Waters™

应用纪要

Fast and Sensitive *In Vitro* Metabolism Study of Rate and Routes of Clearance for Ritonavir Using UPLC Coupled With the Xevo QTof MS System

Jose Castro-Perez, Kate Yu, John Shockcor, Henry Shion, Emma Marsden-Edwards, Jeff Goshawk, Yasuhiro Yamada, Motoji Oshikata

Waters Corporation, Showa University

Introduction

A typical *in vitro* metabolic study includes the investigation of both the rate and metabolic routes of the parent drug. A desirable analytical protocol for this type of study needs to provide both the analytical speed and the sensitivity to detect metabolites at substrate concentration levels that mimic *in vivo* conditions.

In this application note, we present a simple and generic strategy for metabolite identification studies that utilizes UPLC® and a new, highly-sensitive hybrid quadrupole oa-TOF benchtop mass spectrometer: the Waters® Xevo™ QTof MS (Figure 1). This mass spectrometer follows Waters instrument design philosophy of Engineered Simplicity™ to provide enhanced performance together with ease of use.

The Xevo QTof MS features high desolvation temperature (up to 650 °C), optimized gas flow dynamics, and efficient ionization to accommodate high LC flow rates and highly aqueous solvents, common in reversed-phase LC conditions. As a result, the Xevo QTof MS combines both speed and sensitivity to

provide the best interface for UPLC/MS applications.

Figure 1. The Xevo QTof MS System.

To prove the power of this technology, an *in vitro* study of ritonavir (Figure 2) was conducted. Ritonavir is an antiretroviral drug from the protease inhibitor class used to treat HIV infection and AIDS. An intelligent Metabolite ID Workflow (Figure 3) developed by Waters was utilized for this study. This workflow takes full advantage of the speed and sensitivity offered by pairing the ACQUITY UPLC® System with the Xevo QTof MS, and maximizes productivity by utilizing intelligent software algorithms in MassLynx™ Software's MetaboLynx™ XS Application Manager.

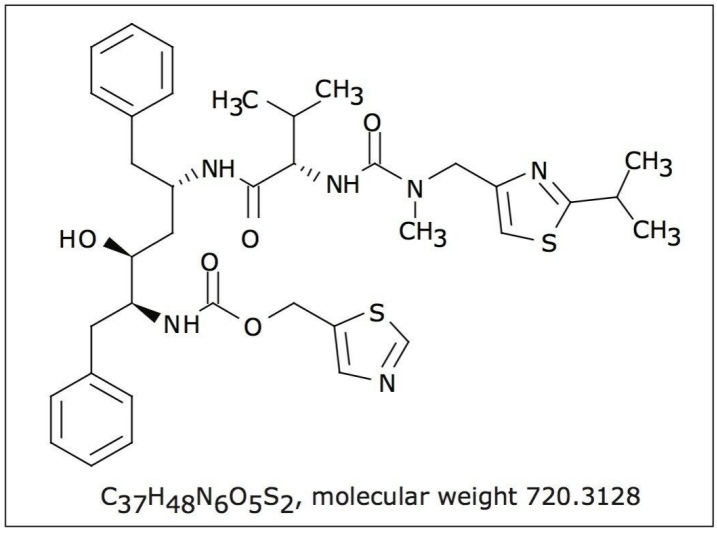

Figure 2. The chemical structure of ritonavir.

