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Nota de aplicación

An Ultra Rapid and Sensitive Strategy for *in vivo* Metabolite Identification for Protopanaxadiol from Rat Bile: UPLC vs. HPLC

Jose M. Castro-Perez, Kate Yu, John P. Shockcor, Henry Shion, Yanguo Sun, Emma Marsden-Edwards, Yuya Wang, Xiaoyan Chen, Dafang Zhong

Waters Corporation, Shanghai Institute of Materia Medica, Chinese Academy of Science

Abstract

This application note demonstrates a simple and effective workflow combining UPLC/TOF-MS^E with MetaboLynx and MassFragment Software for Traditional Herbal (Chinese) Medicine metabolite identification from rat bile.

Introduction

In vivo drug metabolite identification (Met ID) studies encompass some of the most difficult analytical challenges, such as extremely complicated biological matrices and a lack of pure standards for putative metabolites. Utilization of a high resolution chromatographic system plays an important role in separating metabolites from endogenous matrices.

The advent of Waters UltraPerformance LC (UPLC) technology has enabled chromatographic separations to be routinely performed on columns packed with sub-2 µm particles at high linear velocity. Such separations result in high resolution, excellent throughput, and high sensitivity chromatographic analyses.[1-2] These analytical qualities ensure the separation of coeluting metabolites and minimize ESI matrix effects. The use of oa-TOF mass spectrometry offers fast acquisition rates that are compatible with UPLC, high resolution, and exact mass measurement for confident compound identification.

Protopanaxadiol is one of the major sapogenin for ginsenosides[3] (Figure 1). It has been reported that this compound may inhibit cancer cell growth.[4] However, its metabolism in animal or human has not yet been reported. A previously-described UPLC/TOF-MS^E workflow including MetaboLynx and MassFragment Software for metabolite identification[1] was applied to this study and adapted so that an equivalent workflow using a HPLC separation could be compared.

