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應用手冊

Drug Metabolite Identification Using Waters™ SELECT SERIES™ Cyclic™ Ion Mobility Mass Spectrometer and waters_connect™ Application Manager

Adam King, Billy J. Molloy, Russell J. Mortishire-Smith, Robert S. Plumb

Waters Corporation

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Abstract

We describe the use of ion mobility-enabled high-resolution mass spectrometry in combination with powerful informatics tools to characterize the *in vivo* metabolic fate of fasiglifam, a drug withdrawn from clinical trials after it demonstrated the potential to cause liver damage. A total of 15 drug-related metabolites were detected across the time course with an average *m/z* error of –0.77 ppm. The precursor, product ion, and collision cross section data allowed a localization of the site of biotransformation in most cases.

Benefits

- Robust generic data acquisition strategy which enables rapid analysis
- Simple and customizable workflow, which can be tailored to meet user needs

- Tools to facilitate confident metabolite identification

Introduction

Metabolite identification, and subsequent profiling, plays a key role in the drug discovery and development processes. It provides critical information for lead candidate selection pharmacokinetics optimization in early discovery, support of safety assessment studies, and ensuring safety in clinical trials. Drug metabolism scientists must resolve the tension between the need for timely turnaround of results and the requirement that outcomes are generated with sufficient confidence and accuracy. In drug discovery, where the number of new compound entities needing analysis is high, there is need for a higher throughput and relatively generic approach which can be streamlined and used across a variety of compound classes without the need to tailor the methodology for each compound under investigation. In the discovery stage, relatively little is known about the metabolic fate of the molecule and so detection/identification must be performed using the mass spectral characteristics of the compound under investigation, and the likely routes of metabolism for the relevant chemotype.

In contrast, in drug development comprehensive metabolite detection/identification is more important than throughput, thus there is a need for expert systems that allow strong data mining and structural elucidation tools. There may be previous knowledge of the metabolism of the compound from *in vitro* studies or early animal studies. Under these circumstances, a combination of targeting known metabolites and characterizing novel metabolites are applied to fully characterize the fate of the molecule for the regulatory package.

Thus, there is a need for an integrated, automated acquisition, and processing system for drug metabolite detection, mining, and identification. Here, we describe the combination of ion mobility-enabled high-resolution liquid chromatography (LC-MS) with an intelligent, data-driven, structural assignment workflow.

Ion mobility MS, using the Waters SELECT SERIES Cyclic IMS combined with the rapid high-resolution chromatography provided by ACQUITY™ UPLC™ offers a powerful flexible platform for both discovery and development metabolite identification when combined with the Metabolite Identification Application Solution with UNIFI™ via waters_connect. Here, we illustrate the benefit of this solution for the identification of the *in vivo* metabolites of fasiglifam (TAK875) Figure 1, a GPR40 agonist withdrawn from phase III trials due to after the observation of elevated liver enzymes.[1]

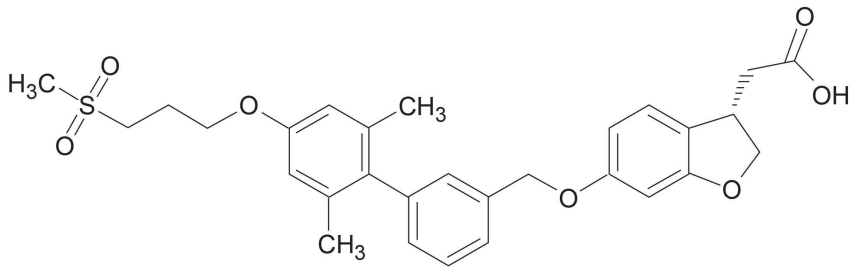


Figure 1. Structure of fasiglifam (TAK875).

Experimental

Sample Description

Male Sprague-Dawley rats were dosed orally with 50 mg/kg fasiglifam as a suspension in 0.5% carboxymethylcellulose in water. Blood was collected from the tail vein immediately prior to dosing and at regular intervals over the following 96 hours. Plasma obtained from blood samples was subjected to protein precipitation *via* mixing with acetonitrile at a ratio of 3:1 (acetonitrile:sample). Following vortex mixing the samples were centrifuged at 13,000 RCF for 10 minutes and transferred to an autosampler vial for analysis. Authentic fasiglifam standard was dissolved in methanol and diluted with 50:50 acetonitrile:water to give a final drug concentration of 100 ng/mL. This sample was used to obtain representative retention time, mass spectrometry (MS), tandem mass spectrometry (MS/MS), collision cross section (CCS), and neutral loss outcomes for the parent compound.

Method Conditions

The authentic standard and extracted rat plasma samples were analyzed using reversed-phase liquid chromatography coupled with negative ion electrospray ion mobility mass spectrometry detection. Briefly, a 1 µL aliquot of the sample was loaded onto a 2.1 x 100 mm ACQUITY™ HSS T3 1.8 µm C_{18} Column, the column was maintained at 40 °C and eluted with a linear solvent gradient of 40–70% 0.1% aqueous formic acid:acetonitrile over ten minutes at a flow rate of 500 µL/min. Mass spectrometry data was collected in negative ion mode over the range of 50–1200 *m/z*, using a collision energy ramp of 20–40 eV and a single pass of the ion mobility cell.