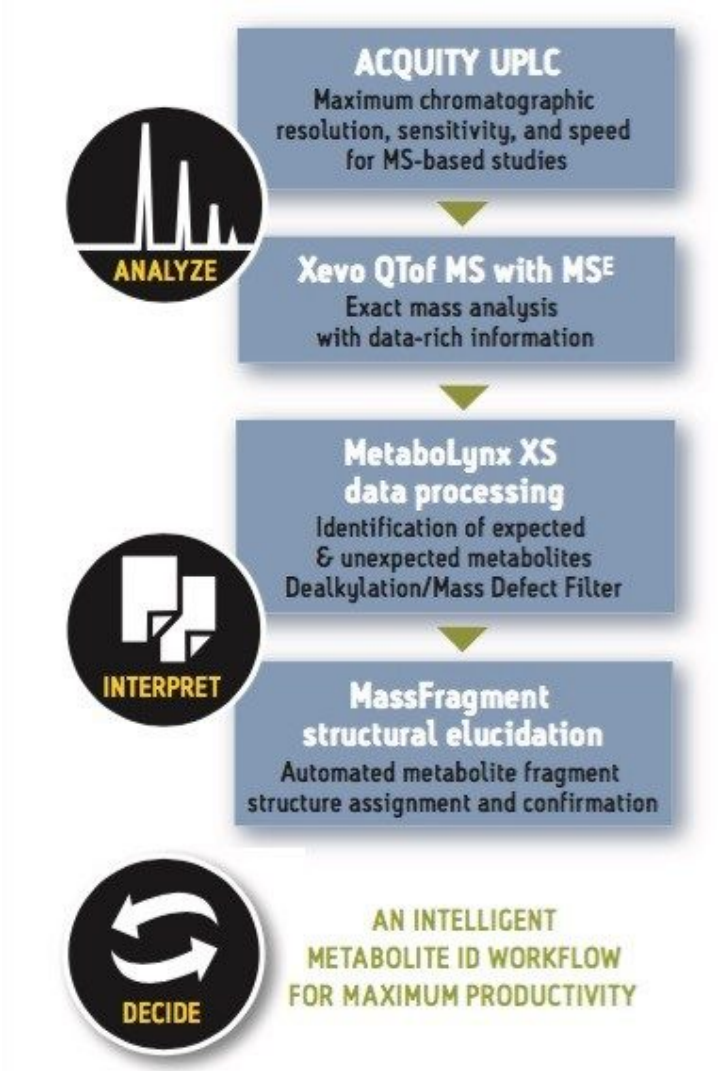

Figure 3. Waters intelligent Metabolite ID Workflow combines ACQUITY UPLC, Xevo QTof MS, and MetaboLynx XS to deliver maximum productivity.

Experimental

Ritonavir was incubated at 37 °C with rat hepatocytes at 1 µM. Multiple time points were taken for the

reaction. The reaction was terminated with equal volume of acetonitrile/methanol (1:1, v/v) to 0, 0.5, 1, 2, and 4 hours respectively for all time points. The samples were centrifuged at 13,000 RPM for 1 minute and the supernatants were directly used for injections.

LC Conditions

LC system:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC HSS T3 Column 2.1 x 100 mm, 1.7 µm, 55 °C
Flow rate:	500 µL/min
Mobile phase A:	Water +0.1% formic acid
Mobile phase B:	MeOH

Gradient Table

Time (min)	Composition (%)	Curve
0	98	
7	20	Curve 6
8	0	Curve 1
11	98	Curve 1

MS Conditions

MS system:	Waters Xevo QTof MS
Ionization mode:	ESI positive

Capillary voltage:	0.5 kV
Cone voltage:	20 V
Desolvation temperature:	550 °C
Desolvation gas:	600 L/Hr
Source temperature:	150 °C
Acquisition range:	150 to 1200 *m/z*
Scan duration:	0.1 sec
Collision gas:	Argon

Data Mangement

Compound screening and identification:	MetaboLynx XS Application Manager
Structural elucidation:	MassFragment™

Results and Discussion

Figure 4 shows the base peak ion chromatogram (BPI) and the extracted ion chromatogram (XIC) for ritonavir at time zero. At 1 µM substrate concentration, the ritonavir peak showed more than 24,000 RMS signal-to-noise ratio. The measured $[M+H]^+$ for the parent drug was 721.3211 (mass error 0.5 mDa, 0.7 ppm).

