas flow: 14 L/min Nebulizer pressure: 20 psig Sheath gas temperature: 400 °C Sheath gas flow: 11 L/min Capillary voltage: 3,500 V Nozzle voltage: 0 V High-pressure RF: 90 V Low-pressure RF: 60 V

Results and Discussion

2D-LC as a Tool to Avoid Signal Suppression and Cross-Talk

Cell supernatants from *in vitro* cell suspension-based assays containing a range of metabolites in the presence of enzyme substrates and inhibitors present complex mixtures. Figure 1 shows the LC/MS/MS analysis of a cell supernatant. The highlighted metabolite M1 coelutes with higher concentrated substrates and inhibitors. In this LC/MS/MS analysis, signal suppression of metabolite M1 due to coelution with higher abundant substrates and inhibitors could occur. For structurally related compounds, cross-talk might also be observed.

To avoid signal suppression and cross-talk, a chromatographic method that allows resolution of metabolites from substrates and inhibitors is required for the analysis of the cell supernatant.

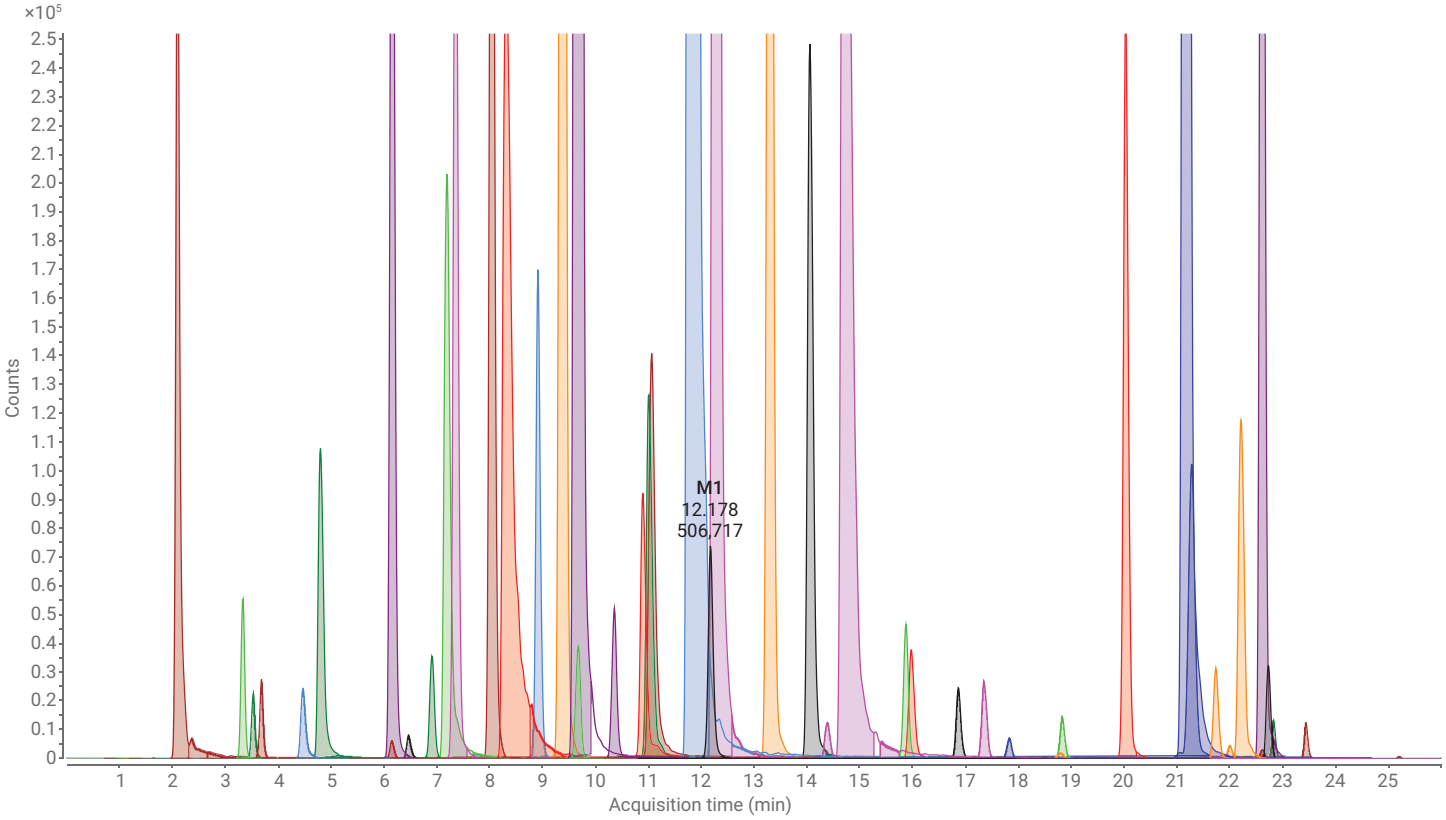


Figure 1. LC/MS/MS analysis of a cell supernatant.

To enable the separation of the metabolite M1 from coeluting substrates and inhibitors, multiple heart-cutting (MHC) 2D-LC was used. Metabolite M1 was heart-cut in time-based mode and transferred to a second-dimension (^{2}D) separation with different selectivity. In the ^{2}D, metabolite M1 was well resolved from the higher-abundant substrates and inhibitors, as shown in Figure 2. The separation of metabolite M1 from higher-abundant substrates and inhibitors can avoid signal suppression and cross-talk. The reduction of signal suppression by further separation of the target compound from the sample matrix is also shown in a previous Application Note[1].

Time-based MHC 2D-LC was used to transfer 16 target metabolites from the ^{1}D to the ^{2}D. This provided further separation from substrates and inhibitors, resulting in an analysis with a total run time of 35 minutes. For one-dimensional separation of the 16 target metabolites from substrates and inhibitors, six different methods with a run time of 25 minutes each were developed (data not shown). In this context, the application of MHC 2D-LC enabled significant time and sample savings.

