

# High Throughput Identification of Irinotecan Metabolites in Tissue Homogenates Using MS<sup>n</sup> and a 10 Hz Cycle Time Linear Ion Trap

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## Overview

**Purpose:** To test the sensitivity and reduced cycle time of a new linear ion trap for the analysis of complex liver extracts and to establish its effectiveness for use in metabolism studies.

**Methods:** Livers from Irinotecan-treated mice were homogenized and acetonitrile-precipitated to produce tissue extracts. The mass spectrometer used is a novel dual cell linear ion trap with improved sensitivity and cycle time.

**Results:** Parent drug and metabolites were detected in liver samples from mice undergoing two different dosing treatments. The results were obtained on a shortened gradient and compressed chromatographic time scale using a single, 8 MS<sup>2</sup> scan event experiment.

## Introduction

A major goal in metabolism studies, is to improve confidence in the identification and quantification of compounds that are the result of *in vivo* transformations. Ideally, this should occur in an automated, unattended fashion, as fast as possible and with the least amount of sample preparation. Unfortunately, sample complexity is high, as drug metabolites are often analyzed from complex matrices such as urine, plasma or tissue extracts. The *cycle time* in an ion trap refers to the time it takes to complete a series of events, starting with sample injection and ending with ion detection. Linear ion traps are ideal instruments for metabolism studies due to their high sensitivity in full scan mode, ability to perform consecutive MS<sup>n</sup> experiments (important for structural elucidation and confirmation) and wide dynamic range (important as sample complexity increases).

Irinotecan (Camptosar<sup>®</sup>, Pfizer) has strong anti-tumor activity against a variety of human tumors as the drug binds to and prevents dissociation of the DNA topoisomerase I complex, which is involved in DNA replication. Irinotecan inhibits enzyme activity and thus the DNA replication process. Nude mice bearing transplanted human head and neck FaDu tumors underwent four different treatments: untreated (control), methylselenocysteine (MSC) alone, irinotecan alone, or both drugs in combination. MSC has been shown to modulate the efficacy of irinotecan against FaDu xenografts<sup>1</sup>. The drug-treated liver samples and controls (without treatment) were used in the present study, while the FaDu tumors were used for MALDI analysis by tissue imaging in a previous work<sup>2</sup> (see also H. Buy et al., Poster Th167, ASMS 2009).

In this study we test the sensitivity and performance of a new linear ion trap, the LTQ Velos<sup>™</sup> (Thermo Fisher Scientific, CA, USA), to sample a chromatographic peak at a fast scan rate for the simultaneous identification of irinotecan and metabolites from complex samples. In view of the results, we discuss its significance for metabolite studies.

## Methods

**Sample Preparation:** Mice were treated with MSC orally, 0.2 mg/mouse/day daily for seven days, then irinotecan (100 mg/kg) was given by i.v. injection, either alone or added to MSC-treated mice. Livers were excised, flash-frozen and kept at -80°C until ready to use. Liver samples were weighed and water added (5% w/v). The mixture was homogenized using disposable pestles and vials. Around 30-35 strokes were sufficient for tissue homogenization. Acetonitrile (2:1, v:v) was added for protein precipitation, vortexed for 1 minute and centrifuged at 6000g for 30 minutes. The supernatants were transferred to centrifuge tubes and their volumes reduced under nitrogen gas. Camptothecin (m/z 393), a homolog that is not a metabolite, was used as internal standard, added prior to protein precipitation as a process control. Prior to analyzing the tissue homogenates, quantitation curves were generated for a 3 component sample: irinotecan C<sub>23</sub>H<sub>38</sub>N<sub>4</sub>O<sub>6</sub> MW 587.14, metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) C<sub>22</sub>H<sub>20</sub>N<sub>2</sub>O<sub>5</sub> MW 392.40 and S-(+)-Camptothecin C<sub>20</sub>H<sub>16</sub>N<sub>2</sub>O<sub>4</sub> MW 348.35 (internal standard) using isocratic elutions (30% organic). All standards purchased from Sigma (St. Louis, MO), solvents were from Fisher Scientific (USA). **Chromatography:** Five µL (full loop) were injected using a Thermo Scientific Accela<sup>™</sup> autosampler and pump, onto a 50mm x 2.1mm, 1.9µm C18 Hypersil Gold<sup>™</sup> column (Thermo Fisher Scientific). Compounds were eluted using a 10% to 35% organic gradient or 10% to 90% organic gradient over 5 or 15 min, respectively, depending on sample complexity. The solvents were: A = acetonitrile/20mM ammonium acetate, pH 3.5 (20:80, v/v) and B = acetonitrile, 0.1% formic acid (FA).