LC Conditions

LC system:	ACQUITY UPLC I-Class
Detection:	ESI- MS
Vials:	Max Recovery Vials
Column(s):	ACQUITY HSS T3 2.1 x 100, 1.8 µm
Column temp.:	40 °C
Sample temp.:	10 °C
Injection volume:	1 µL
Flow rate:	500 µL/min
Mobile phase A:	0.1% aqueous formic acid
Mobile phase B:	Acetonitrile containing 0.1% formic acid
Gradient:	Chromatographic gradient conditions are given in the table below

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0	0.5	60	40	6
0.5	0.5	60	40	6
10	0.5	30	70	6
10.1	0.5	5	95	6
12	0.5	5	95	6
12.1	0.5	60	40	6

MS Conditions

MS system:	Waters SELECT SERIES Cyclic IMS
Ionization mode:	Electrospray Negative Ion
Acquisition range:	60–1200 *m/z*, HDMS^E
Capillary voltage:	2 kV
Collision energy:	Transfer cell ramp: 20–40 eV
Cone voltage:	30 V

Data Management

Chromatography software:	MassLynx™ 4.2
MS software:	MassLynx 4.2

Informatics:	waters_connect 2.0.0.9 with UNIFI 1.9.13

Results and Discussion

Metabolite Identification Workflow

The process of drug metabolite identification involves the development of a data acquisition methodology, acquisition of the data, componentization of the raw data, component review and finally reporting. For drug discovery applications in particular, generic strategies are preferred in order to drive throughput, and to this end the HDMS^E mode of data acquisition is ideal, since it affords precursor, product ion, and CCS outcomes in one simple method. To streamline the data acquisition and analysis process we have developed a simple workflow which combines the analyte characterization capabilities of the Waters SELECT SERIES Cyclic Ion Mobility Mass Spectrometer with the data analysis power of the waters_connect Metabolite Identification Application Solution. The workflow is described in Figure 2 and is divided into 4 simple steps: 1) creation of the analysis method, 2) library entry creation for the parent and known metabolites, and chemically intelligent targeting of unknown metabolites, 3) data filtering and review, and 4) data visualization and reporting.

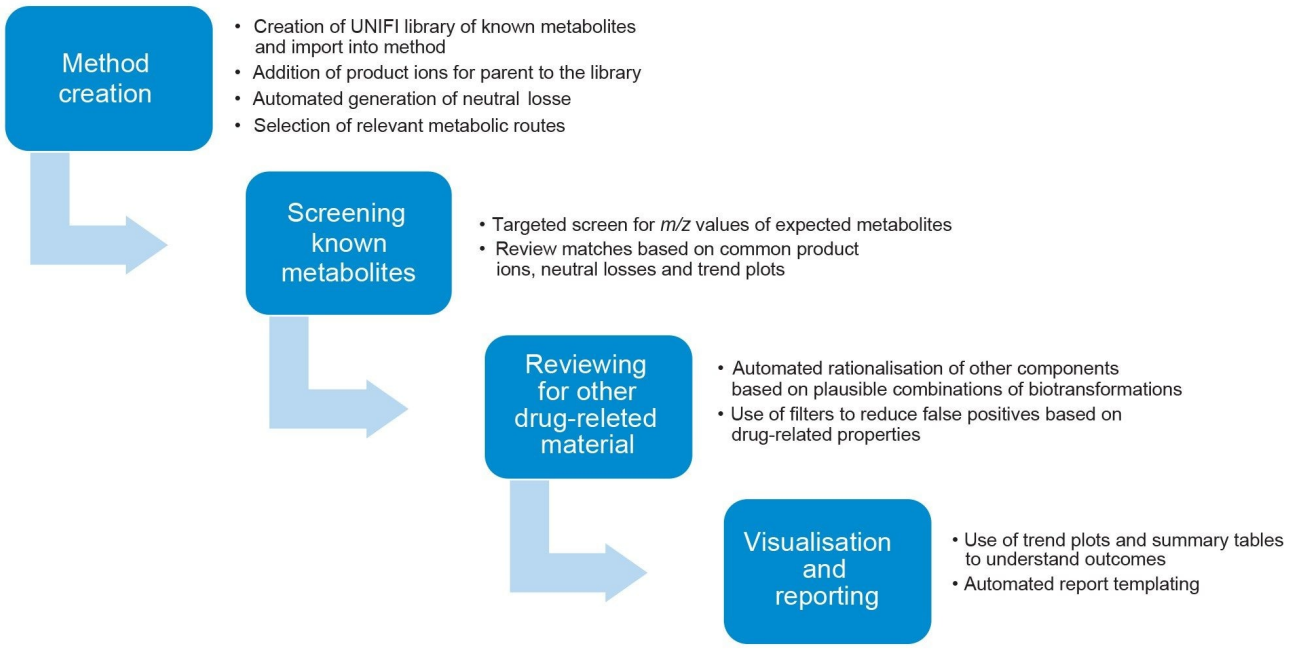
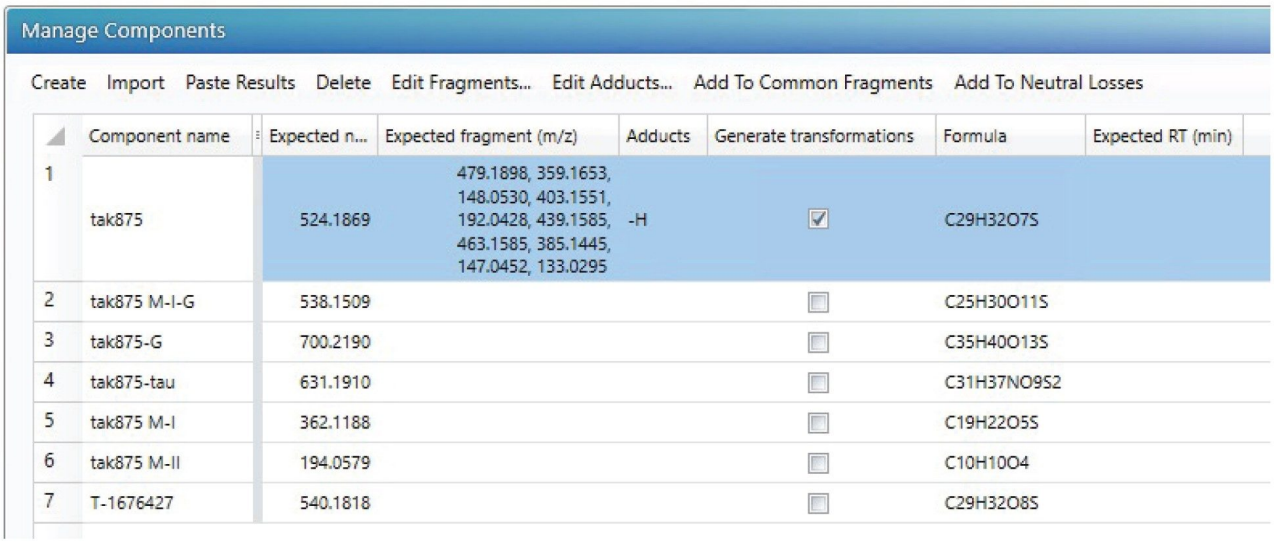