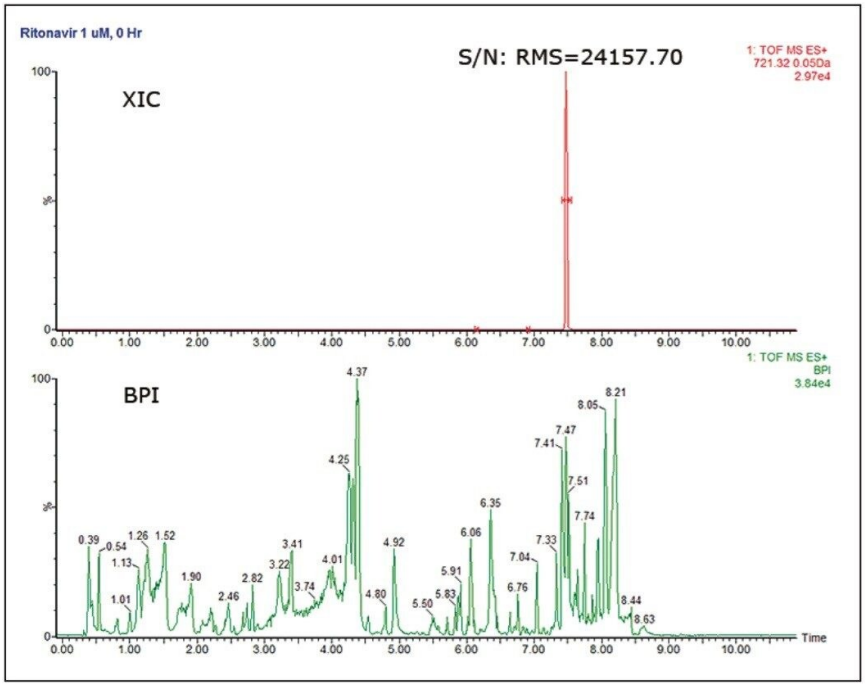

Figure 4. BPI and XIC of ritonavir at time zero with 1 µM substrate concentration.

As demonstrated in Figure 4, in addition to the superb sensitivity, the ritonavir peak obtained from UPLC is 6 seconds wide at the base; at 0.1 sec scan time, more than 15 data points were obtained across the peak. This is sufficient for both qualitative and quantitative analysis.

As indicated in the Metabolite ID Workflow shown in Figure 3, MS^E is used for the data acquisition strategy.[1,2] Here, the mass spectrometer obtains data in two distinct but parallel acquisition functions via rapid switching. One function scans at low collision energy (CE), the other function scans at high collision energy. The resulting raw data file contains two distinct chromatograms, one containing intact *m/z* information, while the other contains fragment *m/z* information. Therefore, from a single LC injection, MS full scan and fragment ion information can be accomplished simultaneously.

The major bottleneck in metabolite identification is data processing and interpretation. The strategy discussed in this work is a structure-driven MetaboLynx XS^3 data processing workflow that incorporates MassFragment for structural elucidation. The entire data processing workflow is displayed below in Figure 5.

