Identification of Drug Metabolites by UPLC/MS with Isotope Pattern Directed Mass Chromatograms and UPLC with Radioactivity Flow Detection

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Wyeth Research
Drug Safety and Metabolism
Collegeville, PA

American Society for Mass Spectrometry
June 2, 2008

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Abstract

Ultra high performance liquid chromatography (UPLC) with its shorter analysis times, narrow peak widths and enhanced peak heights has not been widely accepted in our drug metabolism laboratories due to the requirement for better performing radioactivity flow detectors. In drug metabolism studies, radiolabeled compounds are commonly used to provide a complete metabolite profile. In this study we present an alternative to radioactivity flow detection to screen samples for the presence of metabolites to identify by mass spectrometry. Isotope pattern directed mass chromatograms were generated by software in development by Cerno Bioscience. The narrow chromatographic peak widths (3-6 s) from UPLC analysis were preserved in the isotope pattern directed chromatograms that was diminished by the radioactivity flow detector. Isotope ratios more distinguishable from those of non-related sample components provided the best results. This technique is applicable to both radioactive and stable isotope labeled drug candidates.

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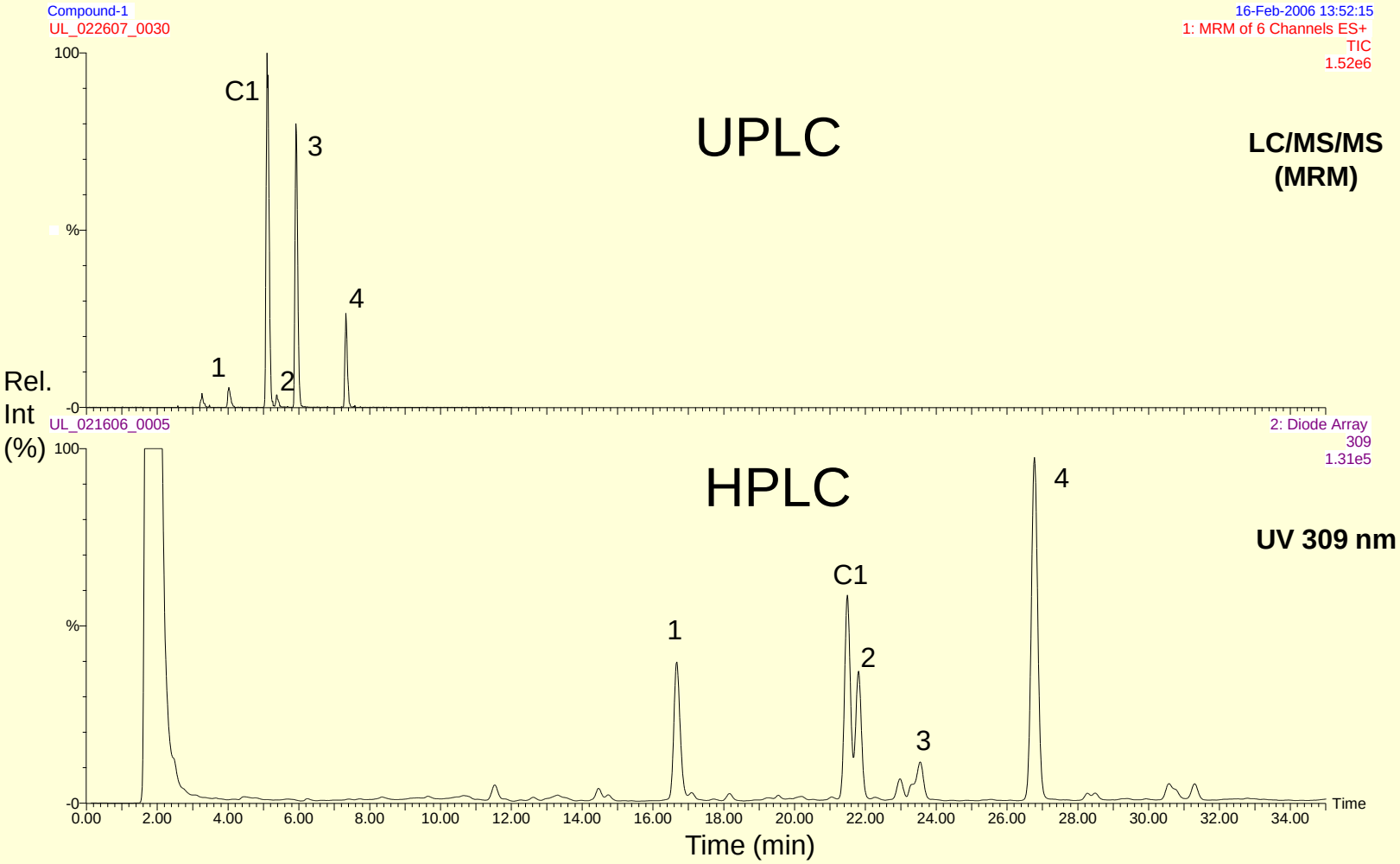
Introduction

Why UPLC for Drug Metabolism ?

Metabolites of Compound-1 (C1) in human plasma – similar sample from different studies



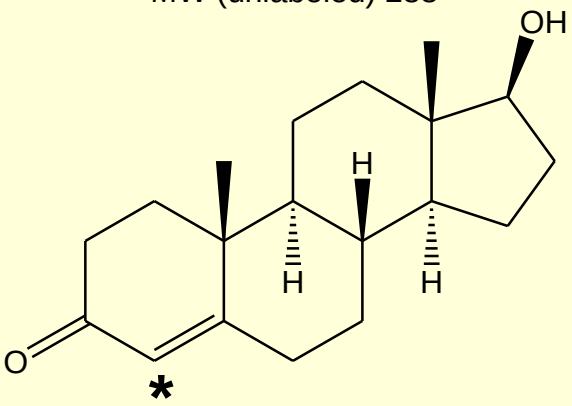
Reduce analysis times to ca. 25% of HPLC, with no compromise in separation

Experimental

Radiolabeled Compounds

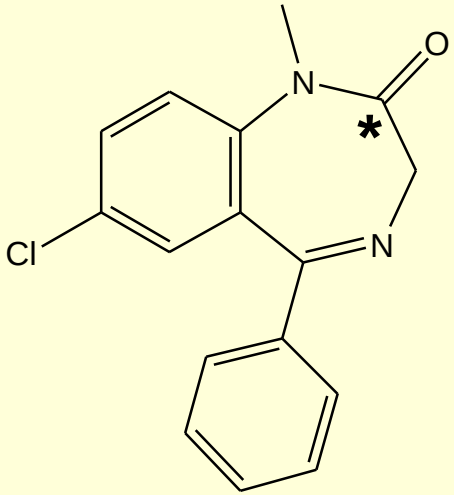
[4-^{14}C]Testosterone

MW (unlabeled) 288



[2-^{14}C]Diazepam

MW (unlabeled) 284



Sample Preparation and Analysis

^{14}C-Testosterone and ^{14}C-diazepam at 100 μM were incubated in rat liver microsomes, then extracted to generate samples for LC/MS analysis. These samples were used for (1) comparison of UPLC with HPLC and (2) to evaluate isotope pattern directed mass chromatograms for metabolite profiling.

LC/MS analysis with UV detection was performed with either a Waters Alliance HPLC or an Acquity UPLC system equipped with a photodiode array detector and interfaced to Quattro Premier XE mass spectrometer. Separations were accomplished with a Phenomenex Luna C18(2) column (150 x 2 mm, 5 μm) for HPLC or a Supelco Ascentis Express C18 column (150 x 2.1 mm x 2.7 μm) for UPLC.

Mass spectra were recorded in continuum mode from *m/z* 100 to 1000 in 0.5 sec.

Radiochromatograms were recorded with an IN/US βRAM Model 3 flow scintillation analyzer equipped with a 250 μL flow cell. A scintillant flow rate of 0.6 mL/min was used.

Post-acquisition data processing to generate isotope ratio pattern directed mass chromatograms was performed with software being developed by Cerno Bioscience.