Figure 2. waters_connect Metabolite Identification Workflow.

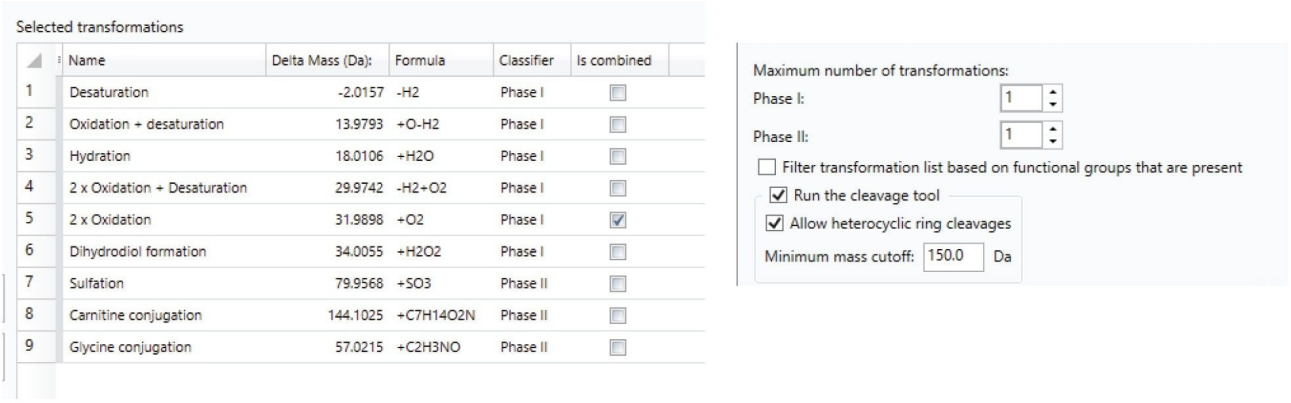
Libraries of Known Metabolites and Generation of Putative Metabolites

Drug metabolism is performed at various stages of the drug discovery and development process, starting in the initial stages when little is known about the metabolic fate of a compound, too late in clinical development when the compound has been extensively characterized both *in vitro* and *in vivo*. The waters_connect Metabolite Identification application enables targeting of known metabolites via the construction of library entries (Figure 3). Here, the structures of known metabolites, together with that of the parent drug, are added to a library which is then used to interrogate the LC-MS data. If available, previously established retention time, product ion and CCS values can be added to the library and included in the screening method. For example, the experimentally observed product ions of fasiglifam have been added to the method, as have the corresponding neutral losses. Along with these known drug metabolites, the data can be examined for novel metabolites using a combination of the structure of the parent compound, and common metabolic functionalization reactions, including both Phase 1 and Phase 2 (Figure 4). The user has the option to determine which target metabolite can be further functionalized via the 'Generate Transformations' checkbox. Once the appropriate list of targets has been created, the acquired LC-IM-MS raw data can be processed to detect and identify drug-related material.