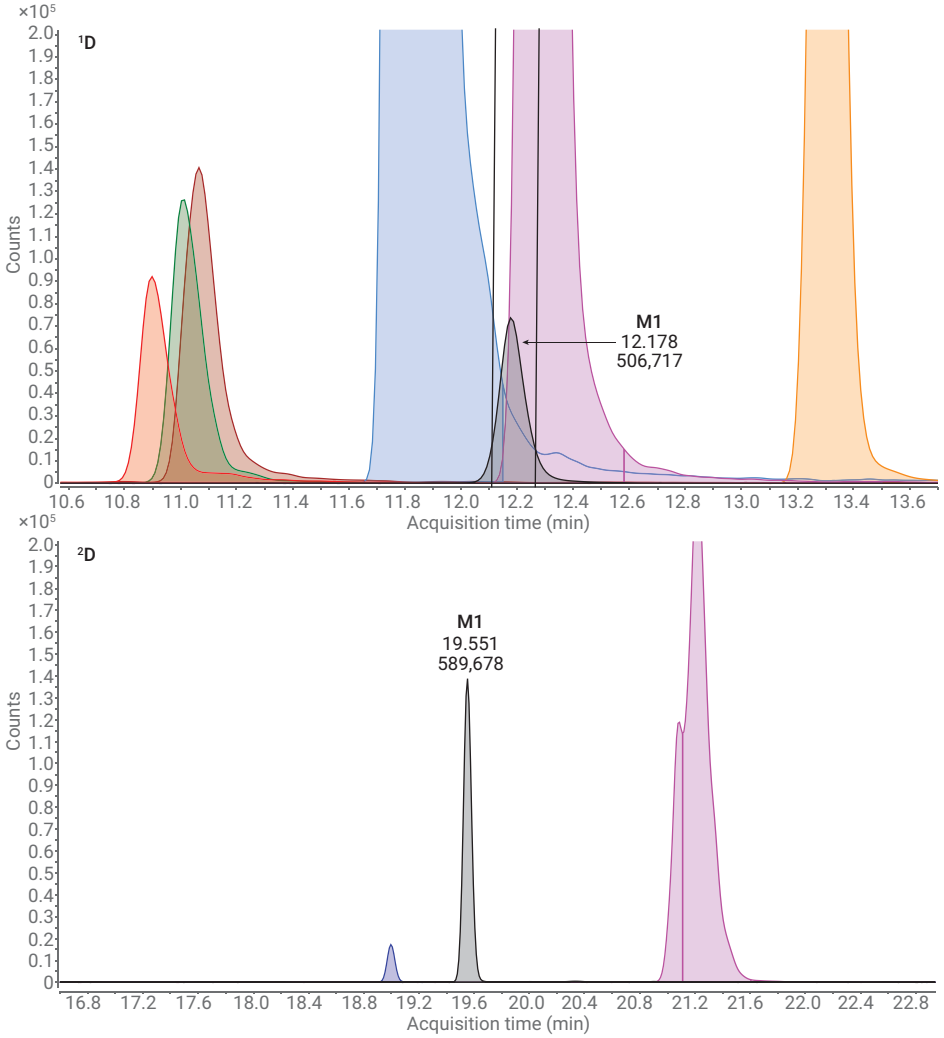


Figure 2. 2D-LC/MS/MS analysis of a cell supernatant.

2D-LC to Increase MS Detection Sensitivity by Solvent Switching

In an *in vitro* cell culture-based assay, one parent compound and one metabolite had to be quantified based on external calibration using an internal standard. For two further metabolites, relative quantification had to be performed because analytical standards were not available. Figure 3 shows the analysis using an existing one-dimensional LC/MS/MS method.

During method optimization, different column and solvent combinations were examined. Figure 4 displays two LC/MS/MS chromatograms obtained during method optimization. In Figure 4A, conditions similar to the existing LC/MS/MS method shown in Figure 3 were used. Under these conditions, all three metabolites (M1–M3) were separated from each other, from the parent compound, and from the internal standard, but MS detection sensitivity was compromised due to the solvent used. Using ammonium acetate and methanol in the mobile phase, MS detection sensitivity was greatly improved, but separation of metabolites M1–M3, the parent compound, and internal standard was compromised, as shown in Figure 4B. The coelution of metabolites M1 and M3 is especially critical because all three metabolites show the same mass transition, and need to be separated chromatographically.

