# Comparing HILIC and RPLC of Morphine Using Agilent ZORBAX RRHD Columns with UHPLC/MS

Application Note

Forensics and Drug Testing


## Author

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## Abstract

UHPLC/MS analyses of morphine and 3 of its metabolites (normorphine, morphine-3-$\beta$-D-glucuronide [M3G], and morphine-6-$\beta$-D-glucuronide [M6G]) are used to compare mass spectrometer sensitivity with reversed-phase liquid chromatography (RPLC) and hydrophilic interaction chromatography (HILIC). Two Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns are used in this comparison, an Agilent ZORBAX RRHD Eclipse Plus C18 for the RPLC method and an Agilent ZORBAX RRHD HILIC Plus for the HILIC method. Each is used with an Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer. Both methods use isocratic elution with an acetonitrile/ammonium acetate buffer mobile phase. Signal-to-noise calculations for the least sensitive M6G peak show HILIC mode allows for more sensitive MS detection due to more efficient spraying and desolvation in the ESI-MS source, as a result of the volatile acetonitrile rich mobile phase used.


## Introduction

Hydrophilic interaction chromatography (HILIC) is gaining popularity in liquid chromatography, particularly for its ability to retain and separate small polar analytes - an area where common reversed-phase liquid chromatography (RPLC) methodology often fails. This novel mode of chromatography results in unique retention mechanisms, because water is used as the strong eluting solvent. HILIC can have distinct advantages over traditional RPLC in LC/MS sensitivity, due to the use of highly organic mobile phases. The highly organic mobile phases have higher volatility than traditional RPLC mobile phases, making HILIC well suited for applications with mass spectrometers.

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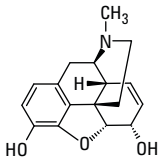
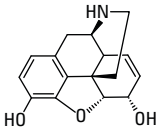
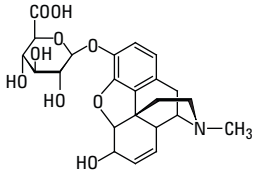
HILIC is used extensively to analyze polar molecules. In this work, morphine and 3 metabolites are analyzed by LC/MS to demonstrate the effectiveness of HILIC with regard to MS sensitivity, compared to RPLC. The compounds of interest are morphine, normorphine, morphine-3-$\beta$-D-glucuronide (M3G), and morphine-6-$\beta$-D-glucuronide (M6G) (Figure 1). Morphine is a powerful opiate analgesic. Normorphine is a major metabolite of morphine; it is a demethylated derivative that can be used to form both 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 effects of morphine [1,2,3].



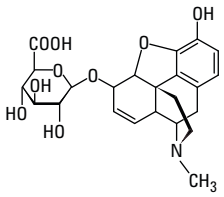

Morphine




Normorphine




Morphine-3-$\beta$-D-glucuronide (M3G)




Morphine-6-$\beta$-D-glucuronide (M6G)

Figure 1. Compounds of interest.

## Experimental

An Agilent 1290 Infinity LC System with an Agilent 6410A Triple Quadrupole Mass Spectrometer was used in this experiment. The setup was optimized for lowest possible extra-column volume with short 0.075 mm id capillaries found in the Agilent Ultra-Low Dispersion Kit (p/n 5067-5189) and with an Agilent LC System Rack (p/n 5001-3726) [4].