Comparison of UPLC and HPLC with UV and Radioactivity Flow Detection

Liquid Chromatography Conditions:

Mobile Phase A: 0.1% formic acid in water (v/v)
Mobile Phase B: 0.1% formic acid in acetonitrile
Flow rate: 0.27 mL/min

UPLC – Short Gradient

Time (min)	A(%)	B(%)
0	75	25
0.8	75	25
6.16	10	90
6.26	75	25
7.76	75	25

UPLC – Long Gradient

Time (min)	A(%)	B(%)
0	75	25
1	75	25
30	10	90
31	10	90
31.1	75	25
33	75	25

HPLC – Long Gradient

Time (min)	A(%)	B(%)
0	75	25
1	75	25
30	10	90
35	10	90
35.1	75	25
45	75	25

Metabolite Profiling by Isotope Pattern Directed Mass Chromatograms

Liquid Chromatography Conditions: The UPLC – Short Gradient described above was used.

Diazepam in RLM: Constant specific activity – varying amounts (DPM) analyzed
Approximate 1:1 isotope pattern

Sample Text	Filename	Specific Activity (μCi/mg)	Specific Activity (μCi/μmol)	Isotope Ratio	Mole Percent Labeled	Actual Rel. Int.
Diazepam in RLM, 2213 DPM	PX_111207_004	59	16.8	1.2:1	27%	86.8%
Diazepam in RLM, 2213 DPM	PX_111207_007			1.2:1		85.9%
Diazepam in RLM, 4427 DPM	PX_111207_005			1.04:1		96.6%
Diazepam in RLM, 4427 DPM	PX_111207_008			1.13:1		88.1%

Testosterone in RLM: Constant specific activity – varying amounts (DPM) analyzed

Sample Text	Filename	Specific Activity (μCi/mg)	Specific Activity (μCi/μmol)	Isotope Ratio	Mole Percent Labeled	Actual Rel. Int.
Testosterone in RLM, 3562 DPM	PX_111207_001	34	9.8	4.1:1	16%	24.4%
Testosterone in RLM, 7124 DPM	PX_111207_002			4.3:1		23.5%
Testosterone in RLM, 25000 DPM	PX_111207_003			3.7:1		27.4%

Metabolite Profiling by Isotope Pattern Directed Mass Chromatograms (continued)

Testosterone in RLM: Constant amount – varying specific activities (isotope ratio) analyzed

Sample Text	Filename	Specific Activity (μCi/mg)	Specific Activity (μCi/μmol)	Isotope Ratio	Mole Percent Labeled	Actual Rel. Int.
Testosterone in RLM - 40751 DPM	PX_012208_0002	17.3	5	7.1:1	8%	14.5%
Testosterone in RLM - 44574 DPM	PX_012208_0003			8.0:1		12.5%
Testosterone in RLM - 21575 DPM	PX_012208_0004	8.8	2.5	13.1:1	4%	7.6%
Testosterone in RLM - 21587 DPM	PX_012208_0005			12.4:1		8.0%
Testosterone in RLM - 10474 DPM	PX_012208_0006	4.4	1.3	17.3:1	2%	5.8%
Testosterone in RLM - 11023 DPM	PX_012208_0007			15.4:1		6.5%

Results and Discussion

Comparison of UV and ^{14}C Chromatographic Peak Area and Height for UPLC vs HPLC

Table 1 summarizes the chromatographic peak areas and heights obtained for the diazepam peak with the three separation conditions evaluated. Corresponding UV and radioactivity chromatograms are shown in Figures 1A and 1B, respectively. UPLC separation times of 5.2 and 9 min were obtained versus the 15 min separation time for HPLC. UV and radiochromatographic peak areas were slightly greater (approx. 10-20%) for UPLC than for HPLC, most likely due to overlapping peaks as the same amount of sample was analyzed.

UV chromatographic peak height increases indicative of the actual advantage obtainable with UPLC were about 550% and 180% greater than the HPLC peak height for the short and long gradients, respectively. The UPLC peak height advantage vs HPLC was reduced to approx. 50% and 20% greater than HPLC for the short and long gradients, respectively, due to mixing in the 250 μL flow cell used in the radioactivity detector.

The flow cell volume and scintillation flow rate were better matched for HPLC in this evaluation, but were kept constant to obtain a better UPLC vs HPLC comparison. Better chromatographic resolution was obtainable for UPLC separations with flow cell volumes and scintillant flow rates better matched to UPLC as shown in Figure 2.

Table 1. Comparison of UPLC and HPLC Peak Area and Height for UV and Radioactivity Detection

Separation	UV Peak Area	Area increase vs HPLC (%)	UV Peak Height	Height increase vs HPLC (%)
UPLC - Short gradient	37720	10.2	1193865	546
UPLC - Long gradient	37478	9.5	523483	183
HPLC - Long gradient	34234	0	184756	0

Separation	^{14}C Peak Area	Area increase vs HPLC (%)	^{14}C Peak Height	Height increase vs HPLC (%)
UPLC - Short gradient	17560	22.3	49371	50.5
UPLC - Long gradient	14335	-0.2	39291	19.8
HPLC - Long gradient	14362	0	32797	0

Peak broadening in ^{14}C detector flow cell reduces UPLC peak height advantage.

Minimal difference in peak areas as expected - same amount analyzed

Partially due to overlapping peaks.

