

HILIC/MS/MS of Morphine and Metabolites with an Ultra Low Dispersion Agilent 1290 Infinity LC System

Application Note

Forensic Toxicology

Abstract

Morphine and 3 of its metabolites (normorphine, morphine-3- β -D-glucuronide [M3G], and morphine-6- β -D-glucuronide [M6G]) are analyzed by LC/MS/MS using an Agilent ZORBAX Rapid Resolution High Definition (RRHD) HILIC Plus, 2.1 × 50 mm, 1.8 µm column. M3G and M6G are isobaric compounds that elute closely. The analysis is performed on a default Agilent 1290 Infinity LC System with standard 0.12 mm id capillaries, and a 1290 Infinity LC System optimized for the lowest possible extra column volume with the 0.075 mm id capillaries found in the Agilent Ultra Low Dispersion Tubing Kit (extra column volume is reduced by >60% compared to the default set up). Both configurations are paired with an Agilent 6410A Triple Quadrupole LC/MS System. Improvements to baseline chromatographic resolution of the isobaric M3G and M6G are evident, as well as improvements in LC/MS/MS sensitivity, leading to easier and more reproducible quantitation.



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Introduction

Small dimension LC columns packed with small particles deliver increased productivity with faster analyses or more resolution. To take full advantage of small UHPLC columns, the LC system extra column volume must be minimized; this can include connecting capillaries, needle seats, heat exchangers, and detector flow cells (if applicable). Peak broadening occurs as soon as the sample is introduced into the LC system, as it travels through the autosampler, to the column, then to the detector, and finally through the detector flow cell. Minimizing this volume is especially critical for small dimension columns as it will account for a higher percentage of the system's extra column volume.

Previous work has shown the benefits of high efficiency columns with LC/MS [1] and the benefits of using ultra low dispersion 0.075 mm id capillaries with a 1290 Infinity LC System [2] separately. This work addresses the use of an ultra low dispersion 1290 Infinity LC System with MS detection and shows the improvements in resolution and sensitivity possible with a challenging isobaric LC/MS/MS separation. The compounds of interest are morphine, normorphine, morphine-3- β -D-glucuronide (M3G), and morphine-6- β -D-glucuronide (M6G) (Figure 1). Morphine is a powerful opiate analgesic. Normorphine is a major metabolite of morphine, a demethylated derivative that can be used to form opioid agonists and antagonists. M3G is a non-active metabolite of morphine, while M6G is the major active metabolite. It is believed that M6G acts as an agonist at the opioid receptors, causing much of the pain relieving analgesic effect of morphine [3,4,5]. M3G and M6G elute closely and are isobaric compounds, making baseline chromatographic resolution essential for good quantitation.

Experimental

A 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole LC/MS System was used in this experiment. Two setups were compared, a default configuration and an optimized configuration for ultra low dispersion. The default configuration used standard 0.12 mm id capillaries to connect the LC modules and the MS. The optimized setup used short 0.075 mm id capillaries found in the Agilent Ultra Low Dispersion Kit (p/n 5067-5189) and an Agilent LC System Rack (p/n 5001-3726), as shown in Figure 2. Morphine

Normorphine



Morphine-3- β -D-glucuronide (M3G)

Morphine-6- β -D-glucuronide (M6G)



Figure 1. Compounds of interest.

Conditions

Column:	Agilent ZORBAX Rapid Resolution High Definition (RRHD) HILIC Plus, 2.1 × 50 mm, 1.8 μm (p/n 959757-901)		
Mobile phase:	A: 10 mM NH ₄ HCO ₂ pH 3.2 B: CH ₃ CN/100 mM NH ₄ HCO ₂ pH 3.2 (9:1)		
Flow rate:	0.4 mL/min		
Gradient:	Hold 100% B for 0.25 min, ramp from 100-55% B in 0.75 min		
Temperature:	25 °C		
Sample:	0.1 μ L injection of 1 μ g/mL each of morphine, normorphine, morphine-3- β -D-glucuronide, and morphine-6- β -D-glucuronide in CH ₃ CN		
MS source:	Positive ESI, capillary 4000 V, drying gas temperature 250 °C, flow rate 11 L/min, nebulizer pressure 30 psi		
MS acquisition:	sition: Dynamic MRM mode (dMRM), delta EMV 200 V, MS cycle time 40 ms, compound MRN transitions are shown in Table 1		
Software:	Agilent MassHunter versions B.03.01, B.02.00, and B.03.01 for data acquisition, qualitative, and quantitative analyses, respectively		



Figure 2. Comparison of extra column volume on an Agilent 1290 Infinity LC System without (left) and with (right) ultra low dispersion optimizations; extra column volume can be reduced by more than 60%.

