CID FOR THE IDENTIFICATION OF DRUG METABOLITES USING UPLC/OA-TOF TECHNOLOGY

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INTRODUCTION

Here we present data using high resolution liquid chromatography coupled with collision induced dissociation (CID) and exact mass measurement with oa-TOF for metabolism studies. Combining the chromatographic resolution of UPLC™ with pseudo MS/MS (CID) and exact mass to determine actual elemental compositions, provides greater confidence in metabolite confirmation. Accurate mass fragment information from CID allows structural information to be derived for the metabolites. When using CID rather than traditional MS/MS, the enhanced resolution of UPLC helps to reduce co-elution and produce clean fragment spectra for easier datainterpretation. When using this approach a routine and rugged hardware configuration is required.

Here we used a dual reference sprayer (LockSpray™) on a Waters LCT Premier XE™ oa-TOF, as shown in Figure 2. LockSpray provides a single reference peak against which subsequent mass spectra are accurately mass measured. Thus, exact mass measurements below 3ppm RMS can be routinely obtained over extended periods of time. The fragmentation is performed by increasing the voltage on a lens within the high pressure region of the transfer optics generating CID. Novel instrumentation and software enables different lens voltage settings to be applied to the analyte and reference acquisition functions. This allows different experiments to be performed using the same lock mass ion. Both MS and pseudo MS/MS experiments can be performed in one analysis by using rapidly alternating MS functions.

Figure 1 A picture showing the Waters LCT Premier XE™ Metabolite ID system solution .

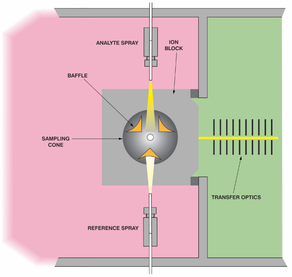

Figure 2. Schematic of the LockSpray™ source in the LCT Premier XE™.

METHODS

Sample Preparation

Human microsomes (S9) with a protein content of 1mg/mL were used to incubate two drug candidates; dextromethorphan and verapamil at a 5µM level. The reaction was stopped by adding an equal volume of ice cold acetonitrile at three time points (15, 30 and 60 minutes). The sample was centrifuged at 15,000 rpm and the supernatant taken for analysis by UPLC/oa-Tof.

UPLC Conditions

System: Waters ACQUITY UPLC™
Column: Waters ACQUITY UPLC™ BEH C18 (100 mm x 2.1 mm,1.7 µm)
Column temperature: 45°C
Flow Rate: 0.6 mL/min
Mobile phase: A: H2O (0.1% HCOOH) B: MeCN (0.1% HCOOH)

Gradient:	Time (mins)	%A	%B	Curve
	0	95	5	1
	15	5	95	6
	18	5	95	6
	18.1	95	5	6

Run time: 21 mins
Injection volume: 10µL

MS Conditions

Mass Spectrometer: Waters® LCT Premier XE™
Ionization Mode: ESI+ at 3kV
Sample cone voltage: 35V
Reference mass: Leucine enkephalin, $[M+H]^+$ = 556.2771
Acquisition Parameters: 50-1000 *m/z;*
0.15 second/spectrum; 0.05 second inter acquisition delay
Resolution: 12 000 FWHM (W mode)
CID voltage: 45V
Non CID voltage: 5V

RESULTS

The generic UPLC gradient described in the experimental section was used to separate the un-metabolised parent compound from the *in vitro* formed metabolites to enable clean spectra to be obtained for easy data interpretation.

Presented in Figure 3 are the major metabolites formed for the *in vitro* incubation of verapamil. The exact mass chromatograms are shown for the un-metabolised parent (A), the metabolites (B) O-demethylation, (C) 2 x O-demethylation (2 isomers C1 and C2) and (D) a metabolic cleavage. The proposed structures are annotated on the chromatograms based upon the exact mass CID spectra obtained. Figures 4 and 5 show the exact mass collision induced dissociation spectra for the un-metabolised parent verapamil and the O-demethylated metabolite respectively. Proposed fragmentation structures shown are based upon exact mass errors, i-FIT (isotopic fit value) and double bond equivalence agreement, all present within MassLynx™ software. Table 1 shows the mass measurement accuracy of 2.5ppm RMS was obtained for the un-metabolised parent, metabolites of verapamil and their respective fragment ions.