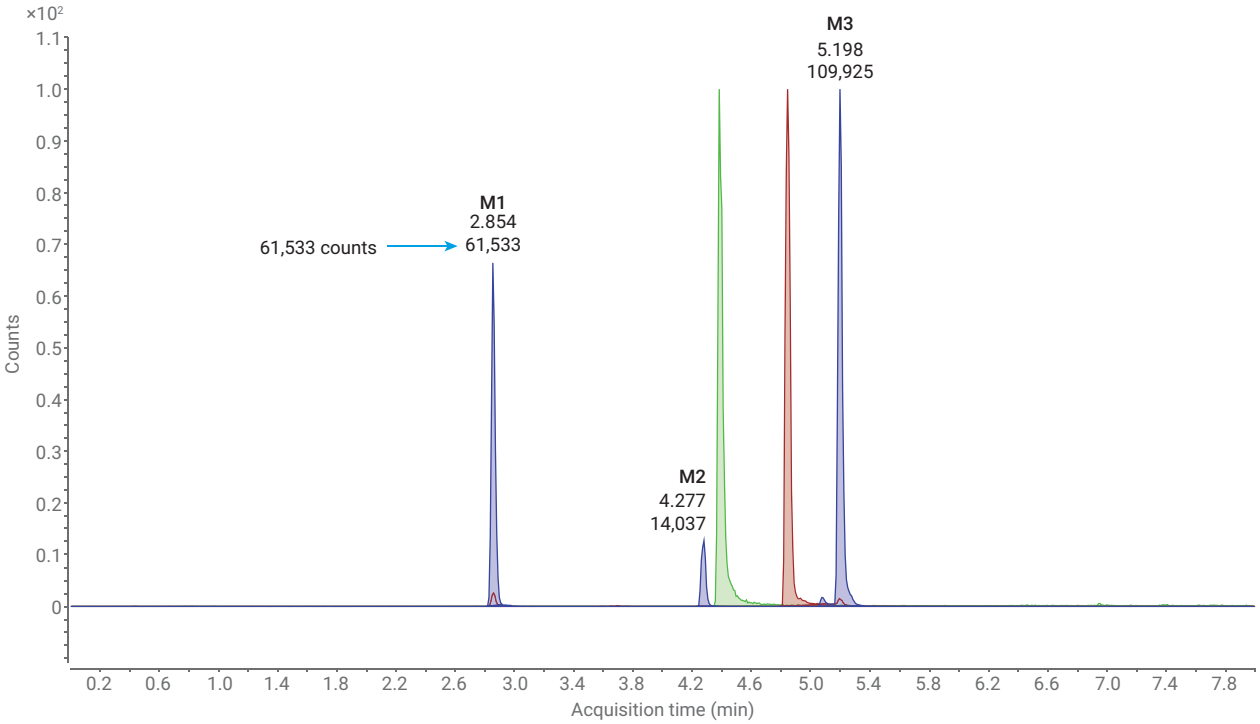
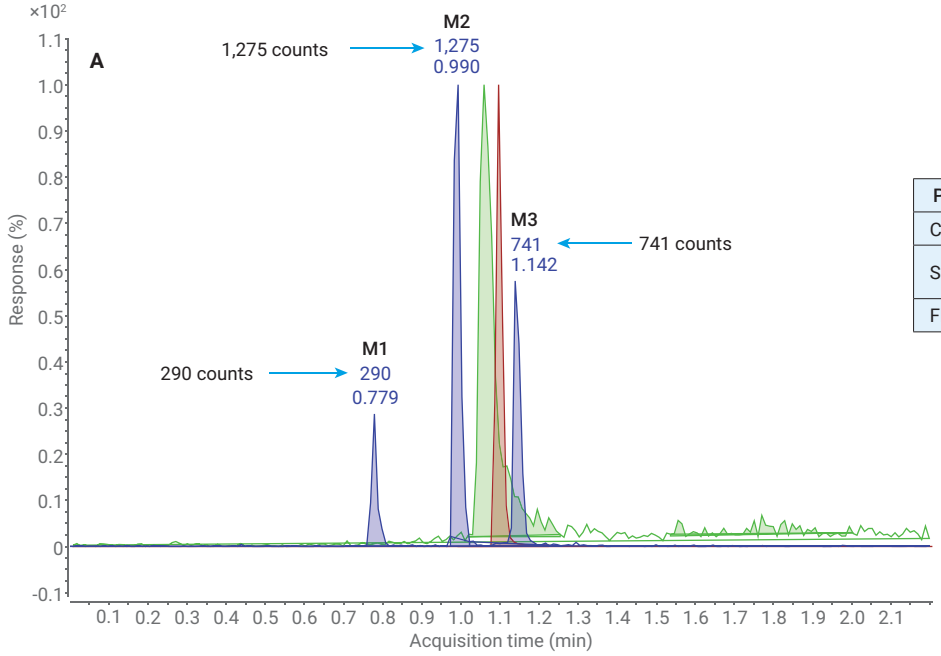