Table 1. Mass spectrometer MRM transitions for analysis of morphine and its metabolites.

Compound name	Precursor ion	Product ion	Fragmentor	Collision energy
Normorphine	272	152	170	65
Normorphine	272	165	170	35
Morphine	286	152	170	65
Morphine	286	165	170	35
M6G	462	286	170	30
M6G	462	201	170	45
M3G	462	286	170	30
M3G	462	201	170	45

Optimized Agilent 1290 Infinity LC System

All 4 analytes were purchased in methanol solutions from Cerilliant and diluted to desired concentrations in acetonitrile. Acetonitrile was purchased from Honeywell. Ammonium formate and formic acid were purchased from Sigma Aldrich. Water used was 18 M Ω Milli-Q water.

Results and Discussion

Narrow 0.075 mm id capillaries from the Agilent Ultra Low Dispersion Kit replace the standard 0.12 mm id capillaries on the 1290 Infinity LC System (ALS→TCC capillary, TCC→MS capillary, and needle seat capillary [0.11 mm id]). The LC is further optimized by rearranging the modules with the Agilent LC System Rack. While the 1290 Infinity LC System is typically stacked with the binary pump on the bottom due to its weight, using an LC Rack allows the pump to be safely located at the top of the stack (from top to bottom: solvent tray, binary pump, autosampler, column compartment, and diode array detector [if applicable]). This permits the use of the shortest possible capillaries. Shorter 220 mm length capillaries connect the autosampler valve to the column inlet and the column outlet to the mass spectrometer (as compared to 340 mm capillaries in the default configuration). For this analysis, the diode array detector and column heat exchangers are not necessary, and are, therefore, removed from the sample path to further minimize dispersion. The result is more than a 60% reduction in extra column volume from the default 1290 Infinity LC System configuration (8.7 μ L) to the optimized configuration (3.1 μ L). See Figure 2 for an illustrative and volumetric comparison of the extra column volume in the default setup versus an ultra low dispersion 1290 Infinity LC System.

Figure 3 shows 2 total ion chromatograms from LC/MS/MS analyses of morphine on a 1.8 μ m Agilent ZORBAX RRHD HILIC Plus column, comparing a default 1290 Infinity LC System configuration with one optimized for ultra low dispersion. The taller, narrower peaks are immediately evident with the low dispersion LC/MS system. Because of the lower volume sample path, there is also a slight decrease in retention time for the low dispersion system.



Figure 3. An overlay of total ion chromatograms from an LC/MS/MS analysis of morphine shows that reducing the LC/MS system extra column volume by >60% generates taller, sharper peaks and improves resolution and MS sensitivity for all peaks.

The extracted MRM chromatograms shown in Figure 4 highlight the importance of optimizing performance of a UHPLC column, by minimizing system dispersion with LC/MS analyses. While the MS can typically isolate coeluting peaks, in this case, M6G and M3G are isobaric and have the same quantitative and qualitative ion transitions, as shown in Table 1, and need to be baseline separated chromatographically. The quantitative ion is shown for each compound in their respective full scale to better reveal the small M6G peak, which is much less sensitive than the other 3 compounds (see the TICs in Figure 3). It is evident that while M3G and M6G are fairly well resolved with the default LC/MS system, reducing the extra column volume improves the resolution substantially, from 1.99 to 2.73. The optimized LC/MS system allows the full resolving power of the sub-2 µm UHPLC column to be exploited, as well as its high efficiency. Reducing the extra column volume by >60% improves the resolution of these 2 peaks by 37%. Sensitivity (signal-to-noise) is also improved by 30%.

Conclusions

The high efficiency of an Agilent ZORBAX RRHD HILIC Plus column, combined with an ultra low dispersion Agilent 1290 Infinity LC System is demonstrated with an LC/MS/MS separation of morphine, normorphine, morphine-3- β -D-glucuronide, and morphine-6- β -D-glucuronide. Minimizing system dispersion allowed for improved baseline resolution of 2 isobaric metabolites of morphine, as well as improved sensitivity. Both allow for easier and more reproducible quantitation.



Figure 4. Extracted MRM chromatograms from an LC/MS/MS analysis of morphine shows that reducing the LC/MS system extra column volume by >60% improves resolution of the isobaric M3G and M6G for easier integration and improves LC/MS sensitivity of M6G.

References

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