FIGURE 1. Schematics of the LTQ Velos, with an S-lens for higher sensitivity and a dual-pressure ion trap for the efficient optimization of ion processes. HPC = high pressure cell; LPC = low pressure cell

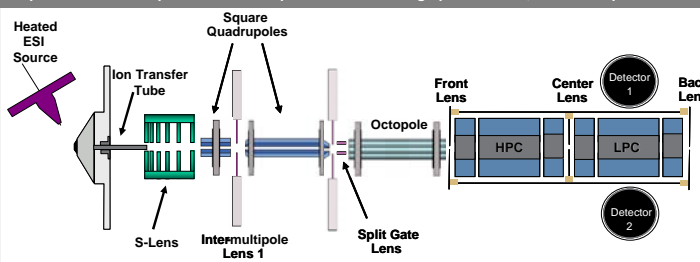
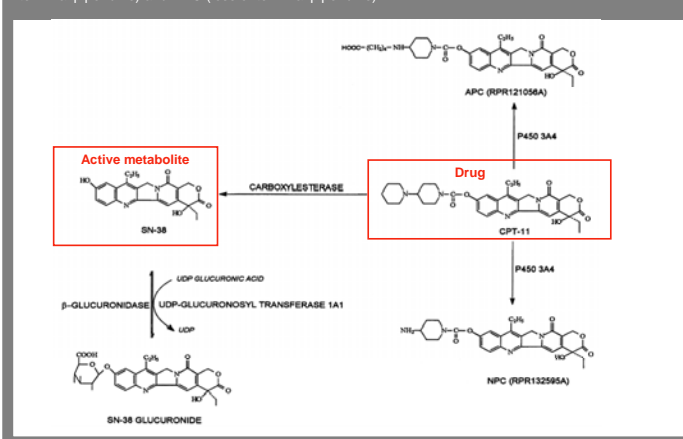


FIGURE 2. Metabolism of Irinotecan into SN-38, SN-38G (glucuronide), APC (double oxidation of terminal piperidine) and NPC (loss of terminal piperidine).



## Methods (continued)

**Mass Spectrometry:** Five replicates of standards and blanks were alternated in an Xcalibur<sup>™</sup> sequence for determining limits of detection and quantitation. Tissue extracts were filtered through 0.2 micron disposable filters prior to LC/MS analysis. An LTQ Velos (Fig. 1) mass spectrometer equipped with a heated electrospray source to aid desolvation (HESI-II<sup>™</sup>, Thermo Fisher Scientific) was used for the measurements. An 8 scan event MS<sup>2</sup> experiment was conducted, with Automatic Gain Control (AGC), 1 uscan and 100 ms MS<sup>2</sup> maximum injection time. Areas under the chromatographic peaks were obtained using Genesis peak detection algorithm and analyzed in Xcalibur.

## Results

The limit of detection for irinotecan was found to be about 100 fg on column in either plasma or buffer (20 µg/L X 5 µL injected) and about 1 pg on column for the SN-38 metabolite. Limits of quantitation have been reported at about 1.5 pg/µL for irinotecan and SN-38<sup>4</sup>. The MS response for SN-38 was found to be lower than for irinotecan in our work and for this reason, SN-38 working solutions were prepared twice taking care to closely follow accepted dilution protocols<sup>4</sup>.

The 1.9 micron LC column coupled to the LTQ Velos, allowed for the screening of complex tissue extracts with shorter analysis times, 15 min gradients compared to the previously reported LC gradients of 35 min<sup>3</sup>.