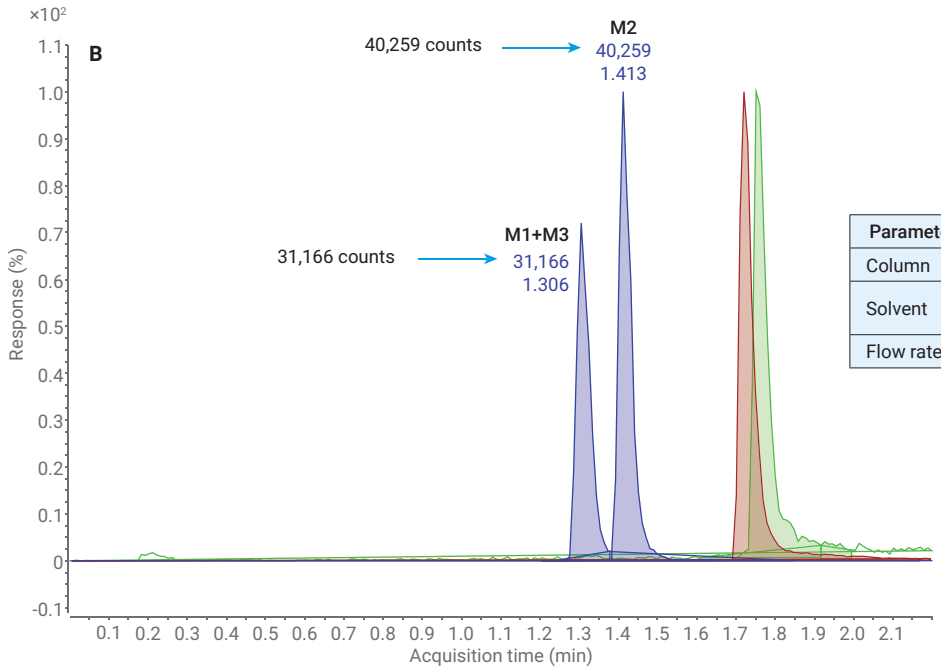