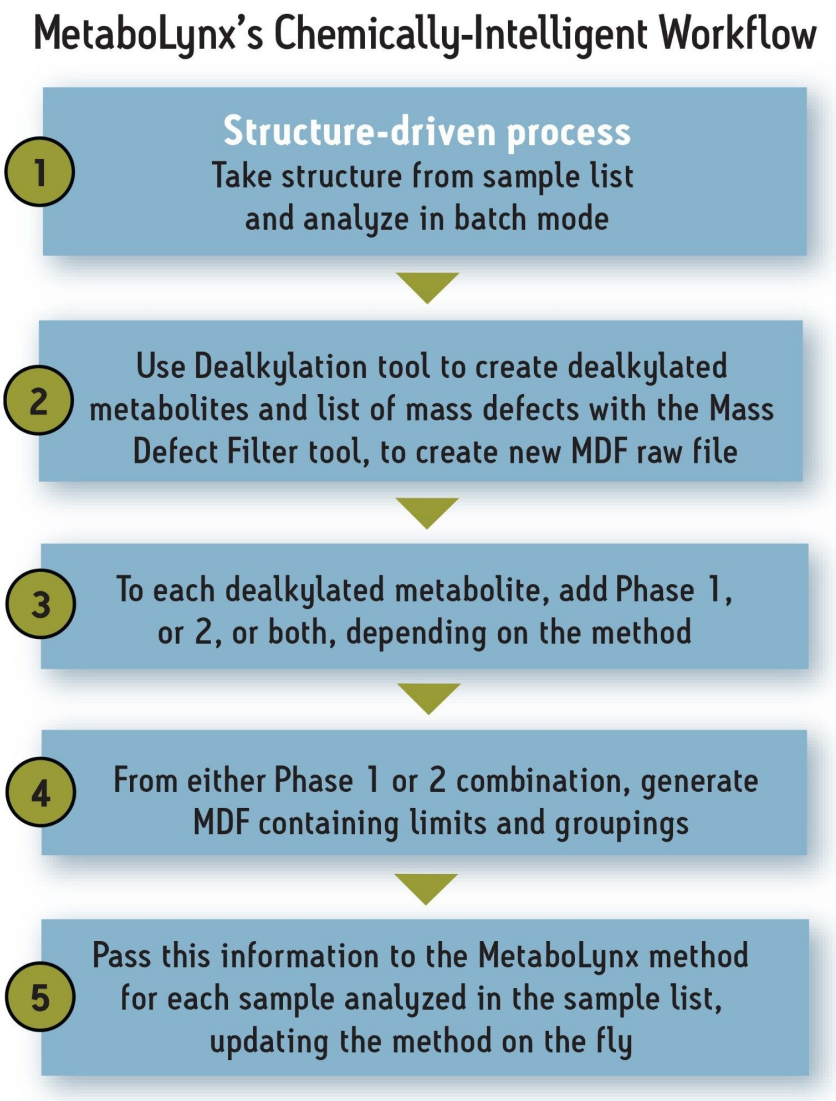

Figure 5. The workflow for chemically-intelligent data processing using MetaboLynx XS.

MetaboLynx XS updates the method on-the-fly according to the structure in question. The results can be interactively reviewed from the MetaboLynx XS browser as shown in Figure 6. Upon opening the browser, the screening results based on the low CE scan are displayed along with the list of potential metabolites, their full-scan MS spectra, XIC chromatograms, as well as the parent drug structure.

The fragment information obtained from the high CE scan is aligned in time and by scan number with

their respective $[M+H]^+$ or $[M-H]^-$ ion in the low energy mode. The potential metabolites identified can be further confirmed by reviewing their respective high energy spectra from the MS Fragment Analysis window as shown in Figure 6.