	Component name	Expected n...	Expected fragment (m/z)	Adducts	Generate transformations	Formula	Expected RT (min)
1	tak875	524.1869	479.1898, 359.1653, 148.0530, 403.1551, 192.0428, 439.1585, 463.1585, 385.1445, 147.0452, 133.0295	-H	☑	C29H32O7S	
2	tak875 M-I-G	538.1509			☐	C25H30O11S	
3	tak875-G	700.2190			☐	C35H40O13S	
4	tak875-tau	631.1910			☐	C31H37NO9S2	
5	tak875 M-I	362.1188			☐	C19H22O5S	
6	tak875 M-II	194.0579			☐	C10H10O4	
7	T-1676427	540.1818			☐	C29H32O8S	

Figure 3. Library of known metabolites used for targeted analysis of data.



	Name	Delta Mass (Da):	Formula	Classifier	Is combined
1	Desaturation	-2.0157	-H2	Phase I	☐
2	Oxidation + desaturation	13.9793	+O-H2	Phase I	☐
3	Hydration	18.0106	+H2O	Phase I	☐
4	2 x Oxidation + Desaturation	29.9742	-H2+O2	Phase I	☐
5	2 x Oxidation	31.9898	+O2	Phase I	☑
6	Dihydrodiol formation	34.0055	+H2O2	Phase I	☐
7	Sulfation	79.9568	+SO3	Phase II	☐
8	Carnitine conjugation	144.1025	+C7H14O2N	Phase II	☐
9	Glycine conjugation	57.0215	+C2H3NO	Phase II	☐

Figure 4. Generation of putative drug metabolites using chemical intelligence.

Identification of Drug-Related Material

One of the significant challenges which exists in metabolite characterization is the identification of drug-related material in the presence of complex endogenous matrix interference. A powerful approach which allows the user to minimize wasted time is the use of custom filters which, when applied in a workflow step, select only those compounds which have a high likelihood of being significant. These can be generalized (having a response

greater than a defined level, being within a retention time which excludes the injection front and high organic wash periods), or compound-specific (have a plausible explanation, contain halogens, have a product ion or neutral loss in common with the parent compound, or are within the allowed mass defect region) (Figure 5).

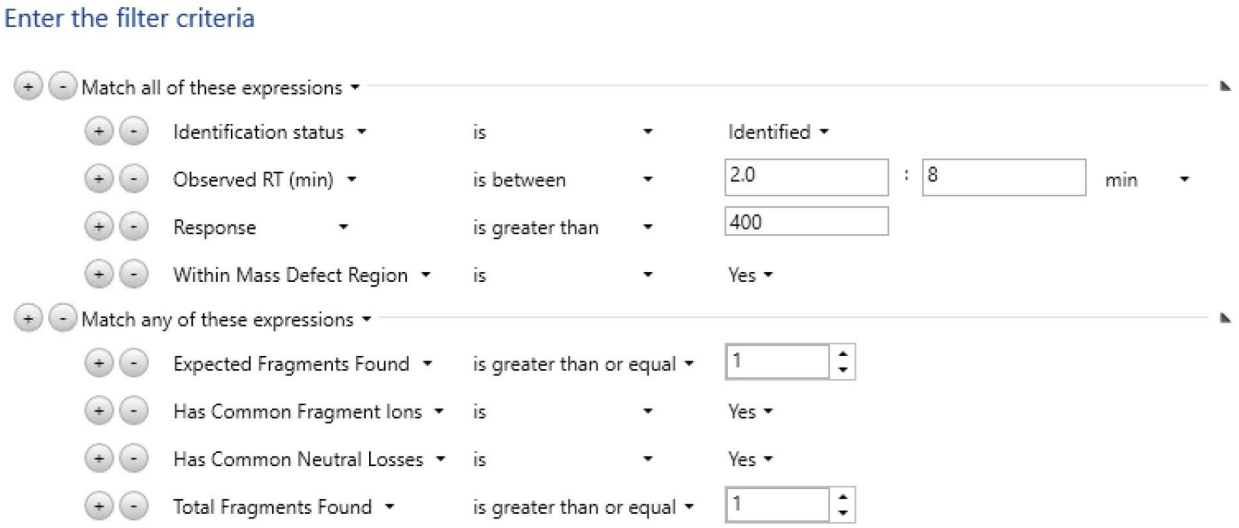


Figure 5. Drug-Related Properties Filter.