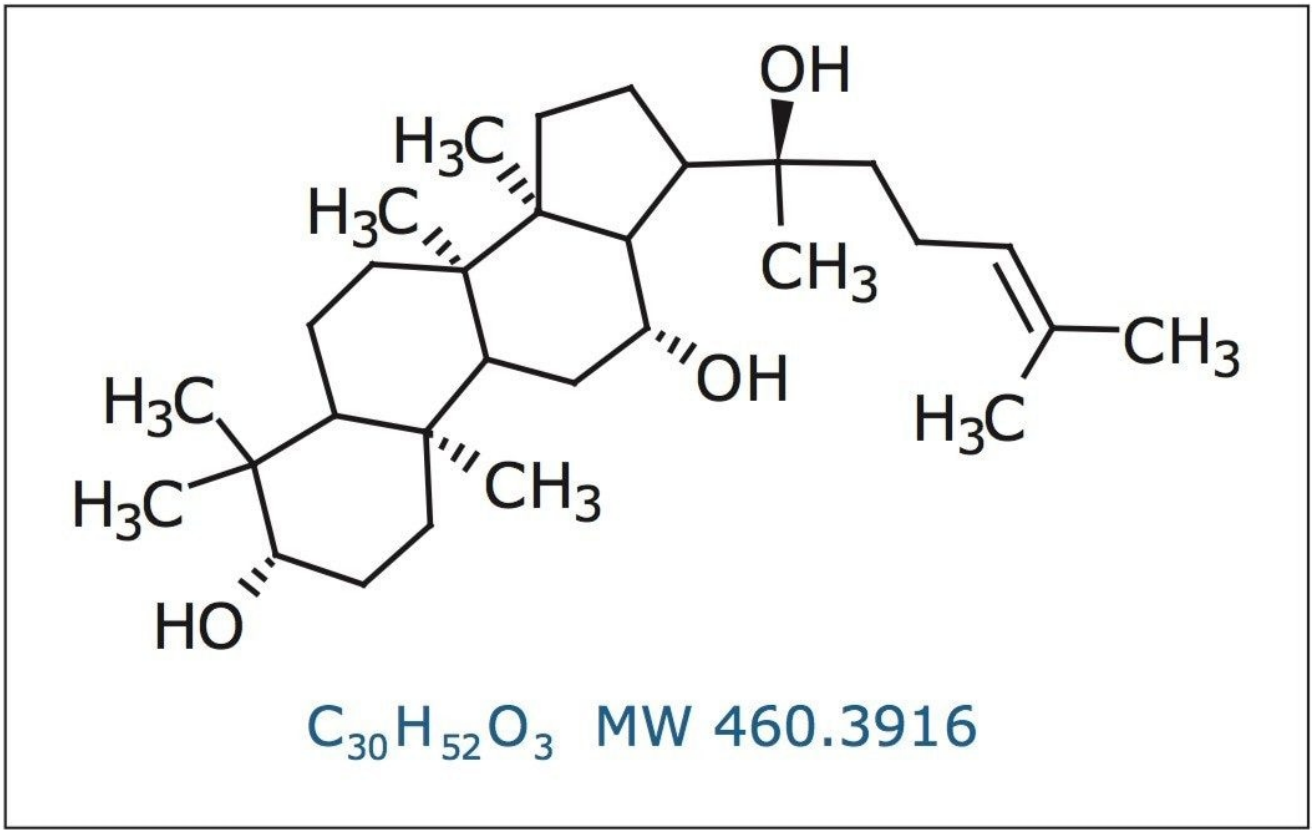

Figure 1. Chemical structure of the protopanaxadiol (PPD).

This application note describes a study that demonstrates the advantages of UPLC over traditional HPLC using TOF-MS detection. An *in vivo* drug metabolism experiment was performed using protopanaxadiol (PPD). A rat was dosed with PPD, with bile subsequently collected and analyzed by both UPLC/TOF-MS and HPLC/TOF-MS.

We show that the higher resolution and sensitivity of UPLC results in the identification of more metabolites, with significantly enhanced signal-to-noise ratios in a shorter timeframe than can be achieved using a comparable workflow using HPLC.

Experimental

Methods

Dosing the Animal

Male Sprague-Dawley rats were fasted for a 12-hour period prior to dosing. Blank bile was collected prior to dosing. The PPD oral dose amount was 100 mg/kg. The dose bile was collected three to six hours after administration of a single dosage.

Sample Preparation

400 µL of MeOH was added into a 200 µL aliquot of rat bile. After being vortex-mixed and centrifuged, the supernatant was evaporated to dryness and reconstituted later with 1 mL of ACN/H_2O (2:8), centrifuged again at 13,000 rpm, and the supernatant was injected.

MS Conditions

MS system:	Waters SYNAPT HDMS System