FIGURE 3. Irinotecan spiked in mouse plasma or 20/80 buffer (ACN/20mM Amm. Acetate, pH 3.5) normalized by IS (25 pg on column): a) %RSD b) limit of detection is 100 fg on column.

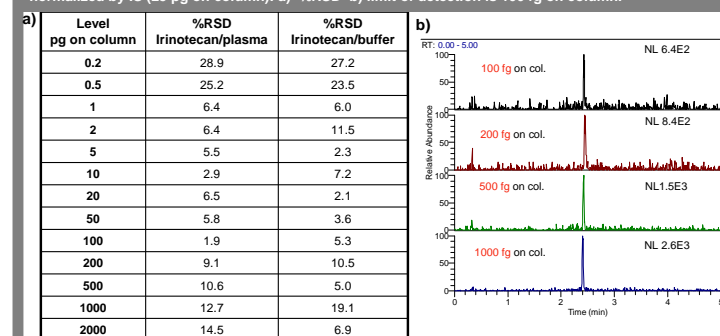


FIGURE 4. Ratios of irinotecan to active metabolite SN-38 in samples from two treatments: irinotecan-dosed and irinotecan plus MSC-dosed animals.

Sample treatment	Area IS Process (Genesis)	Area SN-38 (Genesis)	Area Irinotecan (Genesis)	(Area irinotecan)/(area SN-38)*	Liver weight (g)	Comments
Irinotecan-treated						
Liver 1a, LCMS 1	84,324	76,928	678,157	8.8	0.1655	LCMS 1 and 2 = same sample, diff runs
Liver 1a, LCMS 2	79,578	81,526	788,939	9.7		
Liver 1b, LCMS 1	87,505	25,142	360,392	14.3	0.1355	Liver 1a and 1b = 2 samples from same liver
Liver 1b, LCMS 2	123,907	28,652	327,982	11.4		
Irinotecan + MSC						
Liver 2a, LCMS 1	78,773	44,581	47,927	1.1	0.0702	Liver 2a and 3b = diff liver samples
Liver 3b, LCMS 1	90,118	20,653	87,345	4.2	0.0627	
Control	78,984	ND	ND			

\*All samples were reduced to about 1 mL in volume, filtered through 0.2 micron filters and analyzed without further dilution.

## Results (continued)

Figure 4 shows chromatographic area ratios for drug to metabolite of interest, SN-38. The amount of irinotecan in liver samples is, on average, about 10 times more abundant than SN-38, for the irinotecan-only dosed animals. This agrees with mouse plasma and liver levels reported in the literature<sup>4</sup>, where concentrations maintain a ~10:1 ratio (irinotecan:SN-38) as both are metabolized with time. However, liver samples from 'drug plus MSC' treated mice display a lower ratio of drug to SN-38 metabolite as compared to the drug-only treatment. Less drug appears to remain in liver tissue for 'drug plus MSC' treated animals, while the metabolite remains at about the same abundance level. This is an important result as selenium compounds such as MSC have been shown to offer protection against toxicity induced by chemotherapeutic agents<sup>1</sup>. Selenium compounds at the same time have been shown to increase the cure rate of both irinotecan sensitive and resistant human tumor xenografts in nude mice.

Figures 5 and 6 show the tremendous benefit of using a faster cycle time ion trap for metabolism studies. An experiment consisting of 8 scan events was set up using Xcalibur software, with each a different MS/MS, corresponding to expected compounds in the sample. The 8 MS/MS experiment repeats 10 times during a typical chromatographic peak, as shown in Figure 6a. In this example the cycle time is shown to be about 3.3 Hz (80 MS<sup>2</sup>/24 sec), due to the long 100ms injection time chosen.