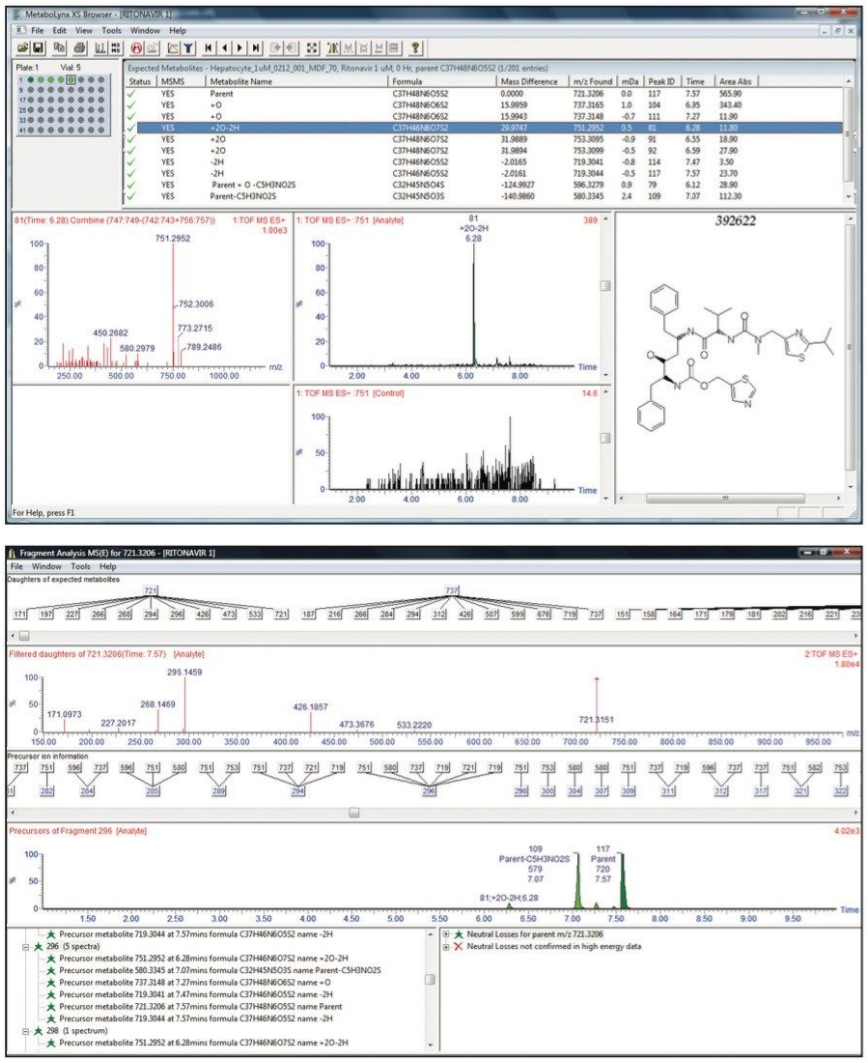

Figure 6. The MetaboLynx XS browser shows the results of metabolite ID for ritonavir, with an interactive results review window above, and the Fragment Analysis window below.

For structural elucidation, the fragment ions can be imported from the Fragment Analysis window directly into MassFragment. The structural elucidation results obtained from MassFragment provide the

proposed fragment structures along with the exact mass information (Figure 7).