Worked Example

To illustrate the power of the Waters SELECT SERIES Cyclic Ion Mobility Mass Spectrometer combined with the waters_connect Metabolite Identification application software, this platform was used for the analysis of plasma following the oral administration of fasiglifam to Sprague-Dawley rats at 50 mg/kg. The protein-precipitated plasma was analyzed by reverse-phase UPLC-MS with detection in the negative ion ESI mode. The mass spectrum obtained for the parent compound is shown in Figure 6. In the upper trace, Figure 6(i), we see the deprotonated parent ion for fasiglifam *m/z* = 523.1809, and in the lower trace, diagnostic MS/MS fragment ions at *m/z* = 479.1899, 403.1549, 359.1650, 319.1340, 192.0427, and 148.0527 (Figure 6(ii)). A blue icon by a product ion indicates that the software has assigned a plausible structural explanation for that product ion based on systematic disconnections of the structure.

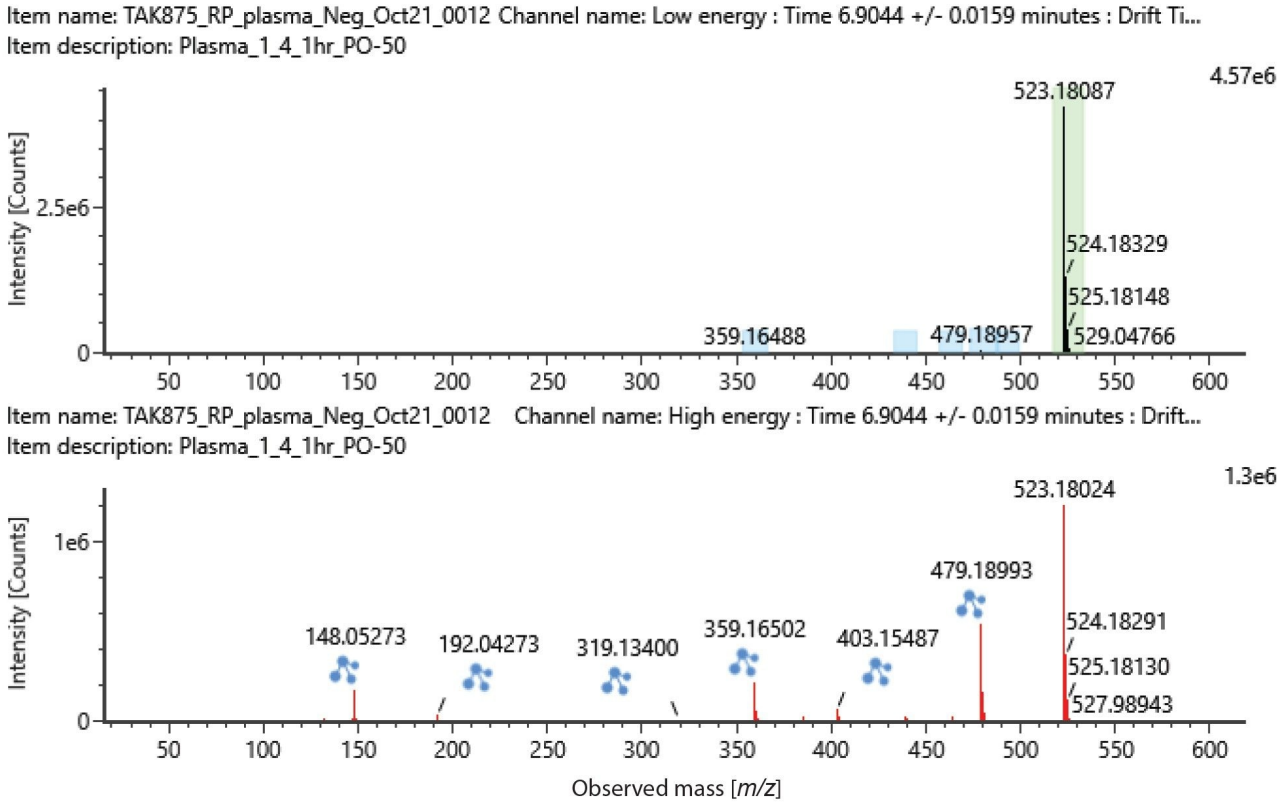


Figure 6. Precursor (upper trace) and product ion (lower trace) spectra of fasiglifam.

The major and previously reported metabolites of fasiglifam were identified using a combination of targeted screening using library entries, diagnostic fragment ions, common neutral losses and established metabolic cleavage, functionalization, and conjugation reactions. For example, the glucuronide metabolite of fasiglifam, eluting with a retention time t_R = 6.26 minutes with a deprotonated molecular ion *m/z* = 699.2113 is shown in Figure 7, with diagnostic fragment ions at *m/z* = 523.1786, corresponding to a neutral loss of the glucuronide moiety, and the glucuronide itself as a product ion. Complete analysis of these samples resulted in the detection of the previously reported metabolites[2,3] together with several new, and previously unreported metabolites, in which the biotransformations appear to be associated with the substituted dihydrobenzofuran moiety. The structure of these new metabolites is currently under further investigation.