Presented in Figure 6 are the major metabolites formed for the *in vitro* incubation of dextromethorphan. The exact mass chromatograms and proposed structures are shown for the un-metabolised parent (W), the metabolites (X) O-demethylation, (Y) N-demethylation and (Z) N,O-demethylation. Figures 7 and 8 show the exact mass collision induced dissociation spectra for the un-metabolised parent verapamil and the N,O-demethylated metabolite respectively. The proposed structures for the fragments are annotated. Table 2 shows the mass measurement accuracy of 2.7ppm RMS was obtained for the un-metabolised parent, metabolites of dextromethorphan and their respective fragment ions.

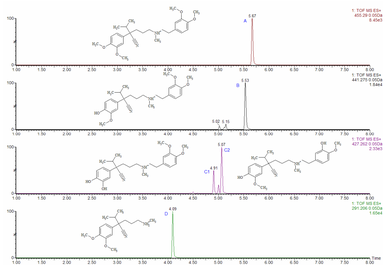

Figure 3 Exact mass chromatograms for the major metabolites formed for the in vitro incubation of verapamil, at T=30mins.

Compound	Formula	m/z theoretical	m/z observed	ppm	mDa
Verapamil (A)	C27H39 N2O4	455.291	455.2915	1.1	0.5
Verapamil fragment 1	C18H27N2O2	303.2073	303.2077	1.3	0.4
Verapamil fragment 2	C10H13O2	165.0916	165.0907	-5.5	-0.9
O-Demethylation (B)	C26H37N2O4	441.2753	441.2743	-2.3	-1.0
O-Demethylation fragment 1	C17H25N2O2	289.1916	289.1927	3.8	1.1
O-Demethylation fragment 2	C10H13O2	165.0916	165.0912	-2.4	-0.4
2xO-Demethylation (C1)	C25H35N2O4	427.2597	427.2605	1.9	0.8
2xO-Demethylation (C1) fragment	C10H13O2	165.0916	165.0919	1.8	0.3
2xO-Demethylation (C2)	C25H35N2O4	427.2597	427.26	0.7	0.3
2xO-Demethylation (C2) fragment	C9H11O2	151.0759	151.0759	0.0	0.0
Metabolic Cleavage (D)	C17H27N2O2	291.2073	291.2071	-0.7	-0.2
Metabolic Cleavage fragment	C16H22NO2	260.1651	260.1643	-3.1	-0.8
			RMS	2.5179	0.6513

Table 1 Table to show the exact mass errors for verapamil, its metabolites and their associated fragments at T=30mins.

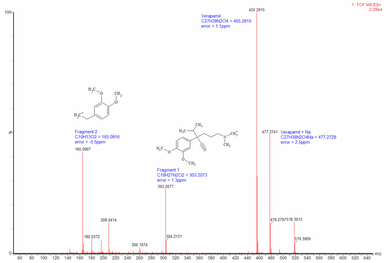

Figure 4 Exact mass spectra for the CID of the un-metabolised parent drug verapamil, at T=30mins.

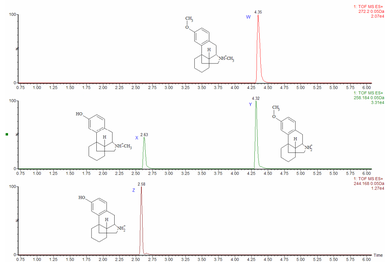

Figure 6 Exact mass chromatograms for the major metabolites formed for the in vitro incubation of dextromethorphan, at T=30mins.

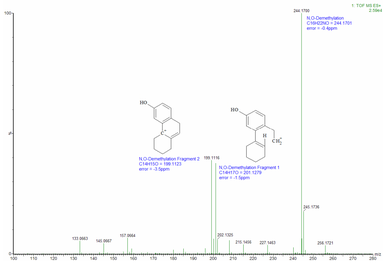

Figure 8 Exact mass spectra for the CID of N,O-demethylation of dextromethorphan, at T=30mins.

