Evaluation of Ion Mobility Enabled Collisional Cross Section Measurements for the Differentiation of Acyl and Phenolic Glucuronide Metabolites

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Introduction

Acyl glucuronidation is a common metabolic conjugation reaction of carboxylic acid drugs. Under physiological conditions, these reactive metabolites can isomerise by acyl migration, transferring the acyl group from the C-1 position of the glucuronic acid ring to the C-2, C-3 and C-4 positions, resulting in the formation of isomeric acyl glucuronides. They can also undergo spontaneous hydrolysis and nucleophilic substitution reactions with proteins. The formation of these metabolites can, in some cases, raise concerns over drug safety due to potential toxicity of the acyl glucuronide conjugates. Regulatory guidance (FDA, MIST, ICH M3 (R2)) recommends that if toxic compounds, such as acyl glucuronides, form then "additional safety assessment may be needed"[1, 2]. Therefore the ability to distinguish between acyl, migrated acyl and non-acyl glucuronides during metabolite identification studies would provide important information in the drug's development program.

Ion Mobility Mass Spectrometry (IMS) offers the potential to discriminate phenolic and acyl glucuronide metabolite isomers based on precise measurements of their ion mobility drift times and collisional cross sectional areas. This evaluation assesses the ability of routine IMS to differentiate between structural isomers of carboxylic acid drug metabolites and provide simultaneous structural information using an HDMS platform.

Methods

Generic carboxylic acid compounds (Naproxen and Diflunisal) (10µM) were incubated with cryopreserved rat hepatocytes at 37°C for 240 minutes. Incubations were terminated with an equal volume of acetonitrile, centrifuged and the supernatant submitted for analysis. For deconjugation experiments, organic solvent was removed from the samples by nitrogen convection prior to incubation with β-glucuronidase at 37°C for 2 hours. For matrix spiked experiments, samples were diluted 1:1 (v/v) with control urine.

Samples were analysed using a Waters ACUITY® UPLC system coupled to a Waters VION™ IMS-QTof mass spectrometer with mobility-enabled non-targeted HDMS^E scan methods (Waters Technologies Corporation). Calibrated ion mobility experiments were used to generate Collision Cross Section (CCS) values for all glucuronide conjugate isomers using UNIFI® software as part of a metabolite identification workflow.

UPLC/MS Method

Column:	Waters HSS T3 C18, 2.1 x 50 mm, 1.8 µm		
Mobile Phase A:	0.1% Formic acid (Aq)		
Mobile Phase B:	Acetonitrile		
Gradient:	Time (min)	A (%)	B (%)
	0.0	95	5
	0.5	95	5
	6.0	50	50
	6.5	5	95
	6.9	5	95
	7.0	95	5
	8.0	95	5
Flow Rate:	650µL min^{-1}		
Column Temperature:	40°C		
Injection Volume:	1-10 µL		
Ionisation Mode:	Negative ion electrospray		
Scan Range *m/z*:	100-1000		

Results

Differentiation of Migrated Acyl Glucuronide Metabolites

Several peaks for acyl glucuronide isomers (*m/z* 405.1191) were observed for Naproxen (structures shown in Figure 1) indicating the position C-1 acyl glucuronide peak and 3 migrated forms, all with identical HDMS^E fragment-ion spectra. The peak eluting at a retention time of *ca* 4.00 minutes was confirmed as the position C-1 acyl glucuronide by comparison with a reference standard and deconjugation experiments with β-glucuronidase. The peaks at retention times of *ca* 3.92, 3.82 and 3.68 minutes were indicative of migrated acyl glucuronide isomers (Figure 2).

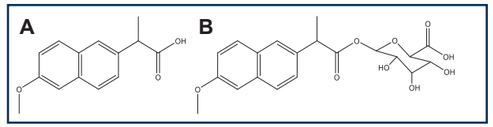

Figure 1. Structures of Naproxen (A) and Naproxen acyl glucuronide (B).

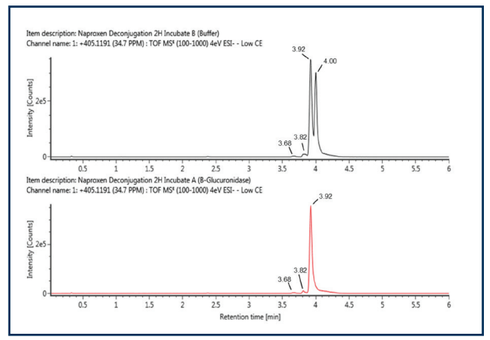

Figure 2. Extracted ion chromatograms for Naproxen glucuronide (*m/z* 405.1191) following incubation in buffer (Top) and incubation with β-glucuronidase (Bottom). The peak at 4 minutes is not detected following incubation with β-glucuronidase, indicating the position C-1 acyl glucuronide.

Automatic calibration of drift time in the mass spectrometer was used to generate Collisional Cross Section (CCS) measurements for the observed molecular ions (Table 1). The data show a consistent, measurable difference in the CCS between the position C-1 acyl glucuronide (216 Å^2) and the migrated forms (212 Å^2), whereas there is no distinguishable difference in CCS between the migrated forms themselves. The precision of the measurements remain consistent in the presence of sample matrix (urine) (RSD 0.08-0.19%).