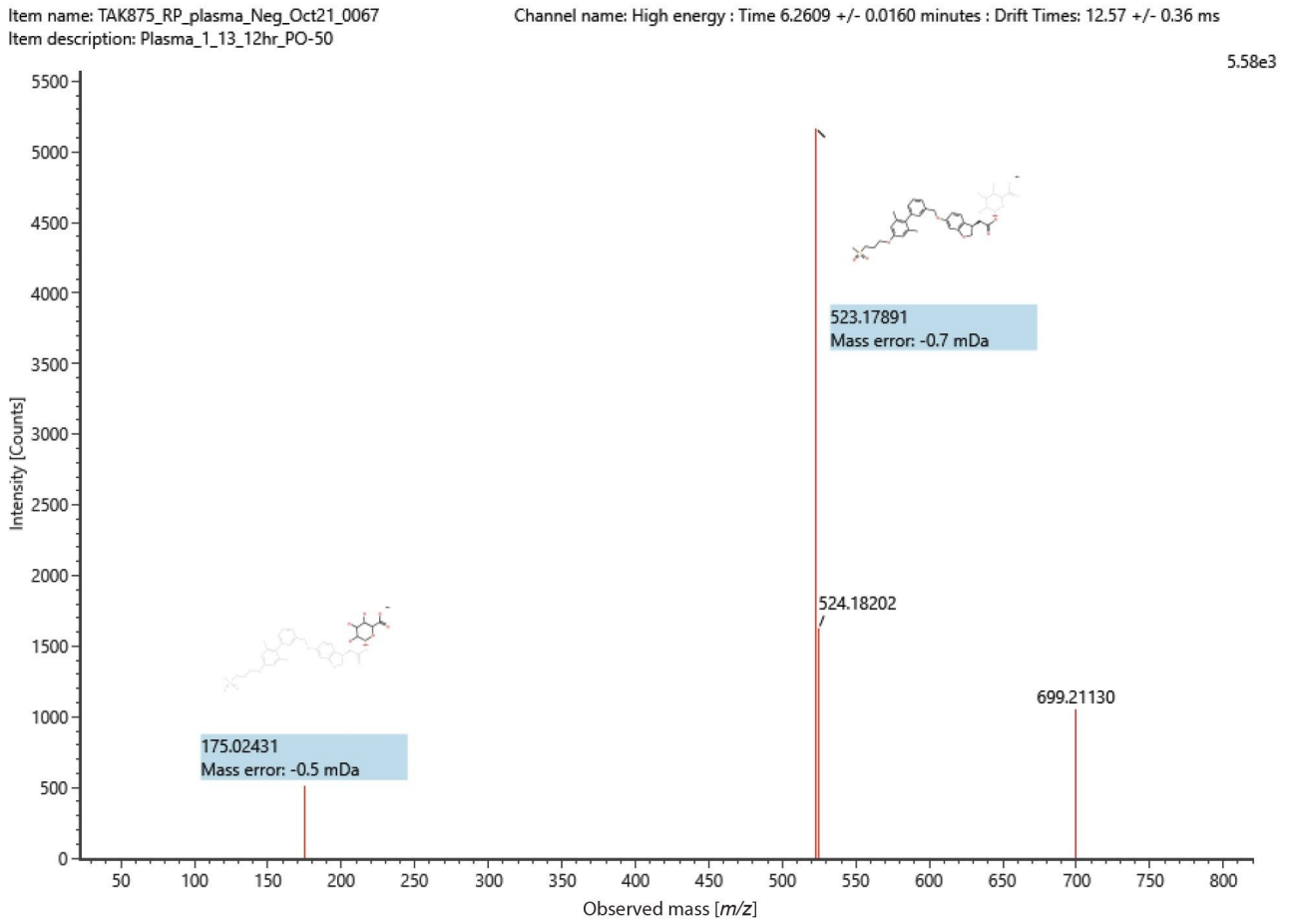


Figure 7. Product ion spectrum of major fasiglifam glucuronide metabolite.

Localization of Site of Metabolism

One of the most challenging, but essential, tasks in drug metabolite identification is the localization of the site of metabolism on the molecule. This can be a time-consuming task requiring scientists with experience in structural analysis and metabolic transformations. The waters_connect Metabolite Identification Application supports this process using systematic bond disconnections and chemical intelligence to suggest or in some cases identify the site of drug metabolism. For example, Figure 8 demonstrates how the software helps the drug metabolism scientist localise the site of metabolism for a previously unreported M-2H+O metabolite of fasiglifam. The software highlights the potential sites of metabolic transformation, with green indicating the most likely sites of metabolism and orange indicating possible, but less likely, sites of metabolism, based on the number of conserved or shifted product ions when compared with the product ion spectrum of the parent drug.

