Utilisation of Ion Mobility Enabled Collisional Cross Section Measurements for the Comparison of Metabolites across Differing Chromatographic Methods

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Introduction

During the conduct of metabolite profiling and identification studies, it is common to analyse samples using multiple chromatographic methods. As studies progress from *in vitro* to *in vivo* and clinical, it is often necessary to further develop chromatographic methods in order to separate and quantify additional metabolites. UPLC methods are ideal for the rapid analysis of non-radioactive samples for early metabolite screening and identification. HPLC methods, on the other hand, with long run times are usually favoured for the analysis of radioactive samples in order to enable suitable dwell times within the radiodetector flow cell for detector sensitivity.

The ability to align and compare the same metabolites, including those that are isomeric, across samples analysed using different chromatographic methods would prove valuable, reducing the need to re-analyse samples using revised methods developed later in the drug development programme and allowing comparisons to be made across studies utilising both HPLC and UPLC methods. Ion Mobility Mass Spectrometry (IMS) offers the potential to discriminate isomeric metabolites based on precise measurement of their ion mobility drift times and collisional cross sectional areas. This experiment assesses the use of IMS to differentiate between structural isomers of metabolites across UPLC and HPLC methods and provide simultaneous structural information using a High Definition Mass Spectrometry (HDMS) platform.

Methods

Nefazodone (10µM), shown in Figure 1, was incubated with cryopreserved rat and human hepatocytes at 37°C for 240 minutes. Incubations were terminated with an equal volume of acetonitrile and centrifuged. The supernatant from the human and rat hepatocyte incubations were combined in order to achieve a more complex matrix.

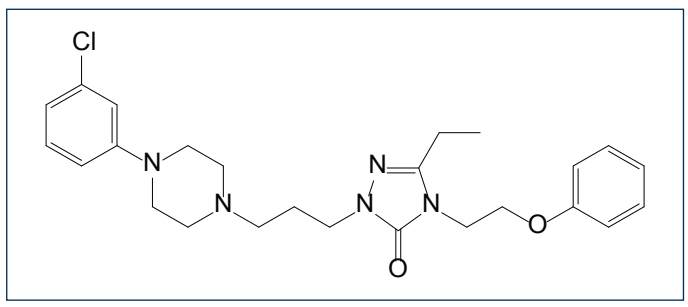

Figure 1. Nefazodone structure.

Samples were analysed using a Waters Acquity® UPLC system coupled to a Waters Vion™ IMS-QTof mass spectrometer with mobility-enabled non-targeted HDMSE scan methods. Calibrated ion mobility experiments were used to generate Collision Cross Section (CCS) values for all metabolites using UNIFI® software (Waters) as part of a metabolite identification workflow.

Method 1 UPLC/MS			
Column:	Waters HSS T3 C18, 2.1 x 50mm, 1.8µm		
Mobile Phase A:	0.1% Formic acid (Aq)		
Mobile Phase B:	0.1% Formic acid in Acetonitrile		
Gradient:	Time (min)	A (%)	B (%)
	0.0	98	2
	5.0	35	65
	5.5	0	100
	6.4	0	100
	6.5	98	2
	8.0	98	2
Flow Rate:	650µL min^{-1}		
Column Temperature:	40°C		
Injection Volume:	3µL		
Ionisation Mode:	Positive ion electrospray		
Scan Range *m/z*:	100-1000		

Method 2 HPLC/MS			
Column:	Agilent Eclipse Plus Hexyl Phenyl, 4.6 x 100mm, 3.5µm		
Mobile Phase A:	25mM Ammonium formate pH4.0		
Mobile Phase B:	Acetonitrile		
Gradient:	Time (min)	A (%)	B (%)
	0.0	98	2
	5.0	98	2
	12.0	60	40
	15.0	0	100
	20.0	0	100
	20.1	98	2
	25.0	98	2
Flow Rate:	1000µL min^{-1}		
Column Temperature:	40°C		
Injection Volume:	3µL		
Ionisation Mode:	Positive ion electrospray		
Scan Range *m/z*:	100-1000		

Results

Automatic calibration of drift time in the mass spectrometer was used to generate Collisional Cross Section (CCS) measurements for all metabolites. Five replicate injections of the test sample were made on each chromatographic method. These measurements show good precision between replicate injections (RSD between 0.05% and 0.42%) and good agreement between chromatographic methods as shown in Table 1.

Correlation of Metabolites across the Different Methods

A total of 29 Nefazodone metabolites were detected across the two methods. In some instances not all isomers were detected using both chromatographic methods; however, where this occurred, CCS measurements were used to confirm which isomer was not detected.