Table 1. CCS Measurements Observed for Position C-1 Acyl Glucuronide and Migrated Forms

		CCS (Å^2)										
		Hepatocyte incubates undiluted		Hepatocyte incubates spiked with Naproxen Acyl Glucuronide reference standard						Mean	ST Dev	RSD %
Position	ca RT (min)											
Acyl Gluc C-1	4.00	-	-	215.66	216.07	216.18	216.06	216.13	216.17	216.05	0.195	0.090
Migrated 1	3.92	211.56	211.78	212.36	212.65	212.5	212.47	212.46	212.17	212.24	0.384	0.181
Migrated 2	3.82	213.42	213.03	-	-	-	-	-	-	213.23	-	-
Migrated 3	3.68	212.17	212.31	212.78	212.05	212.48	212.14	213.23	212.38	212.44	0.392	0.185
Hepatocyte incubates spiked with Naproxen Acyl Glucuronide reference standard, spiked into control matrix (Urine)												
Acyl Gluc C-1	4.00	215.91	216.09	215.81	216.2	216.27	215.92			216.06	0.191	0.089
Migrated 1	3.92	211.82	211.8	211.38	211.2	211.42	211.13			211.41	0.301	0.143

Blank cells were due to low responses for the corresponding glucuronide ions and therefore no CCS measurements were obtained.

The data indicate that routine IMS and CCS measurements could be used to confirm the presence of migrated acyl glucuronide isomers, and differentiate between the position C-1 isomer and the migrated forms, without the use of expensive reference compounds or lengthy deconjugation experiments.

Differentiation of Acyl and Phenolic Glucuronide Metabolites

Several peaks for Diflunisal glucuronide isomers (*m/z* 425.0689) were observed, indicating both phenolic and migrated acyl glucuronide isomers may form (Figure 3). There was little variation observed with the CCS values for the different isomer peaks (*ca* 210.7 Å^2) which was surprising considering the results observed for the Naproxen samples.

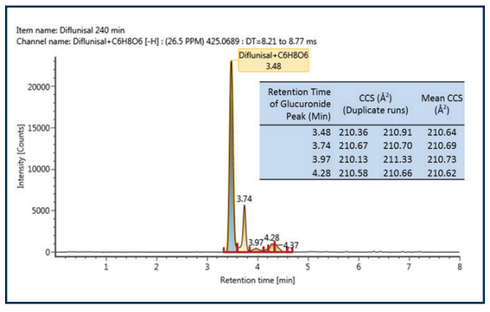

Figure 3. Extracted ion chromatogram for Diflunisal glucuronide (*m/z* 425.0689) and measured CCS values for the molecular ions (insert).

This may be due to the close proximity of the carboxylic acid and the hydroxyl group with the glucuronic acid (Figure 4), and the presence of sterically bulky fluorine substituents, reducing any apparent or potential mobility separations of the positional glucuronide isomers. The effect of this could lead to indistinguishable CCS measurements between the glucuronic acid isomers of compounds with sterically unfavourable conjugation or substituents. Further work is being carried out with similar compounds that contain both hydroxyl and carboxylic acid groups in close proximity to one another, in order to more definitively confirm this theory; in which case this may be a limitation of routine IMS for this type of analysis.

Further analysis is also being conducted in order to ascertain whether CCS values can be used to differentiate between phenolic and acyl glucuronides in compounds which contain hydroxyl and carboxylic acid groups, or sterically bulky substituents, that are not in close proximity.

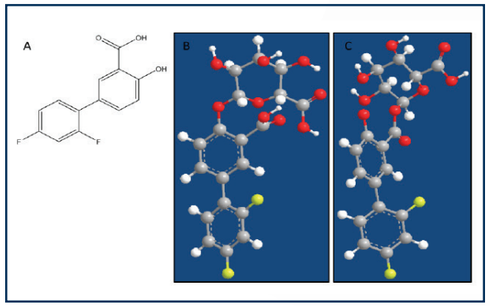

Figure 4. Structures of Diflunisal (A), Diflunisal phenolic glucuronide (B) and Diflunisal acyl glucuronide (C) showing the close proximity of either the hydroxyl group or the carboxylic group to the glucuronide.

Conclusions

- Routine IMS and CCS were successful at discriminating between isomeric metabolites using a measurable parameter CCS other than only mass-to-charge
- IMS was able to confirm the presence of migrated acyl glucuronide isomers and differentiate between the Naproxen position C-1 acyl glucuronide and the migrated forms with consistent accuracy
- CCS measurements remained reproducible in the presence of sample matrix
- IMS was unable to differentiate between phenolic and acyl glucuronides for Diflunisal; possibly due to the close proximity of the carboxylic acid and hydroxyl groups on the molecule
- Further analysis is being conducted to ascertain whether IMS can be used to differentiate between phenolic and acyl glucuronides for other compounds

References

[1] FDA. Guidance for Industry. Safety Testing of Drug Metabolites, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Feb 2008, Pharmacology and Toxicology

[2] ICH Topic M3 (R2) Non-Clinical Safety for the Conduct of Human Clinical Trials and Marketing Authorisation for Pharmaceuticals, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2009

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