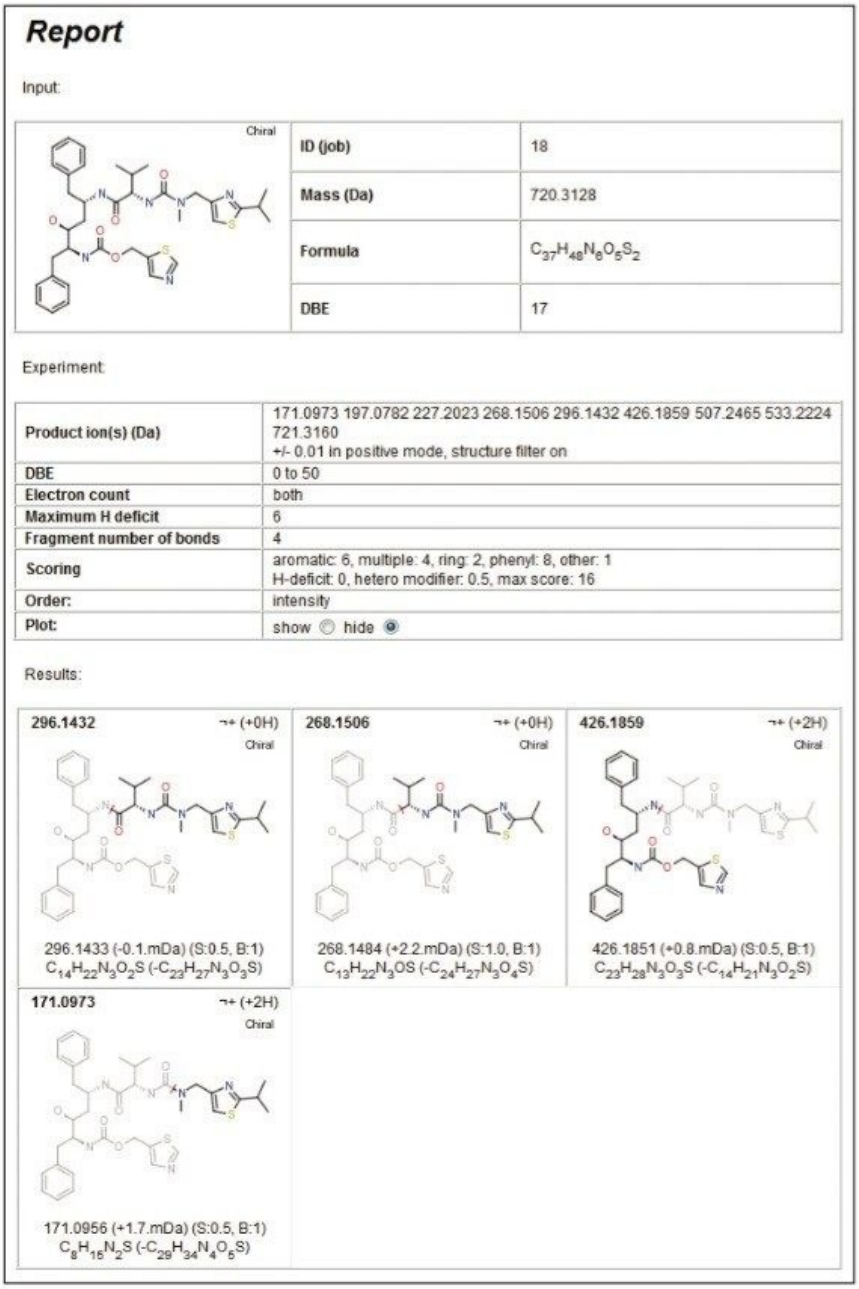

Figure 7. Fragment Analysis window for MSE data review and data input into MassFragment.

As a result, using the UPLC/MS^E data acquisition strategy combined with MetaboLynx XS for data processing, the entire metabolite identification task can be accomplished from a single LC injection. This strategy also allows for a more conventional approach where precursor selection with the quadrupole (Q1) is utilized for MS/MS experiments.

As stated in the introduction, a typical metabolite study in drug discovery includes not only the qualitative identification of the major metabolites, but also a semi-quantitative estimation of the rate of the clearance for the parent drug. With the fast data acquisition obtained from the Xevo QTof MS, even for the narrow LC peaks delivered by UPLC, enough data points are obtained for quantitative estimation of the rate of disappearance for the parent drug and for the identification of metabolites, giving rise to an all-in-one approach that maximizes productivity.

Figure 8 shows an overlay of one of the major metabolites of ritonavir (N-dealkylated metabolite cleaving the 4-methyl-2-(propan-2-yl)-1,3-thiazole motif) over 4 hours with a clear display of the concentration increase over the time.