Compound	Formula	m/z theoretical	m/z observed	ppm	mDa
Dextromethorphan (W)	C18H26 NO	272.2014	272.2013	-0.4	-0.1
Dextromethorphan fragment 1	C15H19O	215.1436	215.1438	0.9	0.2
Dextromethorphan fragment 2	C15H17O	213.1279	213.1285	2.8	0.6
N-demethylation (Y)	C17H24NO	258.1858	258.1852	-2.3	-0.6
N-demethylation fragment 1	C15H19O	215.1436	215.1425	-5.1	-1.1
N-demethylation fragment 2	C15H17O	213.1279	213.1273	-2.8	-0.6
O-demethylation (X)	C17H24NO	258.1858	258.1852	-2.3	-0.6
O-demethylation fragment 1	C14H17O	201.1279	201.1271	-4.0	-0.8
O-demethylation fragment 2	C14H15O	199.1123	199.1118	-2.5	-0.5
N,O-demethylation (Z)	C16H22NO	244.1701	244.17	-0.4	-0.1
N,O-demethylation fragment 1	C14H17O	201.1279	201.1276	-1.5	-0.3
N,O-demethylation fragment 2	C14H15O	199.1123	199.1116	-3.5	-0.8
			RMS	2.7409	0.6007

Table 2 Table to show the exact mass errors for dextromethorphan, its metabolites and their associated fragments at T=30mins.

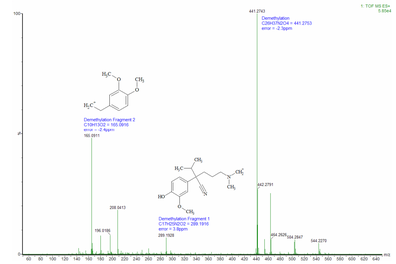

Figure 5 Exact mass spectra for the CID of the in vitro formed O-demethylation metabolite at T=30mins.

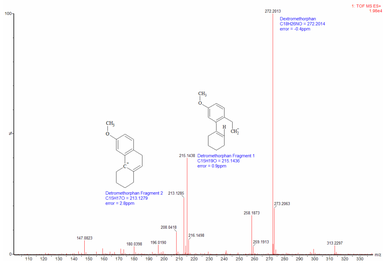

Figure 7 Exact mass spectra for the CID of dextromethorphan, at T=30mins.

DISCUSSION

The parent drugs verapamil and dextromethorphan were incubated *in vitro* using human S9 microsomes. Using UPLC/oa-Tof with full spectra CID ion accumulation with good sensitivity, enables all the major metabolites formed to be determined. Pseudo MS/MS fragmentation was enabled by increasing the voltage on a lens within the high pressure region of the transfer optics generating CID. Novel instrumentation and software enables different lens voltage settings to be applied to the analyte and reference acquisition functions allowing non-CID spectra and CID spectra to be acquired using the same lock mass ion. From the data presented it is shown that mass measurement error within 5ppm error can be obtained for parent ion and the fragment ions produced. The chromatographic resolution produced using UPLC adds futher dimension to selectivity, which could not be achieved using conventional HPLC. As a result single component CID spectra can be produced in combination with full spectra acquistion in one analysis. Also in many cases the combination of accurate mass measurement and i-FIT software resulted in a single elemental composition being determined for the parent ions and fragment ions. The combination of UPLC and oa-TOF maximises the metabolite profile information obtained from a single sample with minimal time.

CONCLUSIONS

- **The highly specific nature of exact mass measurement enables elemental compositions to be rapidly determined providing the correct identification of the metabolites formed.**
- **Greater confidence in identifying the drug metabolites is provided.**
- **Elemental composition of fragments allows rapid identification and structural information to be gained.**
- **Maximum information is obtained rapidly from a single sample.**
- **The powerful combination of the enhanced chromatographic resolution associated with UPLC™ technology with the high resolution capabilities of the LCT Premier XE™ has allowed clean CID spectra to be obtained 2.7ppm RMS.**

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