Substantial increase in UPLC peak heights vs HPLC

Figure 1A. Comparison of UPLC vs HPLC: UV Detection

UPLC – Short gradient

UPLC – Long gradient

HPLC – Long gradient

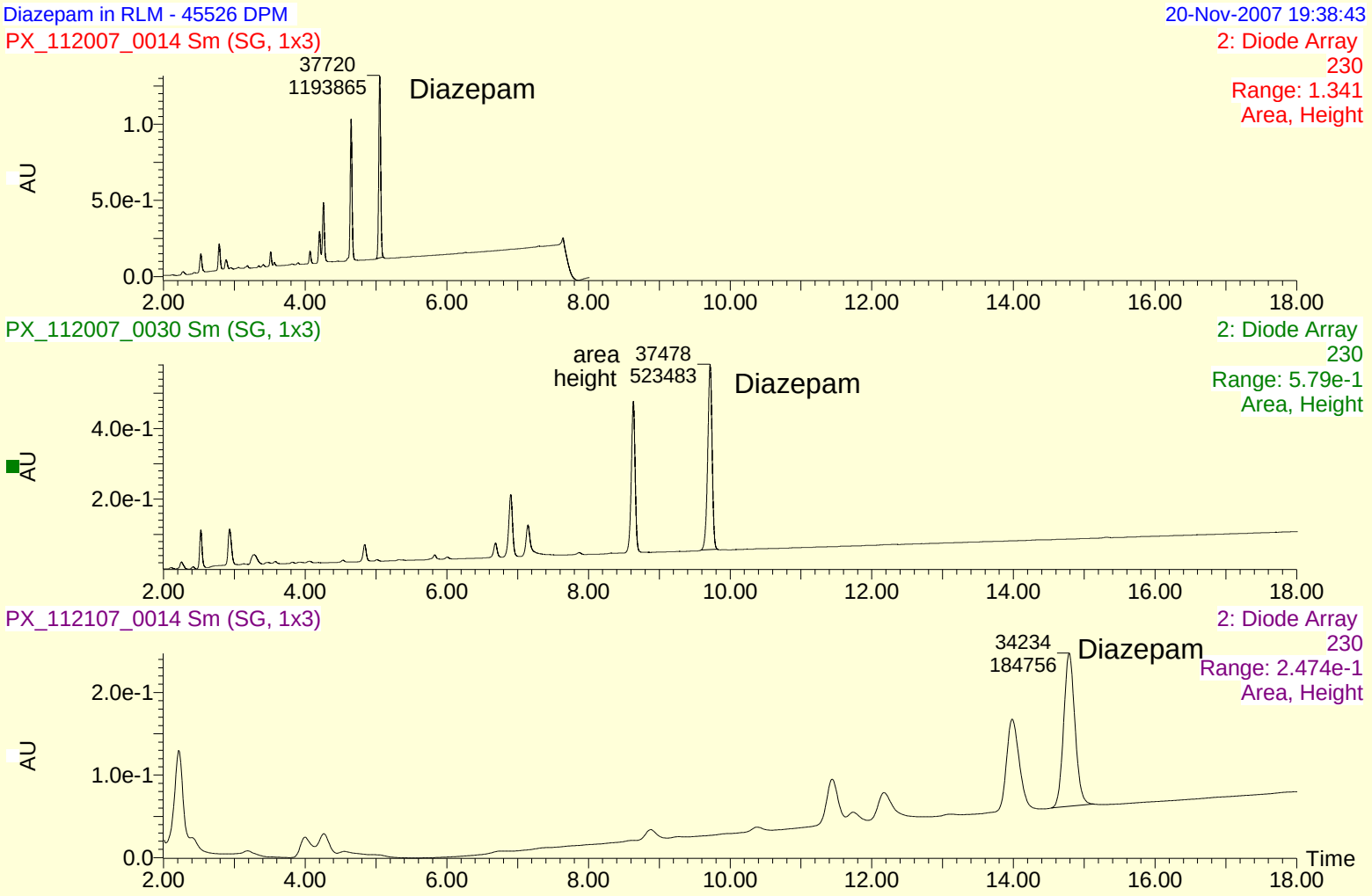


Figure 1B. Comparison of UPLC vs HPLC: Radioactivity Flow Detection

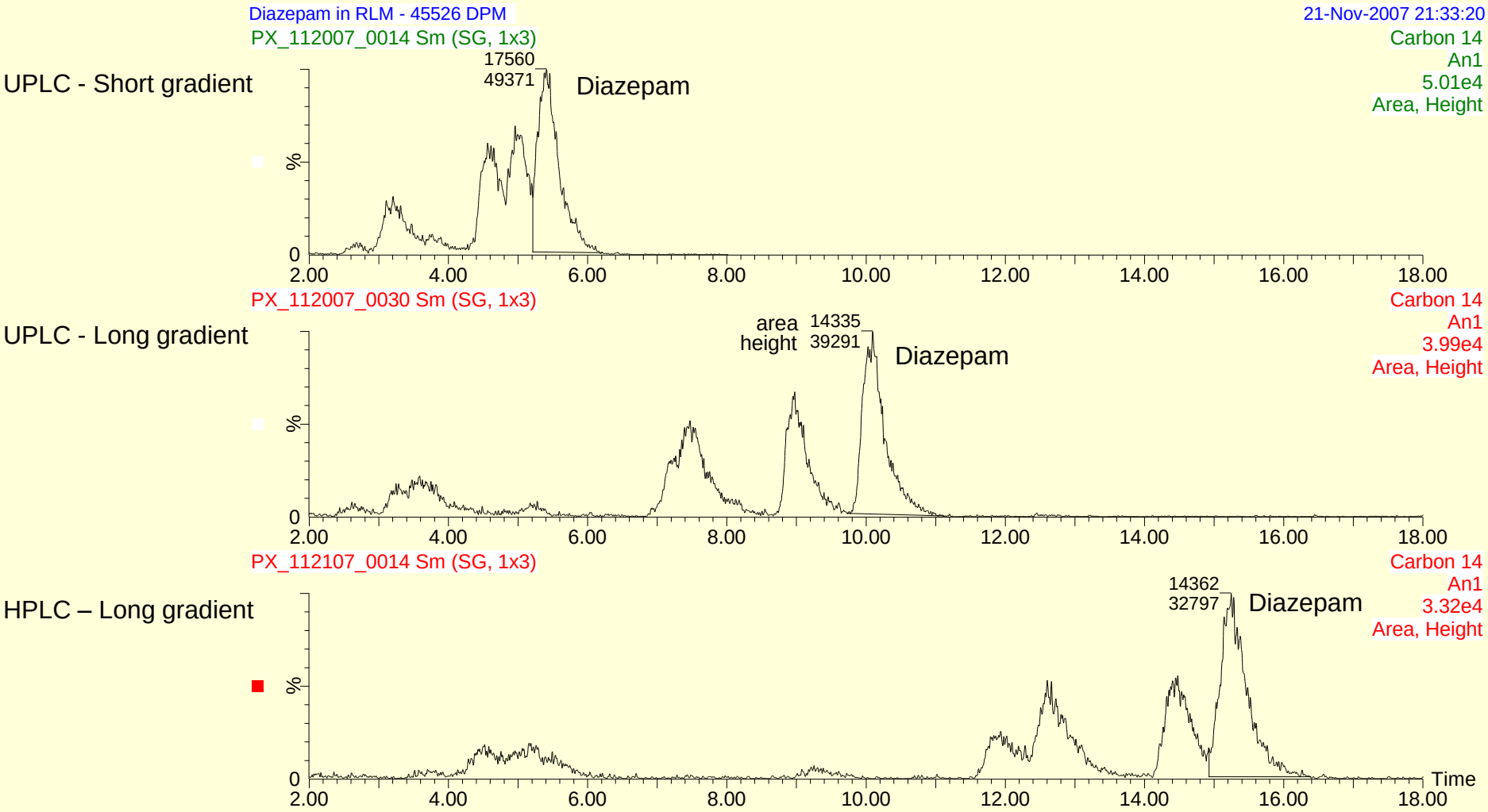
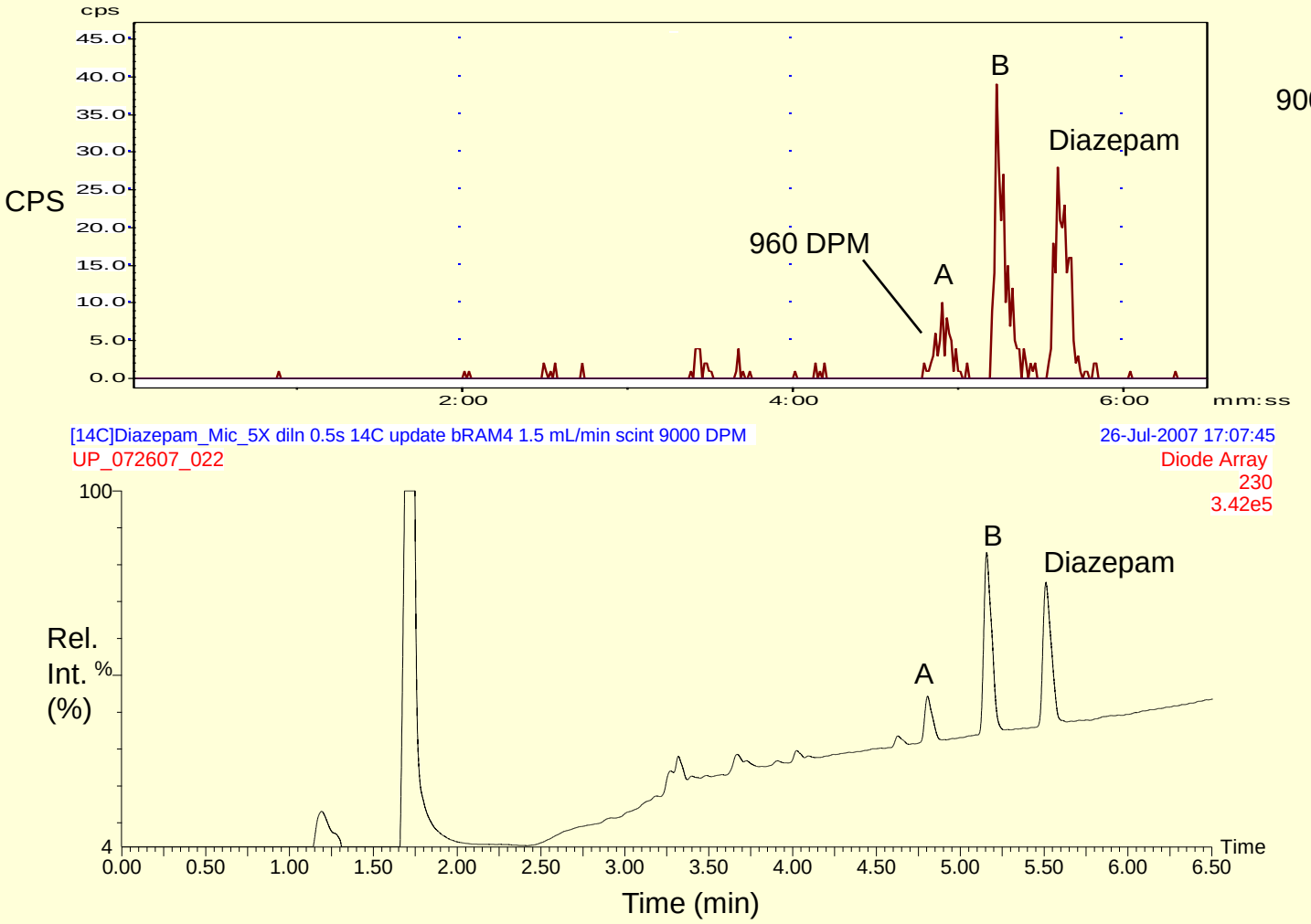