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

### Conditions

| | |
|---|---|
| Columns: | Agilent ZORBAX RRHD HILIC Plus, 2.1 × 100 mm, 1.8 µm (p/n 959758-901)<br>Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902)<br>Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 3.5 µm (p/n 959793-902)<br>Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 5 µm (p/n custom) |
| Mobile phase: | A: 10 mM $NH_4HCO_2$ pH 3.2<br>B: $CH_3CN$/100 mM $NH_4HCO_2$ pH 3.2 (9:1)<br>HILIC 70% B isocratic; RPLC 10% B isocratic |
| Flow rate: | 0.4 or 1.0 mL/min |
| Temperature: | 25 °C |
| Sample: | 2 µL injection of 1 µg/mL each of morphine, normorphine, morphine-3-$\beta$-D-glucuronide, and morphine-6-$\beta$-D-glucuronide; HILIC sample was prepared in $CH_3CN$; RPLC sample was prepared in $H_2O$ |
| MS source: | Positive ESI, capillary 4000 V, drying gas temperature, flow rate and nebulizer pressure vary with mobile phase flow rate and are specified in Table 1 |
| MS acquisition: | Selected ion mode (SIM), delta EMV 200 V, MS dwell time varies with mobile phase flow rate and is specified in Table 1, compound specific parameters are detailed in Table 2 |
| Software: | Agilent MassHunter versions B.03.01, B.02.00 and B.03.01 were used for data acquisition, qualitative, and quantitative analyses, respectively |

Table 1. Mass spectrometer parameters for morphine analyses at various flow rates.

| | 0.4 mL/min | 1.0 mL/min |
|---|---|---|
| Source | ESI+ | ESI+ |
| Delta EMV | 200 V | 200 V |
| MS dwell time | 20 ms | 10 ms |
| Drying gas temperature | 250 °C | 325 °C |
| Drying gas flow rate | 11 L/min | 12 L/min |
| Nebulizer pressure | 30 psi | 55 psi |
| Capillary voltage | 4000 V | 4000 V |

Table 2. Mass spectrometer SIM parameters for morphine analyses.

| | M+H | Fragmentor voltage |
|---|---|---|
| Morphine | 286 | 170 |
| Normorphine | 272 | 170 |
| Morphine-3-$\beta$-D-glucuronide | 462 | 170 |
| Morphine-6-$\beta$-D-glucuronide | 462 | 170 |

## Results and Discussion

### Improvements in RPLC/MS Sensitivity with UHPLC Columns

Comparing traditional 5 µm columns to newer 1.8 µm UHPLC columns shows a substantial improvement in chromatographic performance, specifically regarding peak width, as shown in Figure 2. This RPLC analysis of morphine and its metabolites shows significantly narrower peaks generated by the highly efficient sub-2 µm UHPLC column as compared to the traditional 5 µm column. Method parameters for each of these analyses are identical, including the stationary phase chemistry; the only variable is particle size. This example shows overlays of the individual extracted ion chromatograms (EICs) for each compound in their full scale. Selectivity is maintained across all particles sizes with these ZORBAX Eclipse Plus C18 columns, which could allow for easy method scalability and transferability should the need arise.