Scan range:	100 to 1000 Da
Source temp.:	120 °C
Desolvation temp.:	420 °C

Cone voltage:	15 V
Collision energy (CE):	Low CE: Trap 2 eV/Transfer 0.5 eV High CE: Trap 10 to 25 eV/Transfer 4 eV

HPLC Conditions

Instrument:	Waters ACQUITY UPLC System
Column:	Zorbax Extend-C_{18}, 4.6 x 150 mm, 5 µm
Column temp.:	45 °C
Sample temp.:	4 °C
Mobile phase A:	5 mM NH_4HOAc buffer
Mobile phase B:	AcN
Flow rate:	0.6 mL/minute
Injection vol.:	10 µL

Gradient:

Time (min)	%A	Curve
0	90	
5.5	80	6
10	80	6
10.5	60	6
15	60	6
15.5	40	6
20.0	40	6
20.5	20	6
60	20	6
61	90	1
71	90	1

UPLC Conditions

Instrument:	Waters ACQUITY UPLC System
Column:	ACQUITY UPLC HSS T3, 2.1 x 100 mm, 1.7 µm
Column temp.:	45 °C
Sample temp.:	4 °C
Mobile phase A:	5 mM NH_4OAc buffer
Mobile phase B:	AcN

Flow rate:	0.6 mL/minute
Injection vol.:	10 µL
Data processing:	MetaboLynx XS Application Manager with MassFragment

Gradient:

Time (min)	%A	Curve
0	90	
11	30	6
13	10	1
15	90	1

Results and Discussion

The analysis of protopanaxadiol in bile gave rise to an important number of Phase I and II metabolites. The major biotransformation detected corresponded to the O-sulfate conjugated metabolite. The amount of all O-sulfate conjugates equated to 51% of the total metabolites identified in this study.