Figure 2. UPLC Radioactivity and UV Chromatograms of Diazepam in RLM: Limit of Quantitation



9000 DPM injected

LOQ ca. 960 DPM / metabolite

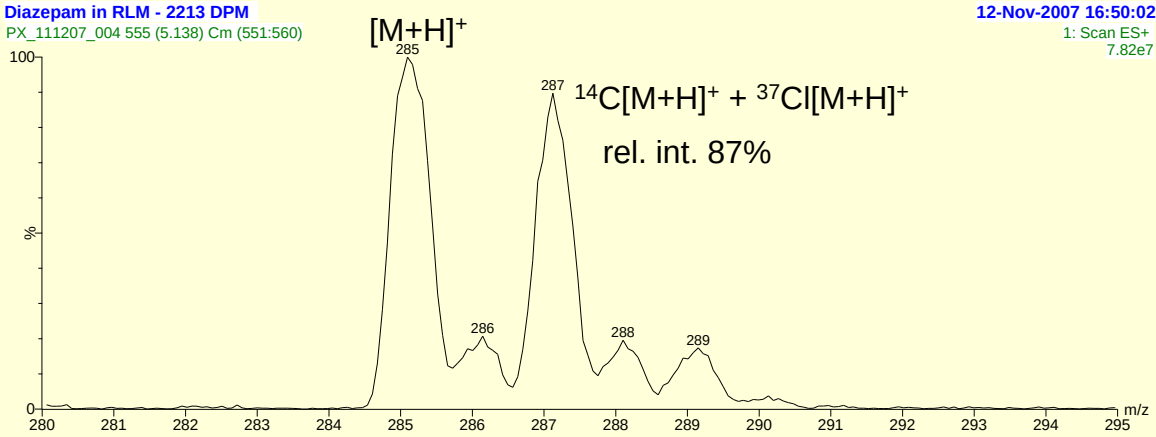
UPLC with Isotope Pattern Directed Mass Chromatograms

The $[M+H]^+$ to $^{14}C[M+H]^+$/ $^{37}Cl[M+H]^+$ isotope pattern evaluated for diazepam (near 1:1) is shown in Figure 3A along with a summed mass chromatogram representative of the metabolites observed. This isotope ratio was also representative of the ratio that would be used in similar work with a stable isotope labeled compound.

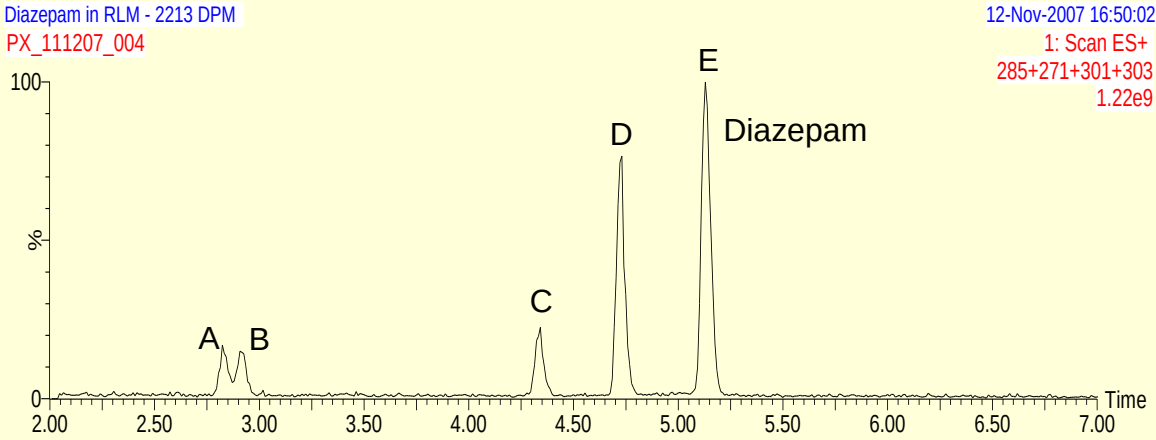
Figure 3B shows the isotope pattern directed mass chromatogram and the corresponding radioactivity flow chromatogram for the diazepam in RLM preparation. The isotope pattern directed chromatogram closely matches the summed mass chromatogram (Figure 3A) and was generated without the need to predict or already know which metabolites were present. Chromatographic peaks in the radioactivity flow chromatogram were broadened due to mixing in the flow cell providing no separation for peaks A and B and inadequate separation for the other metabolites. The radioactivity detector was also operating closer to its limit of quantitation than was the mass spectrometer. Doubling the amount of sample analyzed (Figure 3C) provided no additional information in the radiochromatogram. However, in the isotope pattern directed chromatogram, another metabolite was indicated in front of peak C.

Figure 3A. Diazepam and its Metabolites in RLM: Specific Activity: 59 μCi/mg

Molecular Ions of ^{14}C Labeled Diazepam, 59 μCi/mg



Summed mass chromatogram representative of metabolite profile



Peaks	Metabolite
A, D	Hydroxyl diazepam
B	Dihydroxyl, *N*-desmethyl diazepam
C	*N*-desmethyl diazepam
E	Diazepam

UPLC/MS peak width is approx. 8 s at base.

Figure 3B. Diazepam and its Metabolites in RLM: 2213 DPM Injected

Isotope Pattern Directed Chromatogram

- Narrow UPLC peak width preserved
- All major metabolites observed
- No false positive peaks observed