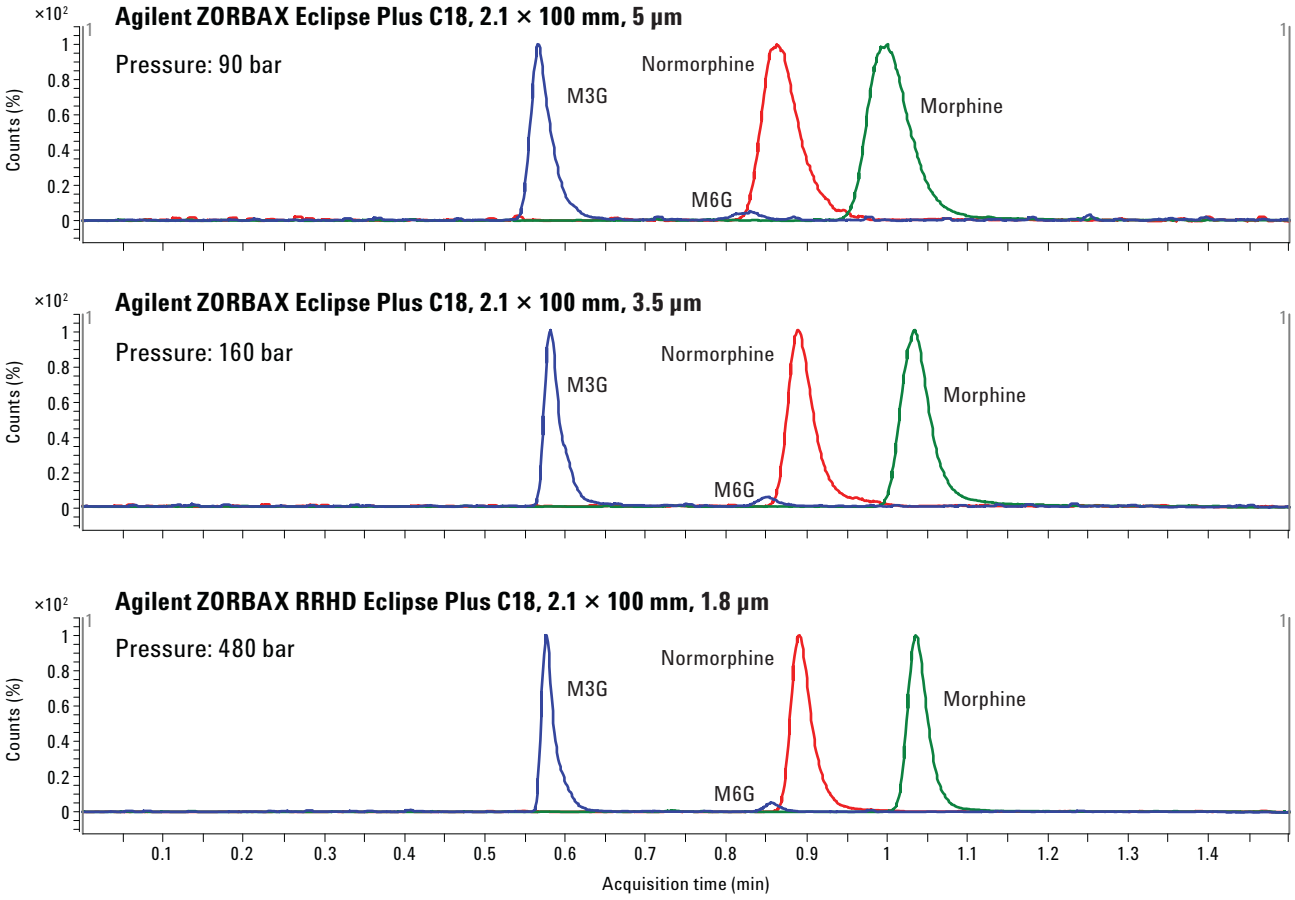



Figure 2. Peak width is improved with high efficiency 1.8 µm UHPLC columns compared to traditional 5 µm columns, while selectivity is maintained; LC/MS method parameters are detailed in the Experimental section.

M3G and M6G are isobaric and, consequently, are detected by the same mass at *m/z* 462. When these EICs are fit to the same scale (Figure 3), not only is the peak width improvement apparent with the smaller particle column, but also the difference in peak height. Comparing the signal-to-noise of the least sensitive M6G peak, shows that the highly efficient sub-2 µm UHPLC column is capable of improving the MS sensitivity by a factor of 5, when compared to a traditional 5 µm column.