Further investigation of the results revealed why UPLC has a noticeable advantage over HPLC for metabolite identification. Figure 2 shows two extracted ion chromatograms (XIC) for the O-sulfate metabolites (*m/z* 557.351) with an extraction window set at ± 30 mDa.

- Nine O-sulfate metabolites were detected by UPLC (Figure 2A).
- Five O-sulfate metabolites were detected by HPLC (Figure 2B).

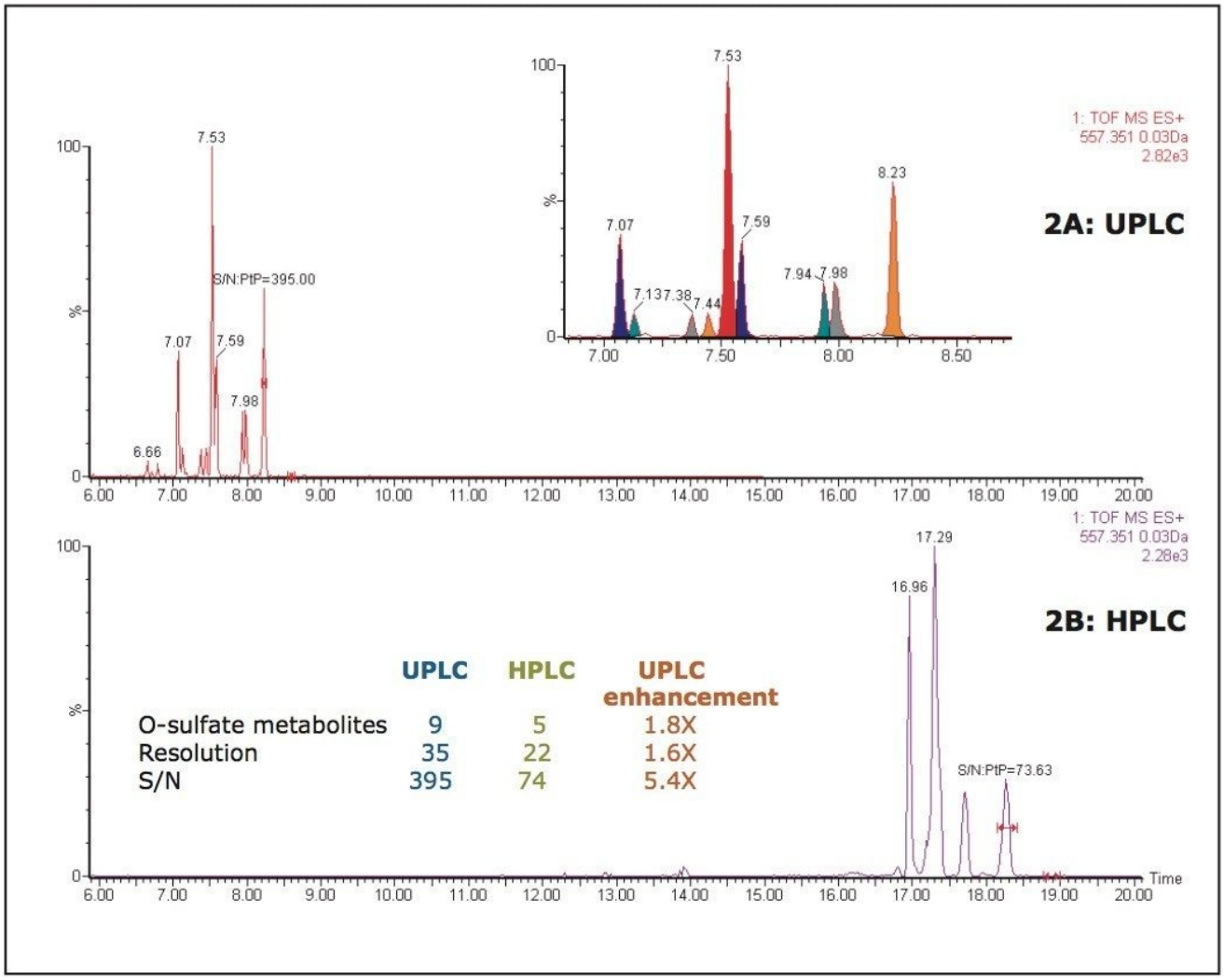

Figure 2. XICs of O-sulfate metabolites for protopanaxadiol, including 2A, the XIC obtained from the UPLC run, and 2B, the XIC obtained from the HPLC run.

In contrast to the HPLC analysis, Figure 2 highlights the power of the UPLC strategy and its additional benefits – the UPLC analysis not only resulted in more identified metabolites, but it also provided a 5.3-fold improvement in signal-to-noise and 1.6-fold improvement in chromatographic resolution.

Table 1 summarizes the comparison of the metabolite identification results between the UPLC and the HPLC methods. From the data shown, it is apparent that with the UPLC approach, simultaneous enhancements in speed, resolution, and sensitivity were observed. As a result, the total number of metabolites identified was significantly higher when utilizing the UPLC separation in both ionization modes (positive and negative ESI).

PPD IN RAT BILE	UPLC	HPLC	ENRICHMENT FACTOR BY UPLC
Metabolites ESI+	39	25	56%
Metabolites ESI-	35	22	59%
LC run time (min)	15	71	4.7X faster

Table 1. Comparison of metabolite identification results obtained from UPLC and HPLC.

The enrichment factor for UPLC vs. HPLC in positive ion mode was +56%, and in negative ion mode, +59%. This may be attributed to the fact that more metabolites and endogenous components were resolved chromatographically, giving rise to an increased degree of separation and a reduction in ion suppression.

Figure 3A shows a chromatogram that combines the extracted masses for all of the metabolites identified using UPLC-MS. The time period between 4 to 12.3 minutes is shown as this is where all of the metabolites were eluted. Figure 3C shows a similar chromatogram for the HPLC-MS data. Note the time scale shown is 11 to 21.5 minutes.