Radioactivity flow chromatogram

- Peak broadening in flow cell
- Operation near low limit of quantitation

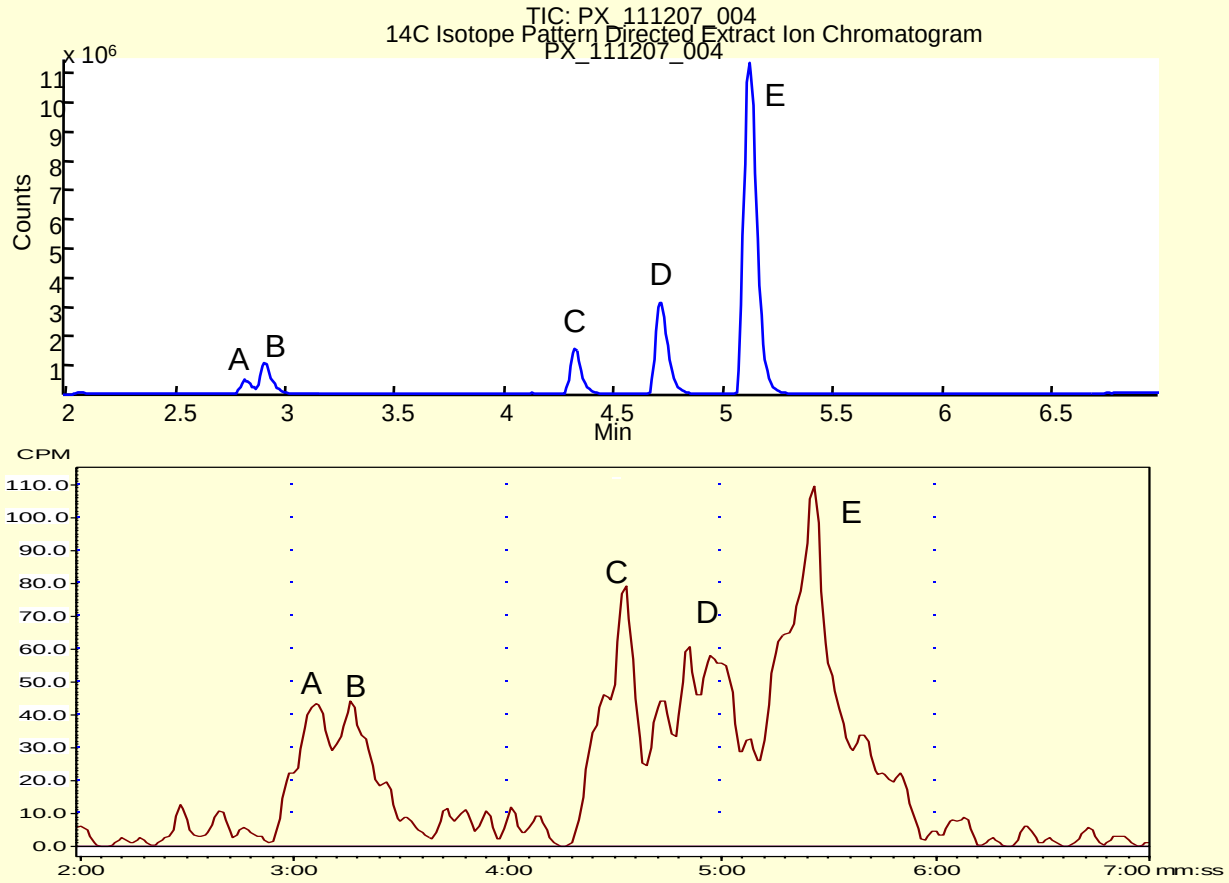
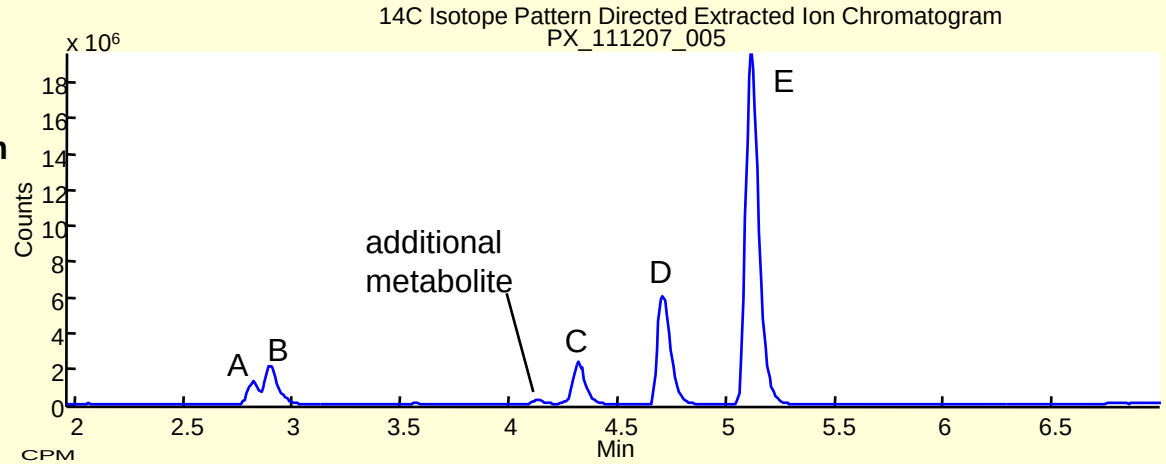


Figure 3C. Diazepam and its Metabolites in RLM: 4427 DPM Injected

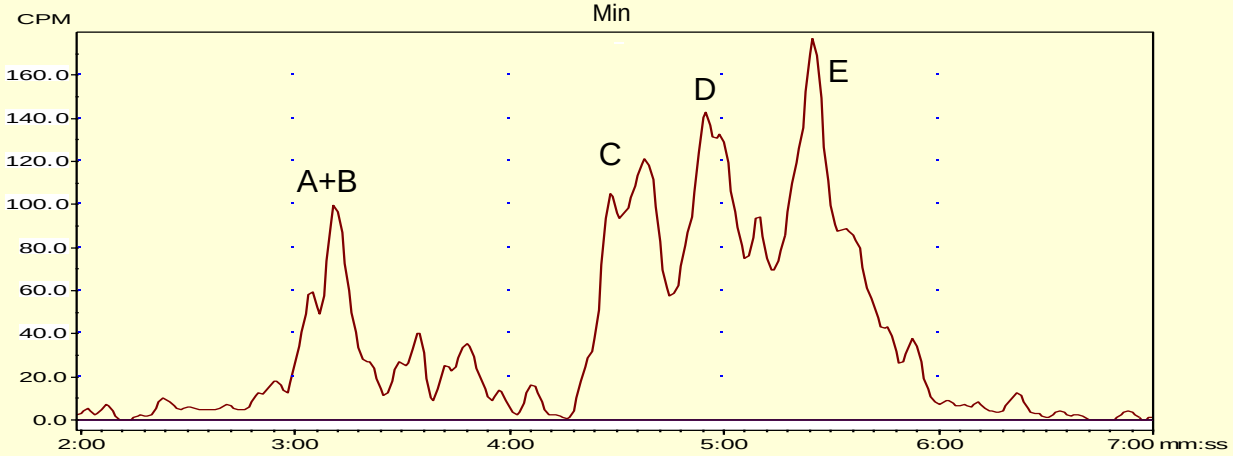
Isotope Pattern Directed Chromatogram

- Narrow UPLC peak width preserved
- All major metabolites observed
- No false positive peaks observed



Radioactivity flow chromatogram

- Peak broadening in flow cell



The high relative intensity (87%) of the $^{14}C[M+H]^+$ + $^{37}Cl[M+H]^+$ ion allows for generation of a

UPLC with Isotope Pattern Directed Mass Chromatograms (continued)

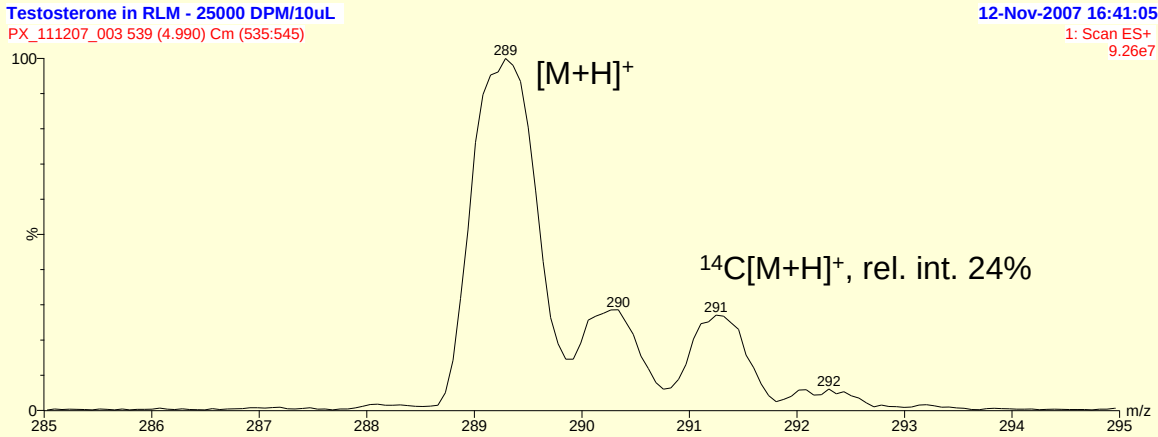
The $[M+H]^+$ to $^{14}C[M+H]^+$ isotope pattern evaluated for 34 μCi/mg testosterone (near 4:1) is shown in Figure 4A along with a summed mass chromatogram representative of the testosterone metabolites observed.

Figure 4B shows the isotope pattern directed mass chromatogram and the corresponding radioactivity flow chromatogram for this testosterone in RLM preparation. The isotope pattern directed chromatogram shows only the most abundant metabolites due to its high "noise level." Only one false positive peak (after peak J) was generated. Chromatographic peaks in the corresponding radioactivity flow chromatogram were broadened due to mixing in the flow cell providing no separation or indication of the less abundant metabolites.