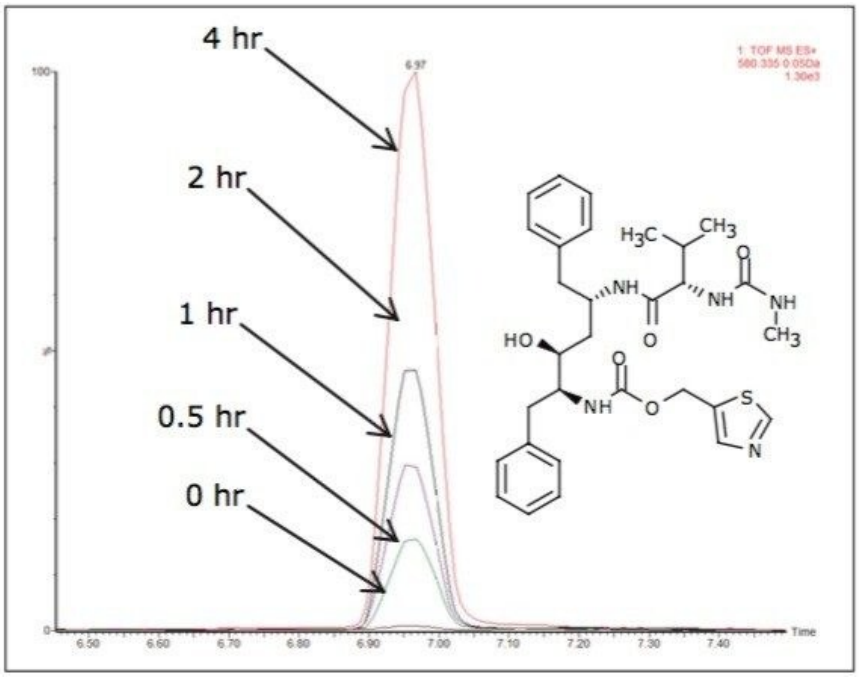

Figure 8. Extracted ion chromatogram (XIC) at different time points for the N-dealkylated metabolite of ritonavir.

Figures 9A and 9B show the disappearance curve for the parent drug and formation rates for the major metabolites, respectively. These were obtained with a single injection of each time point at a low incubation level, which is representative of the detected *in vivo* metabolite concentrations. The intrinsic clearance (CLint) obtained here is 2.09 µL/min/10^6 cells. The routes of the metabolism were also obtained at the same time, again helping to maximize the productivity.