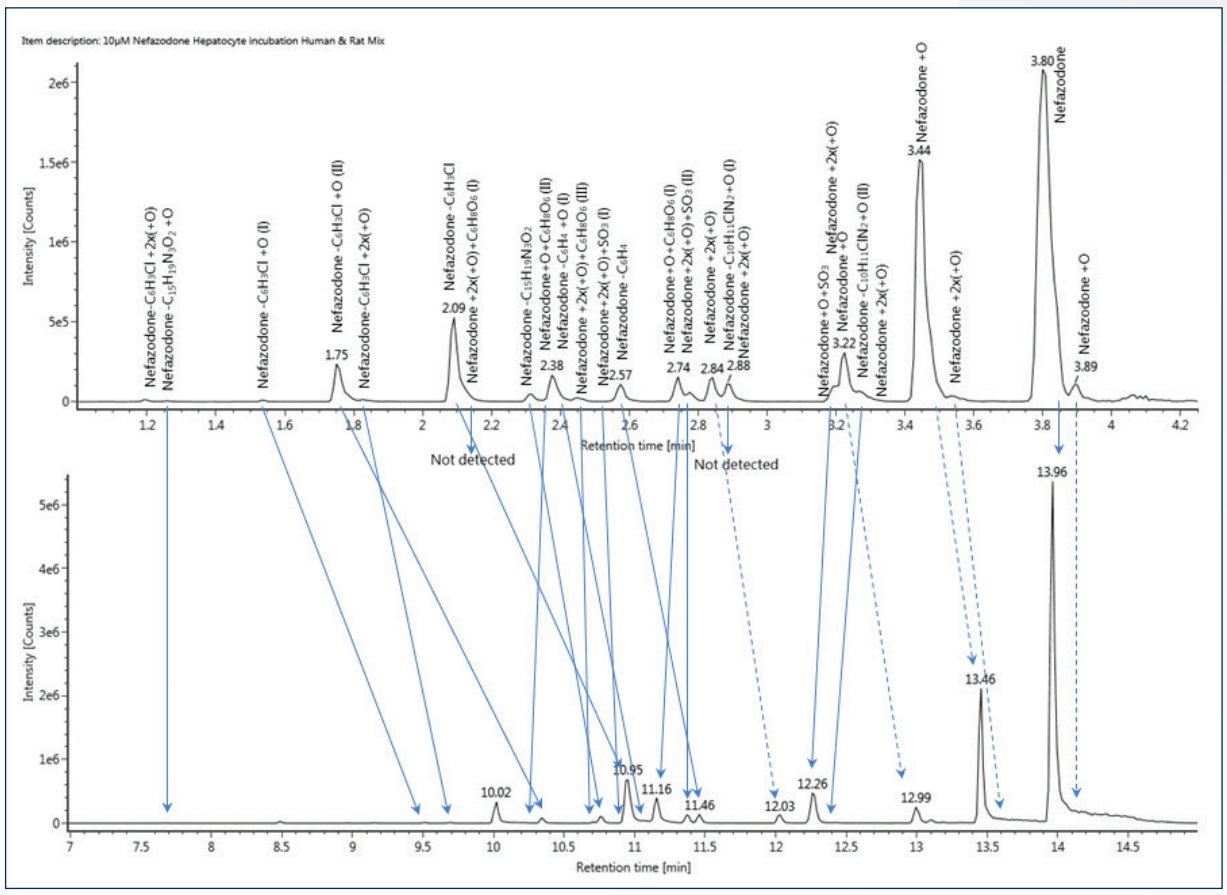

Figure 2. Extracted ion chromatograms of metabolites detected using method 1 (top) and method 2 (bottom) showing metabolites correlated across both methods.

Table 1. Corresponding Nefazodone Metabolites Matched across Method 1 and Method 2 Samples by Comparison of CCS Measurement

		Method 1 UPLC		Method 2 HPLC		
Metabolite	**Theoretical $[M+H]^+$**	**Mean RT (min)**	**Mean CCS (Å²)**	**Mean RT (min)**	**Mean CCS (Å²)**	**Δ% CCS**
Nefazodone	470.2317	3.81	210.28	13.97	210.65	0.18
Nefazodone +O+$C_6H_8O_6$ (I)	662.2587	2.75	255.40	11.17	254.88	-0.21
Nefazodone +O+$C_6H_8O_6$ (II)	662.2587	nd	nd	11.39	239.72	-
Nefazodone +2x(+O)+$C_6H_8O_6$ (I)	678.2536	2.19	252.41	nd	nd	-
Nefazodone +2x(+O)+$C_6H_8O_6$ (II)	678.2536	2.38	258.59	10.35	257.38	-0.47
Nefazodone +2x(+O)+$C_6H_8O_6$ (III)	678.2536	2.46	242.99	10.76	242.72	-0.11
Nefazodone +O+SO_3	566.1834	3.19	228.23	12.27	229.04	0.35
Nefazodone +2x(+O)+SO_3 (I)	582.1783	2.55	224.18	10.94	224.46	0.12
Nefazodone +2x(+O)+SO_3 (II)	582.1783	2.78	232.68	11.38	233.44	0.33
Nefazodone -C_6H_4	394.2004	2.58	196.10	11.47	196.13	0.02
Nefazodone -C_6H_4 +O (I)	410.1953	2.38	198.24	10.98	198.49	0.13
Nefazodone -C_6H_4 +O (II)	410.1953	nd	nd	11.21	198.77	-
Nefazodone -C_6H_3Cl	360.2394	2.10	179.46	10.95	179.82	0.20
Nefazodone -C_6H_3Cl +O (I)	376.2343	1.54	183.86	9.52	183.19	-0.36
Nefazodone -C_6H_3Cl +O (II)	376.2343	1.76	182.49	10.42	182.53	0.02
Nefazodone -C_6H_3Cl +2x(+O)	392.2292	1.83	185.12	9.70	185.00	-0.07
Nefazodone -$C_{10}H_{11}ClN_2$+O (I)	292.1655	2.89	199.40	nd	nd	-
Nefazodone -$C_{10}H_{11}ClN_2$+O (II)	292.1655	3.28	164.07	12.28	164.09	0.01
Nefazodone -$C_{15}H_{19}N_3O_2$	197.0840	2.32	143.13	10.78	142.62	-0.36
Nefazodone -$C_{15}H_{19}N_3O_2$ +O	213.0789	1.27	145.05	7.69	144.58	-0.33