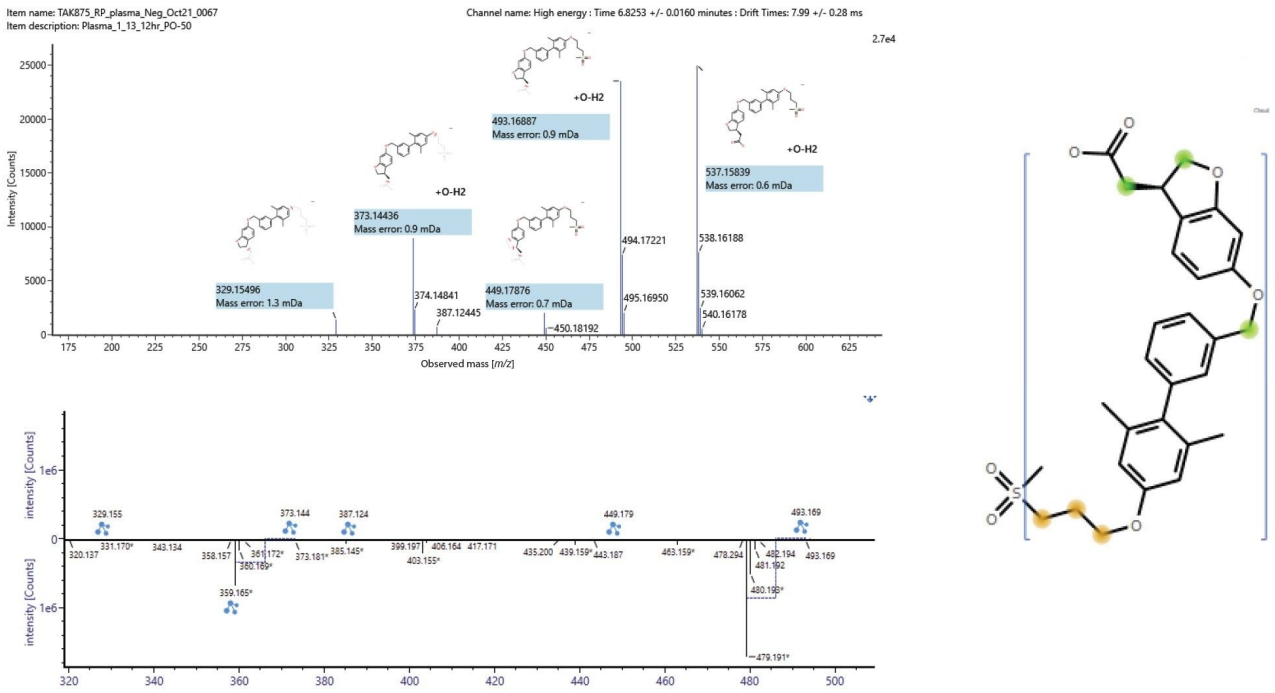


Figure 8. Localization of Site of Metabolism.

Summary Plots and Visualization

The waters_connect Metabolite Identification application facilitates the generation of plots and reports from the reviewed data once an analysis is complete. One of the most useful of these, the summary plot, is depicted in Figure 9, and shows how the concentration of fasiglifam and its major metabolites varies as a function of time post-dose. Here we can see that fasiglifam and the major metabolites follow a similar elimination profile with a maximum concentration observed at 9 h and a gradual reduction in concentration of the metabolites over the following 96 h. However, it is evident that even after 96 h the parent drug and metabolites are still not fully eliminated from the systemic circulation.