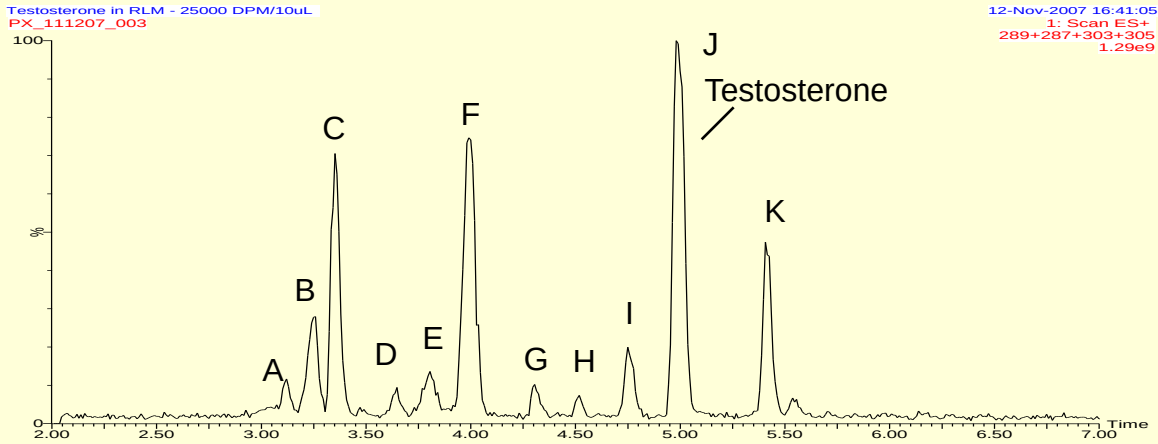
Analysis of lesser amounts of sample, but with the same isotope pattern, are presented in Figure 5 and Figure 6. Results were similar with observation of only the more abundant metabolites and a minimal presence of false positive peaks.

Figure 4A. Testosterone and its Metabolites in RLM: 25000 DPM Injected, 34 μCi/mg

Molecular Ions of ^{14}C Labeled Testosterone, 34 μCi/mg



Summed mass chromatogram representative of metabolite profile



Peaks	Metabolite
A, B, C, D, F	Hydroxyl testerone
B, E, F, G, H	Didehydro hydroxyl testosterone
J	Testosterone
K	Didehydro testerone

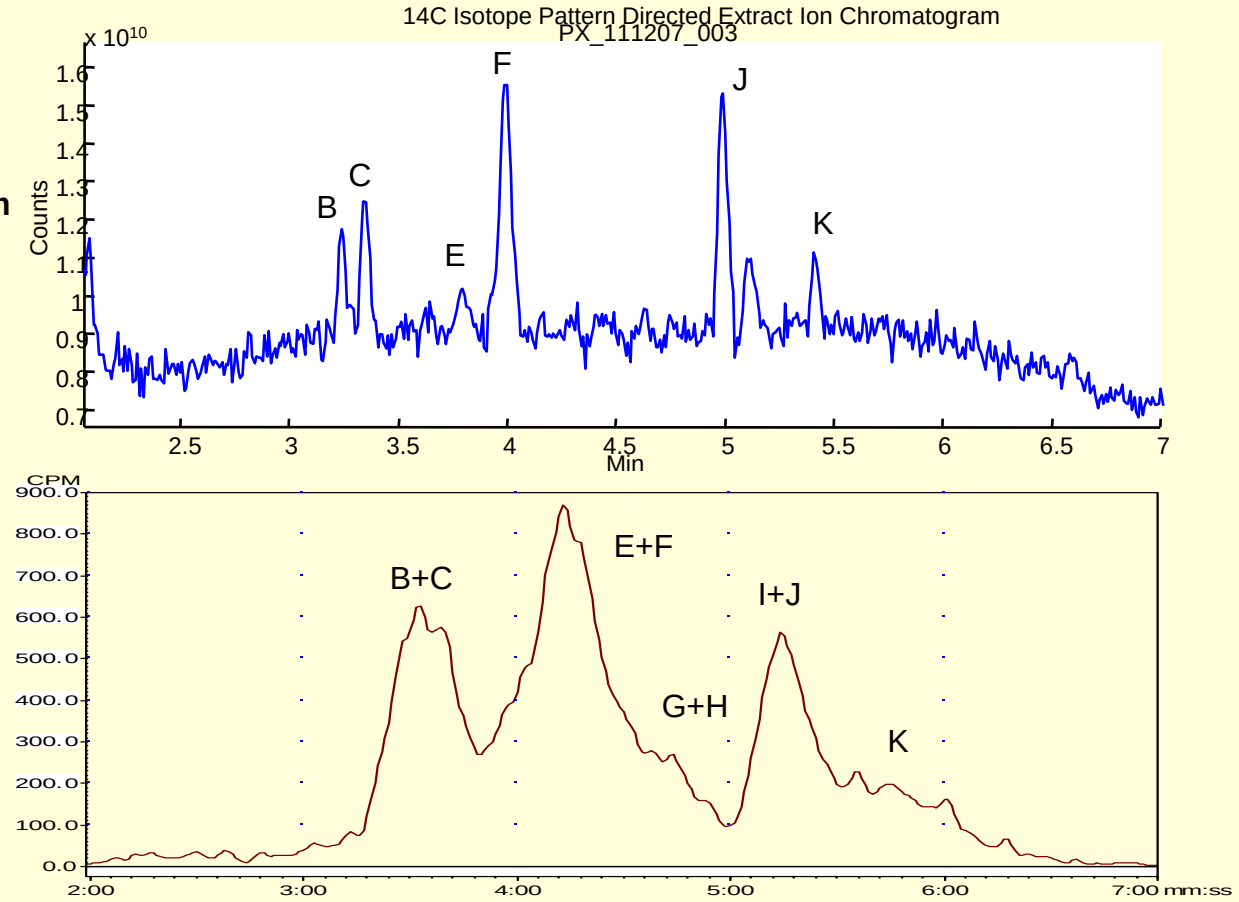
Figure 4B. Testosterone and its Metabolites in RLM: 25000 DPM Injected, 34 μCi/mg

Isotope Pattern Directed Chromatogram

- Narrow UPLC peak width preserved
- Minor metabolites not observed
- Only one false positive peak

Radioactivity flow chromatogram

- Peak broadening in flow cell

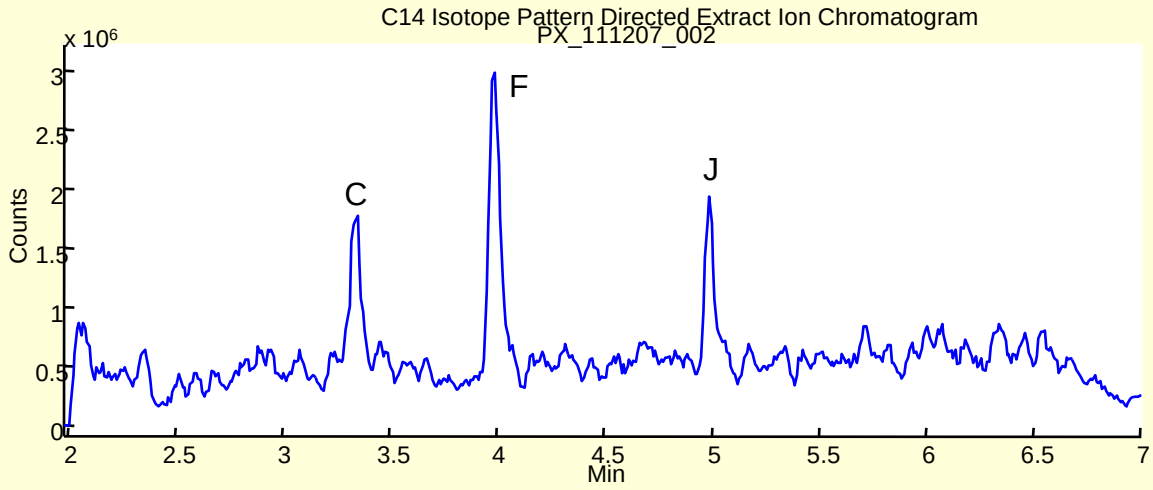


Metabolite profile observable with isotope pattern directed chromatogram.
Advantage of UPLC is diminished by chromatographic peak broadening in radioactivity detector flow cell.