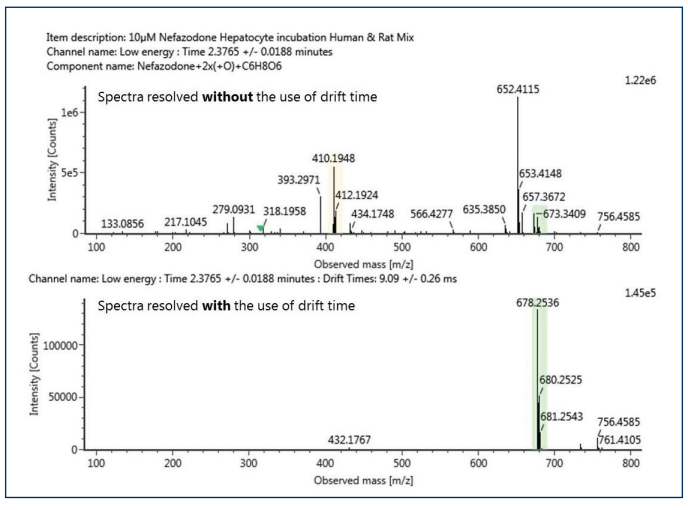

Figure 3. Low energy spectra resolved without the use of drift time alignment (top) and with the use of drift time alignment (bottom).

A total of 19 metabolites were successfully correlated between the two methods using accurate mass and CCS measurements. 13 of these metabolites (many of which were isomeric) could not have been matched by accurate mass alone. Table 1 shows the correlation of metabolites between chromatographic systems.

Some metabolites, Nefazodone +O +Gluc, Nefazodone +2x(+O) +Gluc and Nefazodone -$C_{10}H_{11}ClN_2$ +Gluc, formed isomers, some of which were not observed in both methods, however, using CCS measurements, it could be determined which isomeric forms were not detected across both methods.

Figure 2 shows extracted ion chromatograms of the metabolites detected in the samples from method 1 and method 2. The arrows depict the metabolites that were able to be correlated across both methods.

Three isomers of Nefazodone -C_6H_3Cl +2x(+O) were detected; two observed using method 1 and all 3 observed using method 2. One of these metabolites could be matched across both methods by its CCS measurement (*ca* 185 Å²), however, the CCS values generated for the other two isomeric metabolites were very similar (*ca* 186, 187 Å²), therefore it could not be determined which metabolite was not detected in method 1.

In this experiment, four isomers were detected for two metabolites, (Nefazodone +O and Nefazodone +2x(+O)). There was no significant difference in the CCS measurements between isomers and therefore it was not possible to confidently correlate the isomers across the two methods by CCS measurements and accurate mass alone. Further characterisation of fragmentation patterns and elution profiles enabled these to be further resolved.

Overall the data shows that IMS can be routinely used alongside accurate mass, fragmentation patterns and elution profiles to correlate metabolites across differing methods where there is a measurable difference in the CCS measurements. This could ultimately save repeating experiments performed using earlier methods and would allow the use of the most appropriate methods (UPLC or HPLC) for the type of analysis being performed without losing the ability to compare the results.

Drift Time Alignment

The use of ion mobility drift times allows the differentiation of metabolites from background matrix. Drift time alignment of the ions passing through the drift tube removes background ions and generates much cleaner low and high energy spectra. This provides greater confidence in the assignment of compound related metabolites and associated structures. Figure 3 shows the difference between spectra resolved with and without drift time alignment.

Conclusions

- CCS measurements were consistent and reproducible across sample replicates
- CCS measurements were independent of experimental conditions
- IMS can be used to correlate metabolites across differing methods where there is a measurable difference in the CCS measurements
- Spectra resolved using drift time alignment were cleaner with less background noise

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