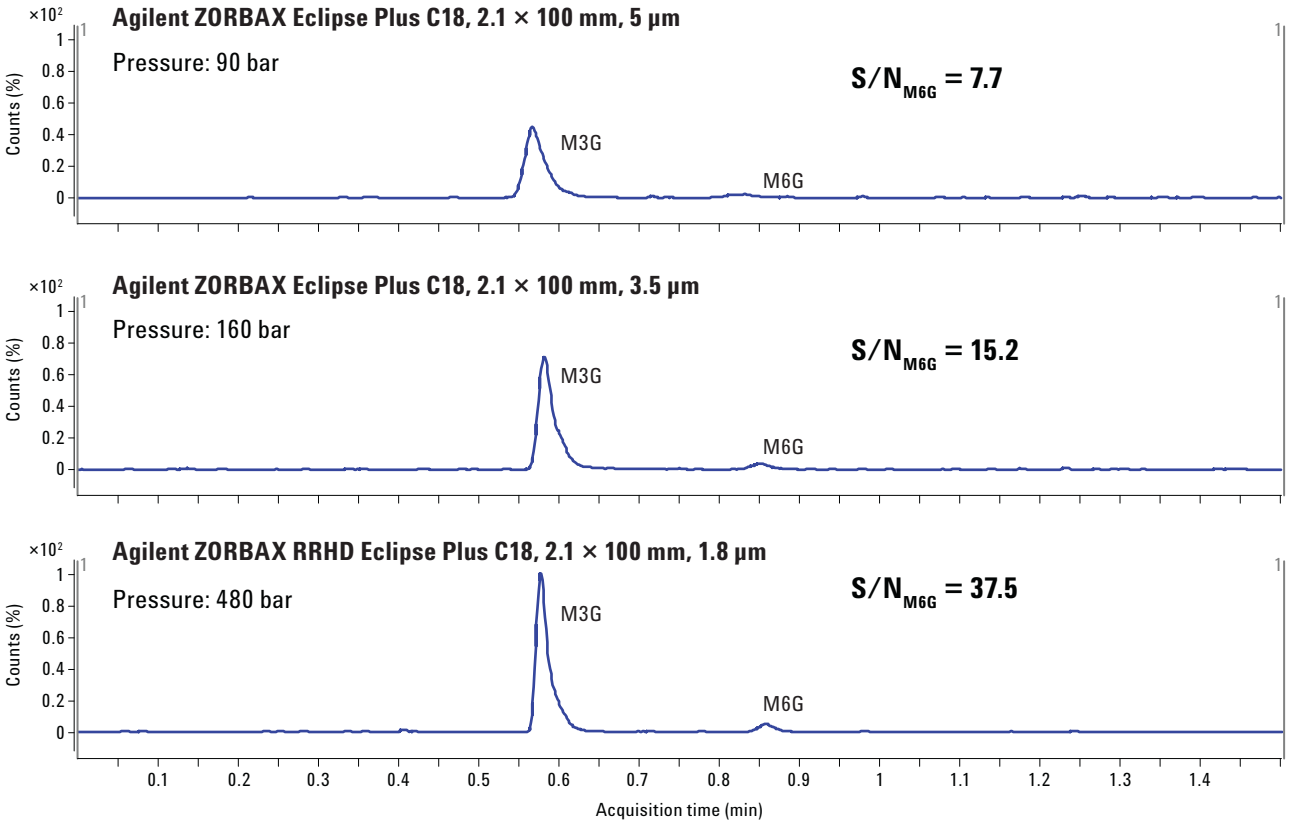



Figure 3. MS sensitivity (signal-to-noise) is improved by a factor of 5 with efficient 1.8 µm UHPLC columns compared to traditional 5 µm columns, while selectivity is maintained; LC/MS method parameters are detailed in the Experimental section.

## Better LC/MS Sensitivity with HILIC Mode

Knowing the improvements possible with UHPLC columns compared to traditional LC columns, the impact of HILIC mode on MS sensitivity is explored. Figure 4 shows side-by-side chromatograms for similar analyses with RPLC and HILIC. Method parameters were kept as consistent as possible to eliminate unfair comparisons between the 2 analyses. The RPLC analysis uses 10% acetonitrile, while the HILIC uses 70% acetonitrile. Sample solvents matched the weak solvent in each case; the HILIC sample solvent was acetonitrile and the RPLC sample solvent was water to eliminate peak shape distortion that could occur if the strong solvents were injected in either mode. The ammonium formate buffer concentration is 10 mM in both the aqueous and organic portions of the mobile phase to eliminate differences in MS detection due to salt concentration. Each analysis is isocratic with similar retention factors to eliminate differences due to gradient focusing or column efficiency. These are overlays of EICs each in their full scale to show the selectivity differences between the C18 and HILIC columns. The HILIC selectivity is complementary to the C18, and almost opposite. An interesting observation is that the HILIC Plus column retains all compounds more than the C18, even though the C18 is run at the minimal suggested organic/aqueous ratio. Using less organic with the C18 column could cause phase collapse or column dewetting, leading to irreproducible or poor chromatography.