Figure 5. Testosterone and its Metabolites in RLM: 7124 DPM Injected, 34 μCi/mg

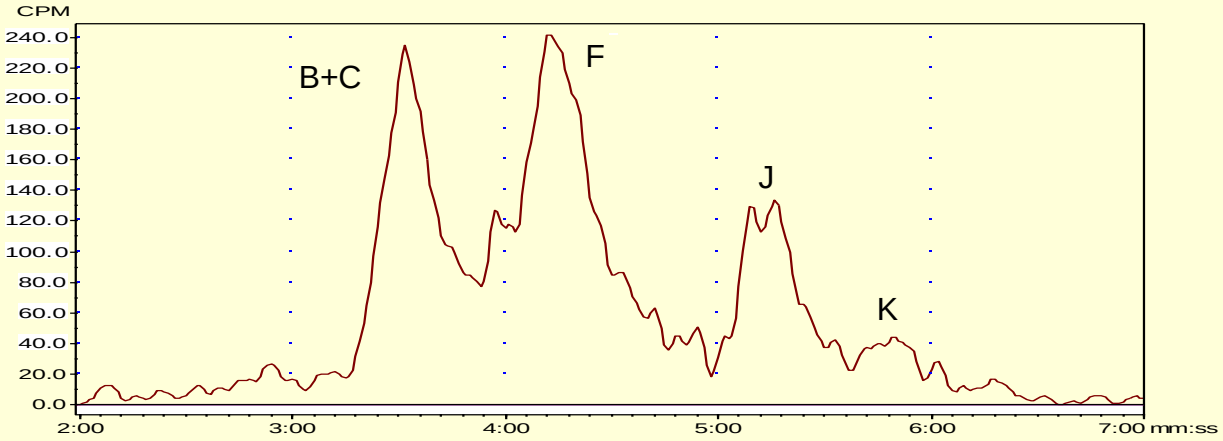
Isotope Pattern Directed Chromatogram

- Narrow UPLC peak width preserved
- Less abundant metabolites missed
- No false positive peaks



Radioactivity flow chromatogram

- Peak broadening in flow cell
- Radioactivity detection more sensitive

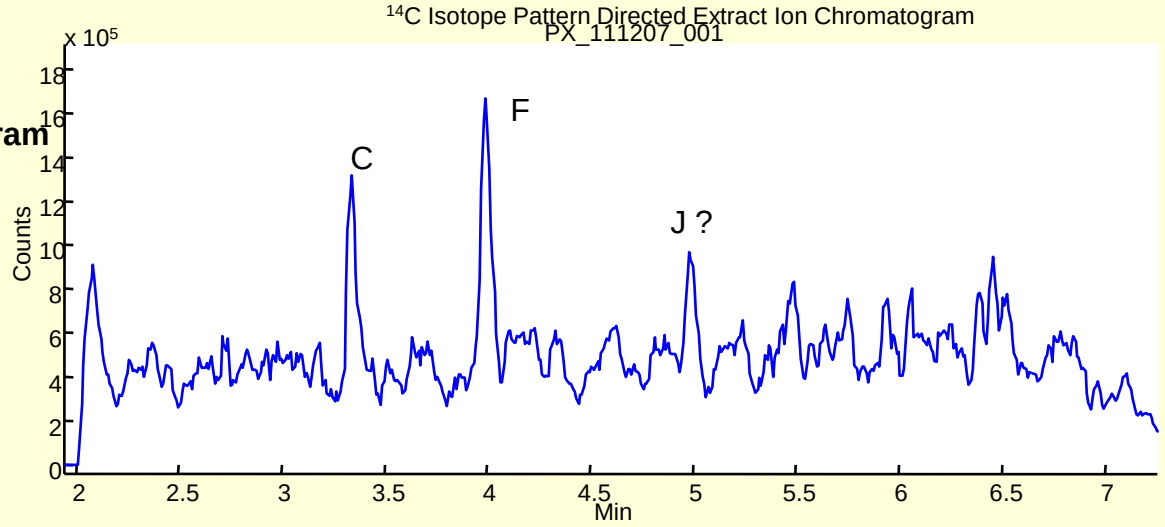


Only 3 of 11 radioactive components detectable in the isotope directed chromatogram due to analysis of an insufficient amount of material.

Figure 6. Testosterone and its Metabolites in RLM: 3562 DPM Injected, 34 μCi/mg

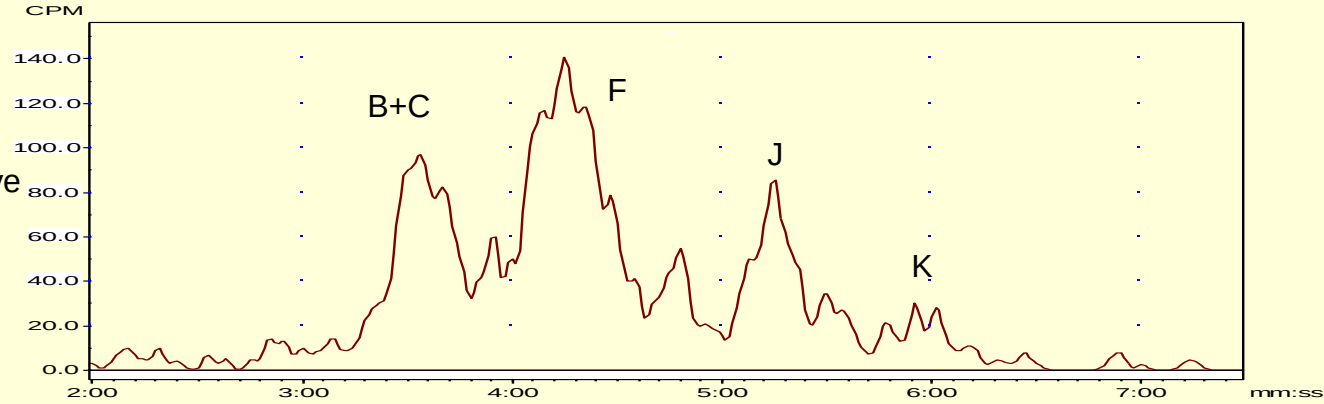
Isotope Pattern Directed Chromatogram

- Narrow UPLC peak width preserved
- Many metabolites missed



Radioactivity flow chromatogram

- Peak broadening in flow cell
- Radioactivity detection more sensitive



Only 3 of 11 radioactive components detectable in the isotope directed chromatogram due to analysis of an insufficient amount of material.

UPLC with Isotope Pattern Directed Mass Chromatograms (continued)

Evaluation of isotope pattern directed mass chromatograms for metabolite profiling with lower specific activities of 17.3, 8.8 and 4.4 μCi/mg testosterone (near 7:1, 13:1 and 17:1, respectively) was performed. A summed mass chromatogram representative all of these testosterone preparations is presented in Figure 7A. Figure 7B shows the corresponding isotope pattern directed mass chromatograms.