Figure 7 shows the extracted ion chromatogram and MS<sup>2</sup> spectra for compounds identified in an irinotecan-treated mouse (no MSC). The APC metabolite and M1 hydroxylated isomer were not detected in any of the liver samples analyzed. An MS<sup>2</sup> fragment ion, indicative of the M1 metabolite at m/z 518, was not taken into consideration as it also appeared in the control sample. Analyzed compounds in Figure 7 are: a) Internal standard, MS<sup>2</sup> 349; b) SN-38 metabolite, MS<sup>2</sup> 393; c) Irinotecan parent drug, MS<sup>2</sup> 587; d) SN-38G glucuronide metabolite, MS<sup>2</sup> 569; e) M2 oxidized metabolite, MS<sup>2</sup> 603; f) NPC metabolite, MS<sup>2</sup> 519; g) M1 oxidized metabolite, MS<sup>2</sup> 603, not detected; h) APC MS<sup>2</sup> 619, not detected.

FIGURE 5. Eight scan event experiment covering 7 expected drug and metabolite compounds plus IS, repeated over the chromatographic run.

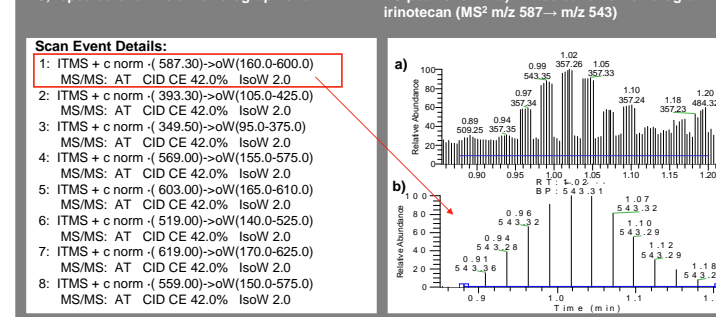
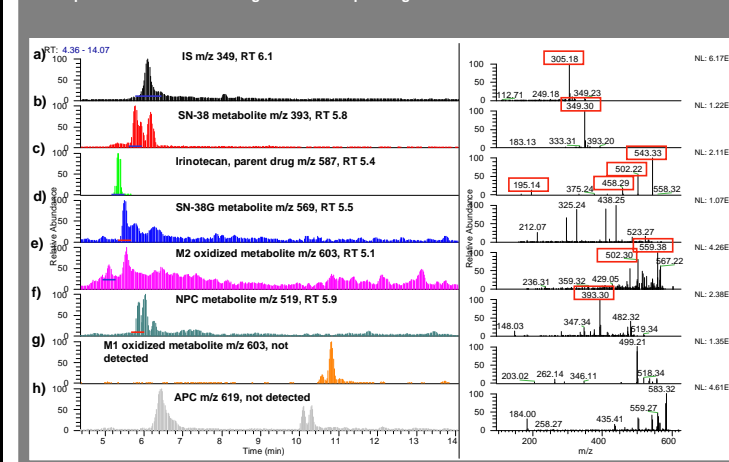


FIGURE 7. Extracted ion chromatogram (stick plots, left) showing the internal standard, irinotecan and four metabolites that were detected and confirmed by MS<sup>2</sup> in liver tissue extracts from dosed mice. MS<sup>2</sup> spectra are shown on the right with corresponding transitions shown in red boxes.



## Conclusions

- ✓ The ratio of Irinotecan to its SN-38 metabolite in the livers of mice treated only with Irinotecan agrees with Irinotecan:SN-38 ratios reported previously for plasma and liver<sup>4</sup>.
- ✓ Less Irinotecan (parent drug) was extracted from liver tissue in 'drug plus MSC' treated mice as compared to those from the drug-only treatment (Fig. 4). These findings suggest that MSC treatment resulted in a reduction of Irinotecan accumulation in the liver, while the ratio of irinotecan:SN-38 decreased (Fig. 4). Since MSC and related Se-containing compounds are known to reduce the toxicity of certain chemotherapeutic agents and to enhance the cure rate of tumors<sup>1</sup>, these findings are relevant to the protective mechanisms of MSC and are being further investigated.
- ✓ The LTQ Velos successfully analyzed compressed chromatographic runtimes and identified drug and metabolites from complex tissue samples: (15 minutes vs. 35 minutes runtime)
- ✓ Future work will focus on the use of MetWorks<sup>™</sup> software and MS<sup>n</sup> spectral trees for differentiation of structural isomers of m/z 603 in conjunction with Mass Frontier<sup>™</sup> software to facilitate spectral interpretation.

## References

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