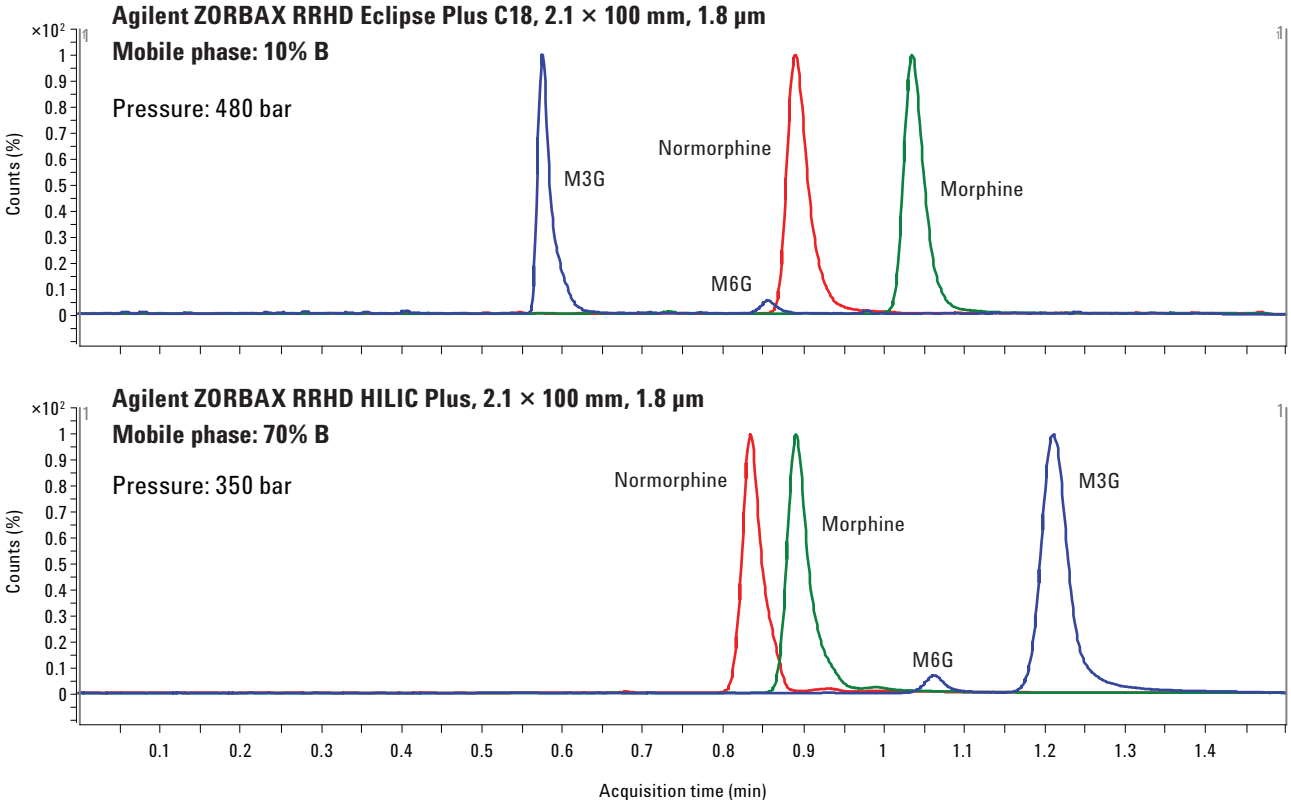



Figure 4. The Agilent ZORBAX RRHD HILIC Plus column offers complimentary selectivity to an Agilent ZORBAX RRHD Eclipse Plus C18 column, almost reversing elution order; LC/MS method parameters are detailed in the Experimental section.