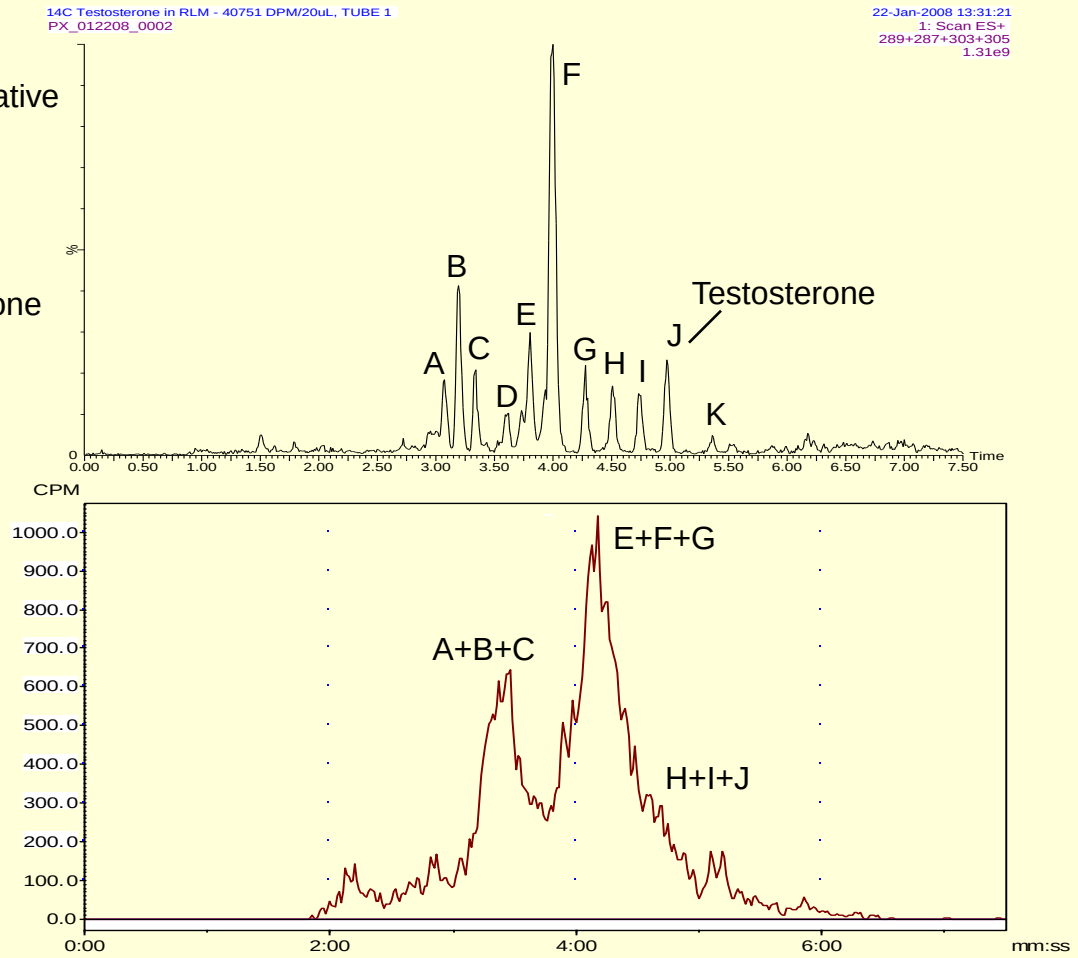
The 17.3 μCi/mg (7:1) ratio (Figure 7B) shows 7 of the 11 testosterone metabolites present. False positive peaks were present at the front and back of the gradient as might be expected for the portions of the chromatogram containing the most endogenous or potentially interfering components.

Isotope pattern directed mass chromatograms for the lower specific activities (8.8 and 4.4 μCi/mg testosterone (near 13:1 and 17:1, respectively) were of no practical use for screening for metabolites. False positive peaks were of the same intensity as metabolite peaks.

Figure 7A. Testosterone and its Metabolites in RLM: Specific Activity Range

Summed mass chromatogram representative of metabolite profile

Peaks	Metabolite
A, B, C, D, F	Hydroxyl testerone
B, E, F, G, H	Didehydro hydroxyl testosterone
J	Testosterone
K	Didehydro testerone



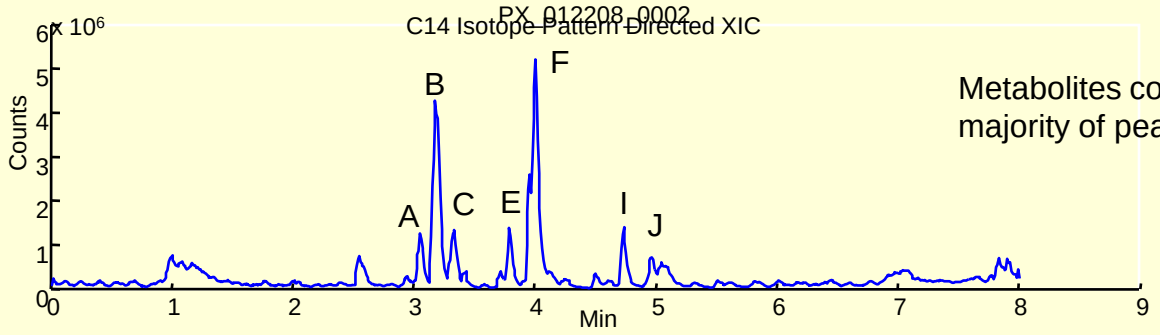
Radioactivity flow chromatogram
40751 DPM injected
17.3 μCi/mg

Chromatographic peak broadening in flow cell diminishes advantage of UPLC

Isotope ratio chromatograms for 17.3, 8.8 and 4.4 μCi/mg shown below (next page). Actual ng/injection was constant.

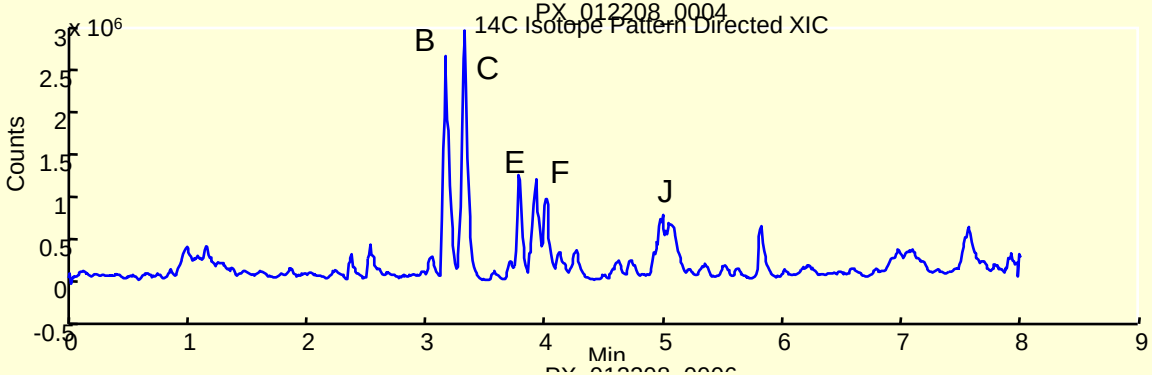
Figure 7B. Testosterone and its Metabolites in RLM: Specific Activity Range: 17.3 to 4.4 μCi/mg

40751 DPM
17.3 μCi/mg
$^{14}C[M+H]^+$ 14.5% rel. int.

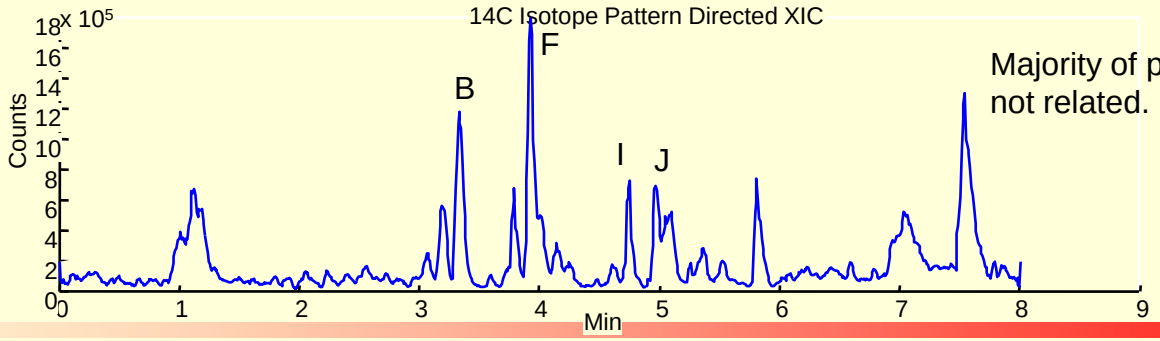


Metabolites comprise majority of peaks.

21575 DPM
8.8 μCi/mg
$^{14}C[M+H]^+$ 8% rel. int.



10474 DPM
4.4 μCi/mg
$^{14}C[M+H]^+$ 6% rel. int.



Majority of peaks are not related.

14.5% (17.3 μCi/mg) appears to be the lowest relative intensity useful for isotope ratio directed metabolite profiling. Lower enrichments leads to numerous unrelated chromatographic peaks.

Summary

- **UPLC significantly reduces analysis time (4X) for metabolite profiling.**
- **Online radioactivity detection slightly more sensitive with UPLC than for HPLC with same detector conditions**
- **UPLC narrow peak width advantage diminishes in radioactivity detector flow cells.**
- **Isotope patterns more distinguishable from patterns for endogenous compounds (1:1) ratio provide the best results for metabolite profiling.**
- **A 7:1 isotope ratio was the lowest limit usable for metabolite profiling of in vitro samples. Higher enrichments provide more reliable results with fewer false positive and missed metabolites.**

Acknowledgements

- **Ming Gu, Cerno Bioscience**
- **Phil Hurst, IN/US**