Figure 3. LC/MS/MS analysis of one parent compound (brown trace), its three metabolites (M1-M3, blue trace), and one internal standard (green trace) in a sample from an *in vitro* cell culture-based assay.



Parameter	Value
Column	Hybrid silica C18 column, 2.1 × 50 mm, sub-2 µm
Solvent	A) 0.1 % Formic acid in water B) 0.1 % Formic acid in acetonitrile
Flow rate	0.500 mL/min



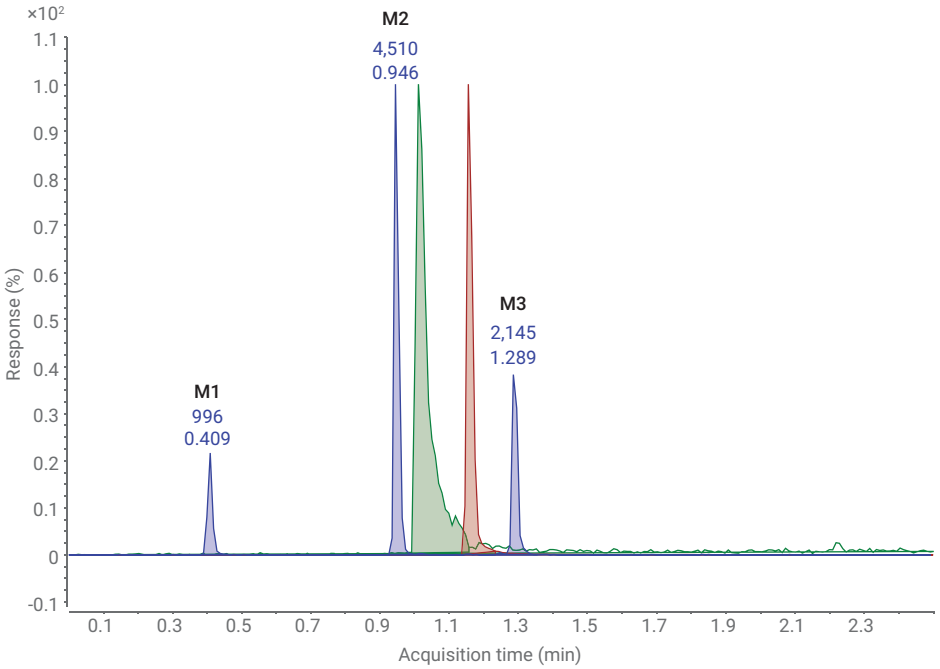
Parameter	Value
Column	Non-endcapped silica C18 column, 2.1 × 50 mm, sub-2 µm
Solvent	A) 5 mM ammonium acetate and 5 % methanol in water B) Methanol
Flow rate	0.500 mL/min

Figure 4. Chromatograms obtained during LC/MS/MS method development.

To take advantage of both the separation achieved using formic acid in water and acetonitrile in the mobile phase, as well as the MS detection sensitivity achieved using ammonium acetate and methanol in the mobile phase, the two separations were combined in a 2D-LC setup using multiple heart-cutting. In the ^{1}D, the flow rate was increased to 0.9 mL/min to increase analysis speed. Figure 5 shows the final ^{1}D separation.

The five target peaks were transferred to the ^{2}D using time-based heart-cutting. In the ^{2}D, a fast gradient was performed with a ^{2}D cycle time of 1.0 minute to trap the compounds on the ^{2}D column and elute them in the ^{2}D solvent to the MS for increased detection sensitivity. Figure 6 shows the ^{2}D chromatogram obtained after 2D-LC/MS/MS analysis of a sample from an *in vitro* cell culture-based assay.