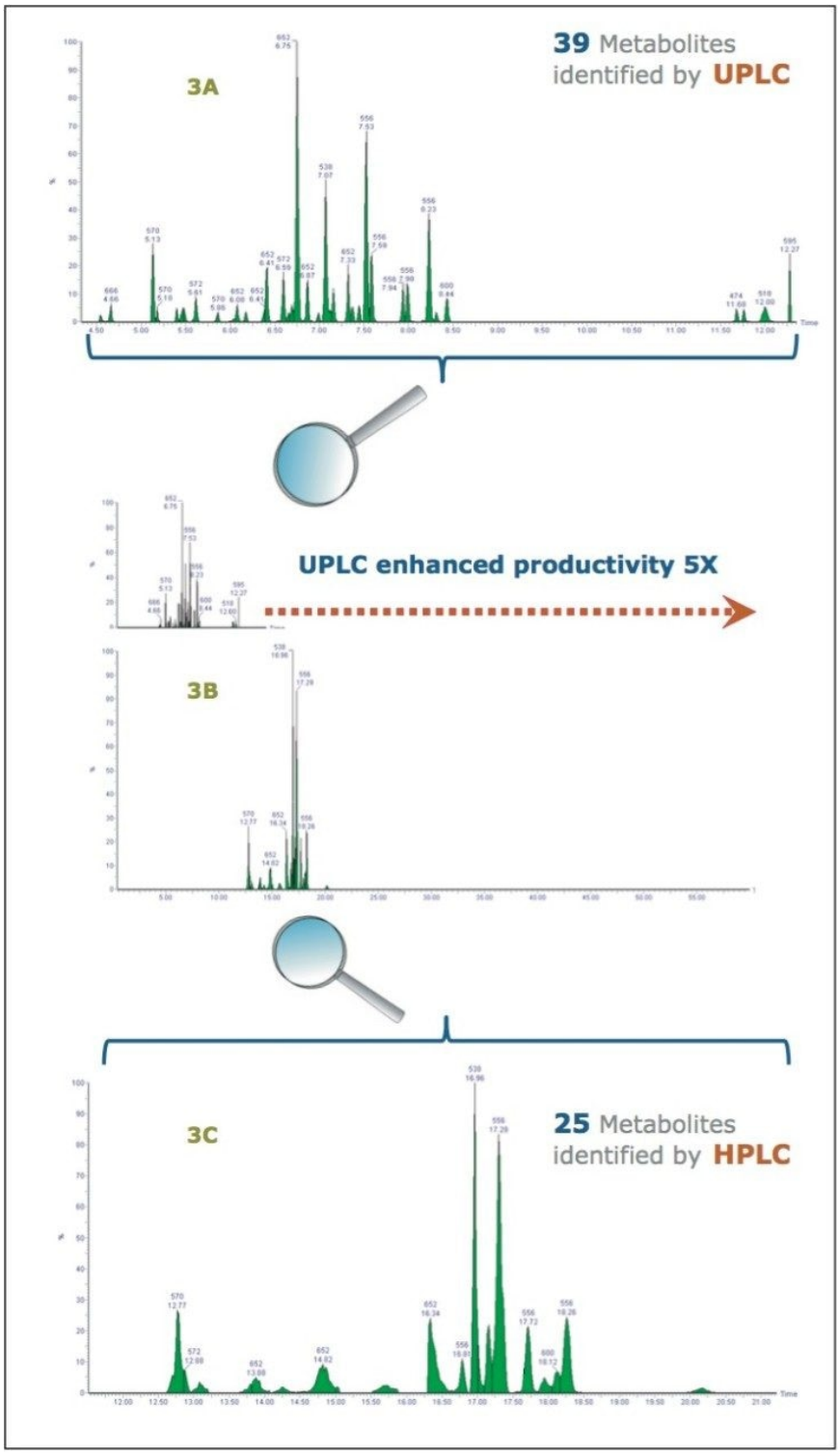

Figure 3. Chromatographic comparison of the UPLC metabolite ID results vs. the HPLC metabolite ID results.

UPLC offers a much better peak fidelity and resolution in a more compact time frame. Figure 3B shows the comparison of the UPLC and the HPLC combined metabolite chromatograms on the same time scale. The total separation time for UPLC was 15 minutes, while the total separation time for HPLC was 71 minutes. The UPLC analysis time was almost five times shorter, and resulted in about a 1.6-fold increase in identified metabolites than the HPLC methodology.

Thus, UPLC enables enhanced productivity with better quality data.

Conclusion

This application note demonstrates a simple and effective workflow combining UPLC/TOF-MS^E with MetaboLynx and MassFragment Software for Traditional Herbal (Chinese) Medicine metabolite identification from rat bile. Compared with HPLC, the UPLC approach delivered much higher chromatographic resolution (1.6X increase) in a shorter time frame. In turn, this directly translates to more productivity with more samples to be analyzed per unit time (5X increase), and with more metabolites being identified (1.6X increase).

The enrichment factor obtained from UPLC over HPLC for the detection of metabolites was the direct result of enhanced chromatographic resolution. This subsequently means that it is possible to obtain better and more conclusive MS/MS data from isobaric metabolites that would have otherwise coeluted. Therefore, this comprehensive workflow for metabolite identification enables the scientist to be more adept in the structural elucidation step.

References

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720002781, September 2008