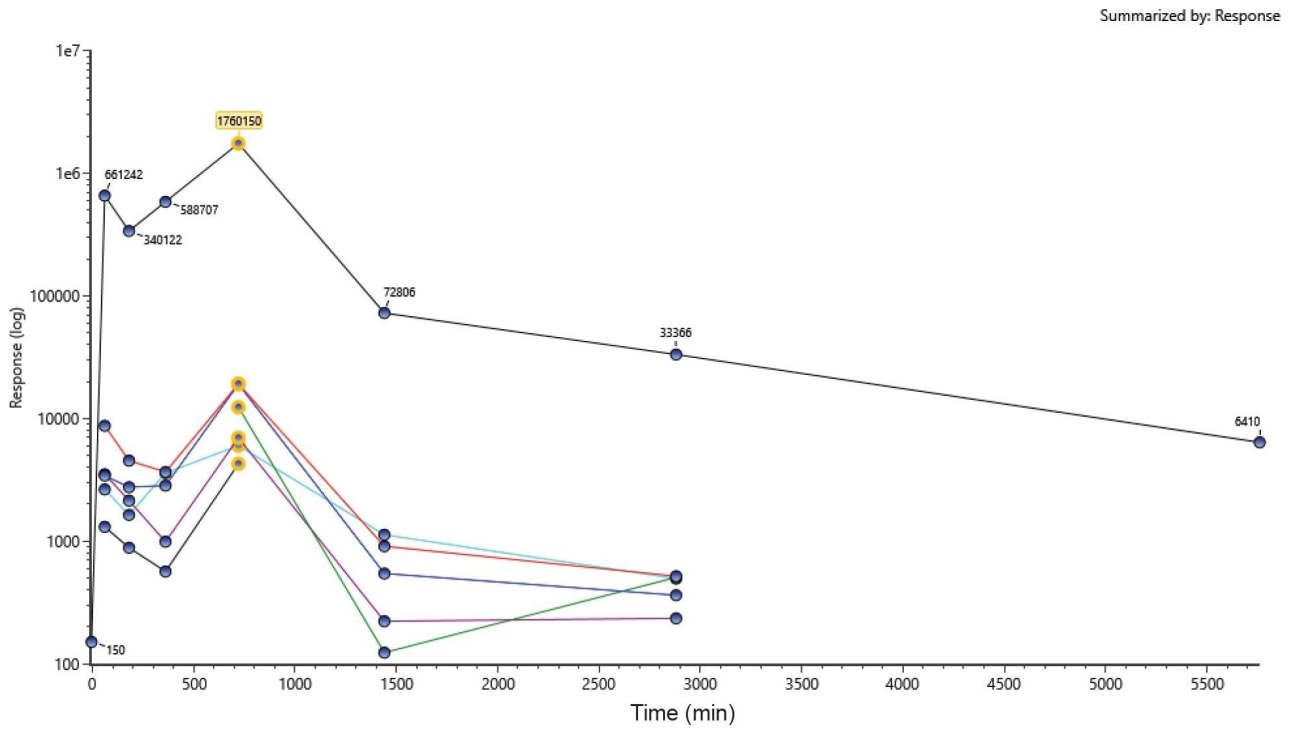


Figure 9. Summary plot of drug and metabolite concentration with sampling time.

Figure 9. Summary plot showing log (response) for the parent drug and a variety of observed metabolites as a function of time post dose (h). Reporting waters_connect has a powerful and flexible reporting function allowing complete customization of outputs based on customer needs. For example, Figure 10 shows a summary table of the metabolites observed in the 9 h sample, automatically labelled in order of increasing retention time.

Item name: TAK875_RP_plasma_Neg_Oct21_0067, Sample position: , Replicate number: 1

Label	Component name	Formula	Observed m/z	Mass error (ppm)	Observed RT (min)	Observed CCS (Å²)	Has Common Neutral Losses	Has Common Fragment Ions	Total Fragments Found	Expected Fragments Found	Response
M1	tak875 M-I	C19H22O5S	361.1111	-1.2	6.03	199.70	Yes	No	1	0	1908
M2	tak875+O	C29H32O8S	539.1739	-1.1	6.22	219.80	Yes	Yes	6	1	6020
M3	tak875-G	C35H40O13S	699.2113	-0.5	6.26	246.26	No	No	1	0	2523
M4	tak875+O2	C29H32O9S	555.1687	-1.2	6.32	222.93	No	Yes	4	2	4281
M5	tak875+O-H2	C29H30O8S	537.1585	-0.7	6.34	220.17	Yes	No	3	0	1036
M6	tak875+O2	C29H32O9S	555.1688	-1.1	6.41	224.27	Yes	No	1	0	787
M7	tak875-tau	C31H37NO9S2	630.1836	-0.2	6.44	238.40	Yes	No	1	0	3834
M8	tak875+O	C29H32O8S	539.1743	-0.3	6.46	220.79	Yes	No	5	0	3812
M9	tak875+O2	C29H32O9S	555.1690	-0.7	6.53	221.09	No	No	12	0	19155
M10	tak875+O	C29H32O8S	539.1738	-1.2	6.54	220.25	No	No	7	0	1162
M11	tak875+O-H2	C29H30O8S	537.1586	-0.5	6.60	219.97	Yes	No	5	0	3173
M12	tak875+O-H2	C29H30O8S	537.1583	-1.1	6.83	218.96	Yes	No	5	0	12403
M13	tak875-CH2(cleavage)	C28H30O7S	509.1636	-0.8	6.83	214.82	Yes	No	4	0	2277
M14	tak875-H2	C29H30O7S	521.1636	-0.6	6.98	217.82	Yes	Yes	8	2	19205
M15	tak875-H2	C29H30O7S	521.1638	-0.3	7.06	218.05	Yes	Yes	7	1	6973

Figure 10. Automatically generated report object documenting the metabolites found in the 9 h sample.

Conclusion

This combination of a data-rich, generic, and robust data acquisition method, combined with powerful analysis tools allows highly complex datasets to be reduced to a set of tractable outcomes which can be reviewed in a relatively short period of time by the drug metabolism scientist. Known metabolites can be included in the analysis method, and a systematic search for other likely metabolites is facilitated by the use of powerful filters which rank components based on their drug-like properties. Ion mobility-enabled mass spectrometry allows clean product ion spectra to be generated without the need to create complex data-dependent acquisition schemes, and further affords compound-specific CCS values which can be used to distinguish isobaric metabolites and track metabolites across chromatographic methods.

References

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3. Kogame A, Lee R, Pan L, Sudo M, Nonaka M, Moriya Y, Higuchi T, Tagawa Y. Disposition and Metabolism of the G Protein-Coupled Receptor 40 Agonist TAK-875 (Fasiglifam) In Rats, Dogs, and Humans. *Xenobiotica*. 2019;49(4):433–445.

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