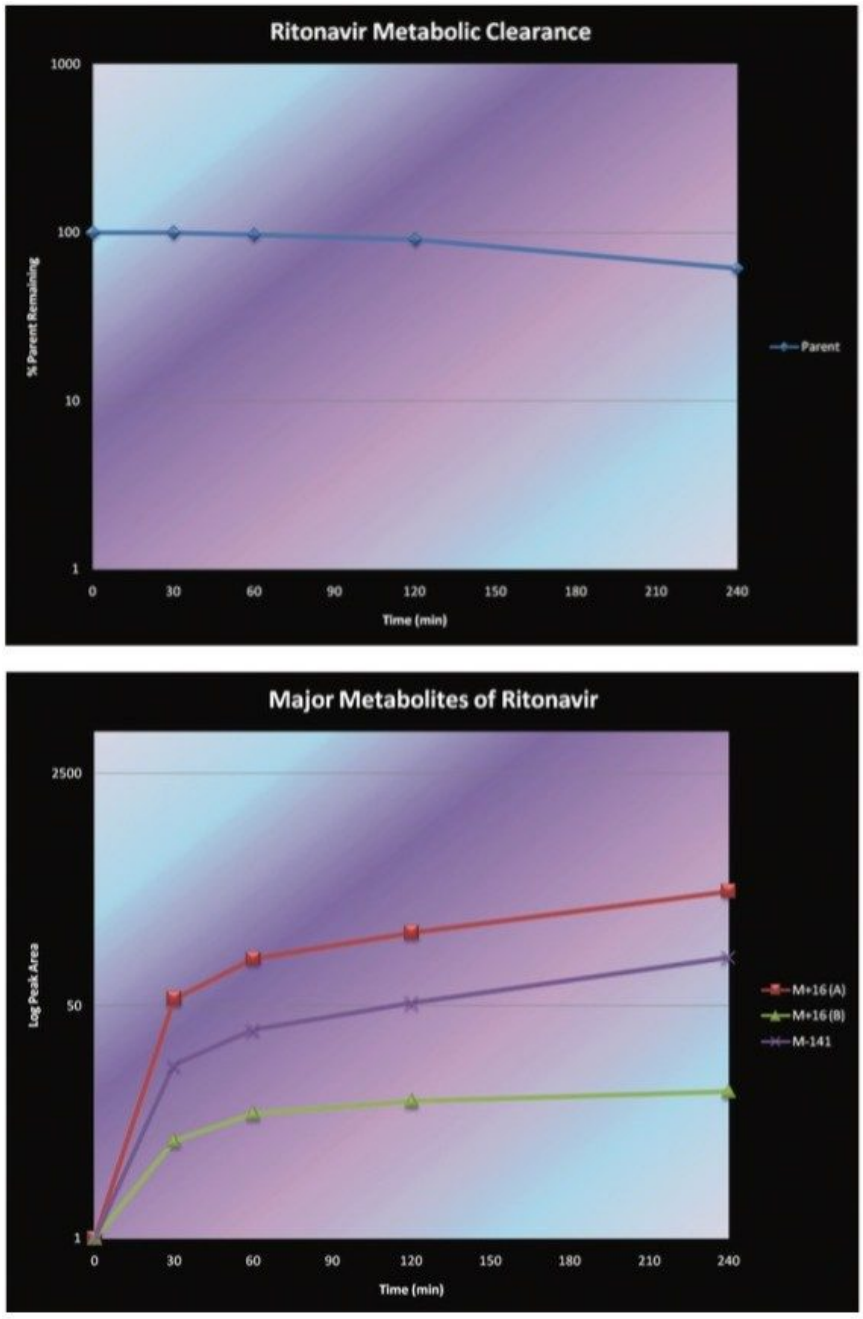

Figure 9. A) Disappearance curve for ritonavir. B) Formation rates for its major metabolites.

Conclusion

A fast, sensitive, and efficient Metabolite ID Workflow utilizing ACQUITY UPLC with the Xevo QTof MS, combined with MetaboLynx XS data processing, has been demonstrated to provide maximum productivity for metabolite identification.

The Xevo QTof MS, with its innovative Engineered Simplicity design that makes exact mass MS more accessible, offers the best interface to UPLC. In addition to its exact mass capability, the Xevo QTof MS delivers high data acquisition speeds, up to 20 spectra/sec, and provides superb sensitivity in full MS scan and MS/MS modes.

By using this UPLC/MS^E data acquisition strategy, along with the chemically-intelligent MetaboLynx XS data processing workflow, the complete metabolite identification task can be accomplished from a single LC injection extremely rapidly. With multiple time-point samples injected, the rate and routes of metabolism of a target drug can be obtained with ease at very low incubation levels. This unique combination of an efficient Metabolite ID Workflow with powerful instrumentation and software enables laboratories to utilize resources more effectively, and decrease time to market.

References

1. Bateman K, Castro-Perez JM, Wrona M, Shockcor JP, Yu K, Oballa R, Nicoll-Griffith D. Rapid Commun. *Mass Spec*. 2007; 21 (9): 1485–96.
2. Mortishire-Smith RJ, O' Connor D, Castro-Perez JM, Kirby J. Rapid Commun. *Mass Spec*. 2005; 19 (18): 2659–70.
3. Mortishire-Smith RJ, Castro-Perez JM, Yu K, Shockcor JP, Goshawk J, Hartshorn MJ, Hill A. Rapid Commun. *Mass Spec*. 2009; 23 (7): 939–48.

Featured Products

ACQUITY Premier System <https://www.waters.com/nextgen/global/products/chromatography/chromatography-systems/acquity-premier-system.html>

Xevo G3 QTof <https://www.waters.com/nextgen/global/products/mass-spectrometry/mass-spectrometry-systems/xevo-g3-qtof.html>

MassLynx Mass Spectrometry Software <https://www.waters.com/nextgen/global/products/informatics-and-software/mass-spectrometry-software/masslynx-mass-spectrometry-software.html>

720003025, April 2009