Scaling the glucuronide metabolites to the same scale shows not only a selectivity difference between the C18 column and the HILIC, but also a more intense MS signal in HILIC mode, as shown in Figure 5. The HILIC Plus column uses 70% acetonitrile, compared to the 10% with the C18 column, which is more volatile and allows for more efficient spraying and desolvation in the ESI-MS source, thereby generating a more intense MS signal.

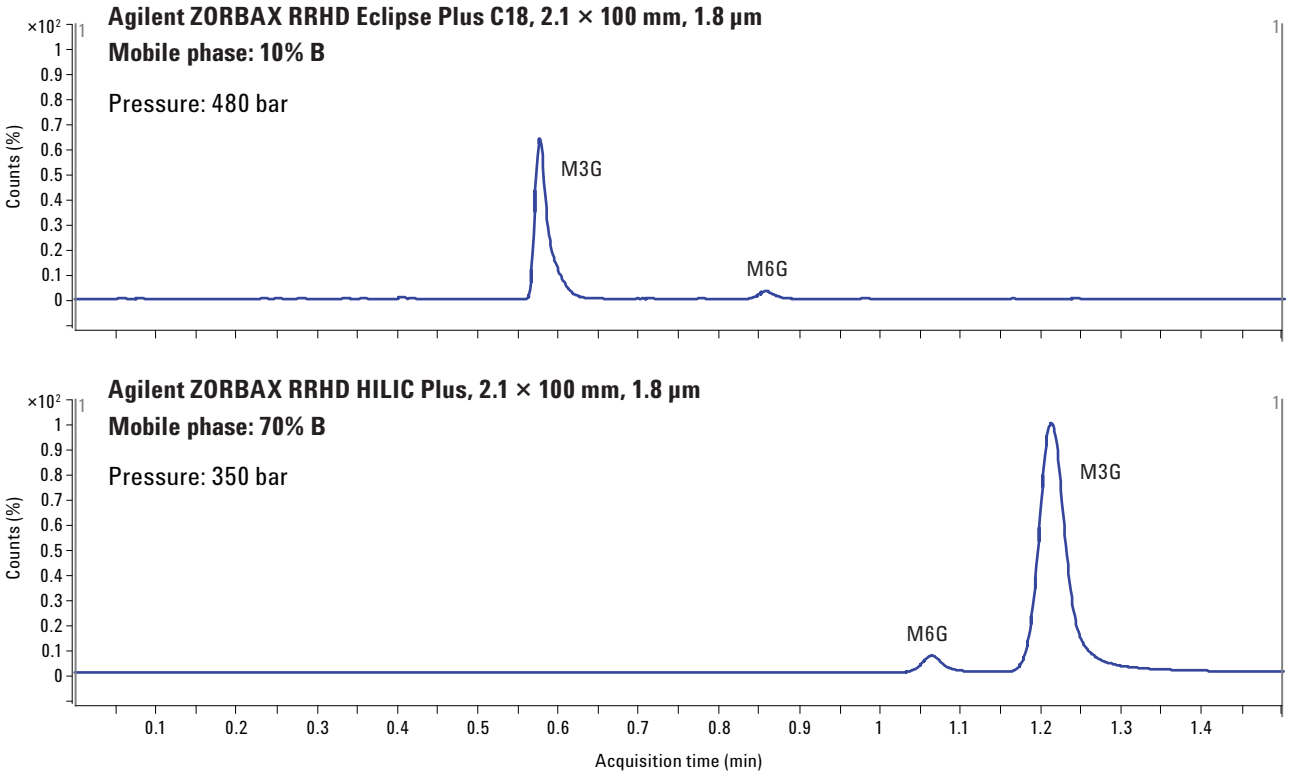



Figure 5. HILIC mode allows for more efficient spraying and desolvation in the ESI-MS source compared to RPLC, generating a more intense MS signal; LC/MS method parameters are detailed in the Experimental section.

A closer look at the M6G peak shows that HILIC mode not only improves signal intensity, but also reduces baseline noise. Figure 6 shows a fourfold improvement in signal-to-noise for M6G in HILIC mode compared to a similar UHPLC C18 method.