Compared to the original 1D-LC/MS/MS method with a signal intensity of 61,533 counts for metabolite M1 (Figure 3), the 2D-LC/MS/MS method, with a signal intensity of 191,646 counts for metabolite M1 (Figure 6), enabled an increase in the MS detection sensitivity by a factor of 15, considering the reduction in injection volume from 5 to 1 µL. The total run time of the analysis was reduced from 9.5 to 6.5 minutes for the 2D-LC/MS/MS analysis.



Parameter	Value
Column	Hybrid silica C18 column, 2.1 × 50 mm, sub-2 µm
Solvent	A) 0.1 % Formic acid in water B) 0.1 % Formic acid in acetonitrile
Flow rate	0.900 mL/min

Figure 5. ^{1}D chromatogram obtained during 2D-LC/MS/MS method development.

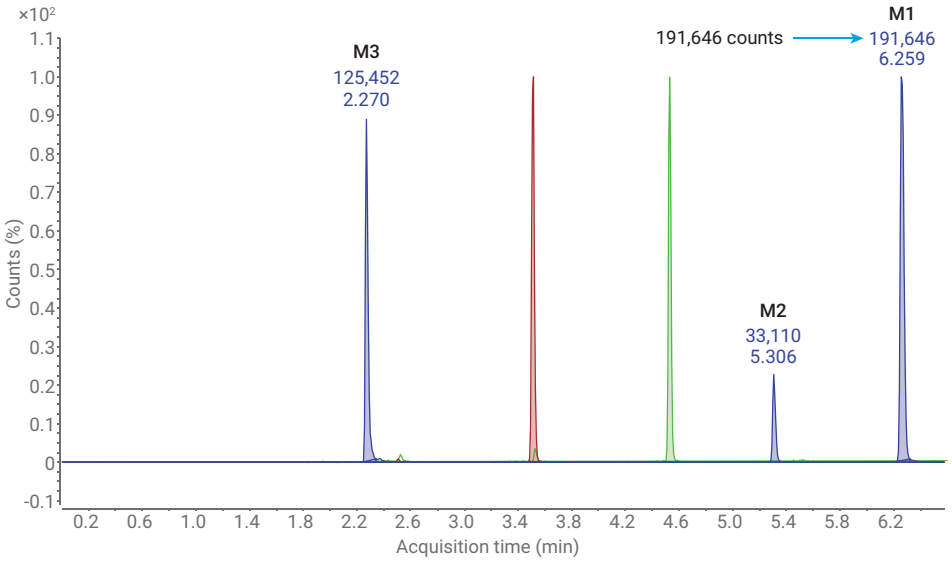


Figure 6. 2D-LC/MS/MS analysis of one parent compound (brown trace), its three metabolites (M1–M3, blue trace), and one internal standard (green trace) in a sample from an *in vitro* cell culture-based assay.

Conclusion

The application of 2D-LC/MS/MS for the analysis of drugs and their metabolites in pharmacokinetic research studies introduces a second-dimension separation, thereby offering the possibility to eliminate coelution and the risk of cross-talk for these complex samples. This was demonstrated for the analysis of cell supernatants from an *in vitro* cell suspension-based assay. The superior separation achieved using 2D-LC allowed the analysis of 16 target metabolites within a total run time of 35 minutes, which offered significant time and sample savings compared to the use of six different methods necessary for 1D-LC/MS/MS analysis of the target metabolites.

The second dimension of a 2D-LC/MS/MS method was successfully used to enhance the signal intensity of MS detection. In such a setup, conditions enabling separation of the target analytes were used in the first dimension, and conditions beneficial for superior MS detection sensitivity were chosen in the second dimension. The introduction of the analytes in a more suitable eluent to the MS source significantly improved quantitation limits.

Reference

1. Vanhoenacker, G.; *et al.* Two-Dimensional LC/MS/MS to Reduce Ion Suppression in the Determination of Cannabinoids in Blood Plasma. For Forensic Use. *Agilent Technologies Application Note*, publication number 5991-7859EN, **2017**.

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