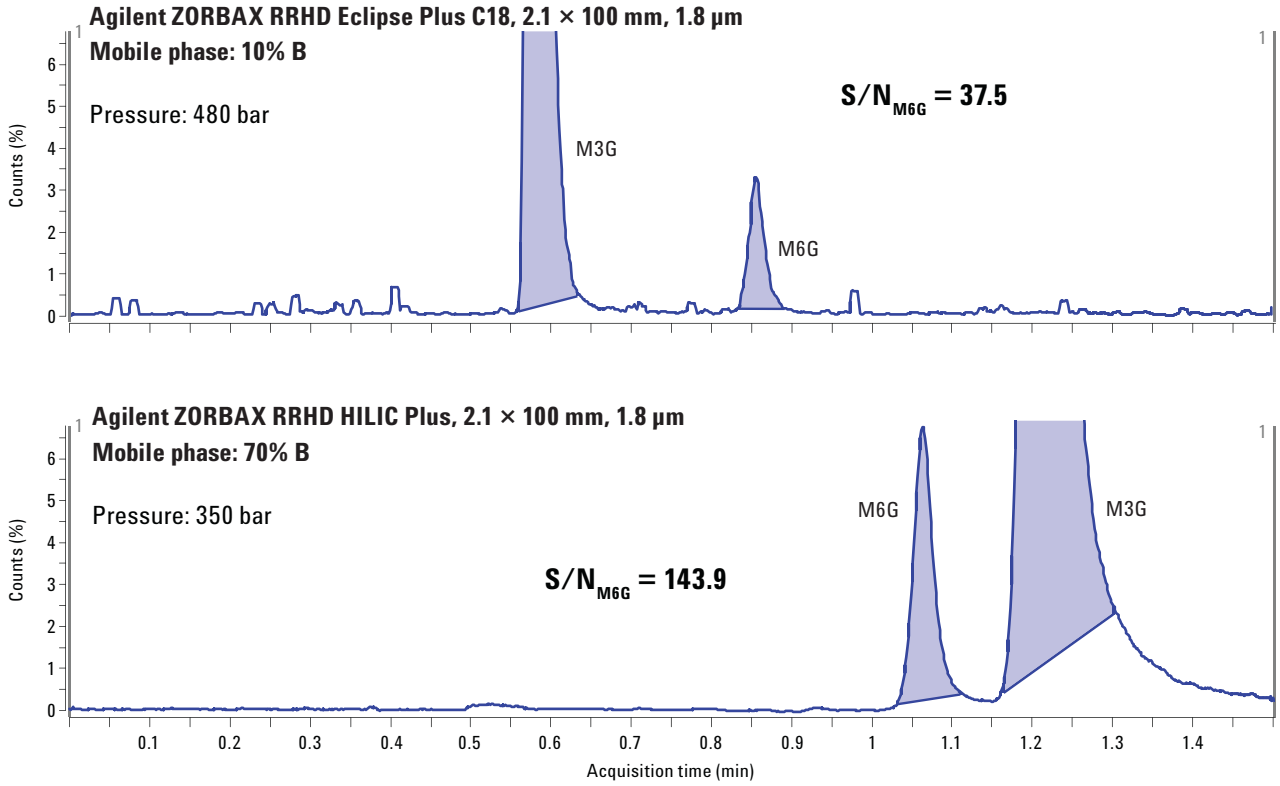



Figure 6. HILIC mode allows for more efficient spraying and desolvation in the ESI-MS source compared to RPLC, generating less baseline noise and a more intense MS signal resulting in a 4× improvement in sensitivity; LC/MS method parameters are detailed in the Experimental section.

To see the combined effects of using HILIC with UHPLC/MS the 1.8 µm HILIC Plus column can be compared to the earlier 5 µm column in RPLC mode. Again, the RPLC chromatogram shows more baseline noise, and when combined with the less efficient 5 µm particle column, the result is a 20x improvement in signal-to-noise for the active morphine metabolite M6G, as seen in Figure 7.

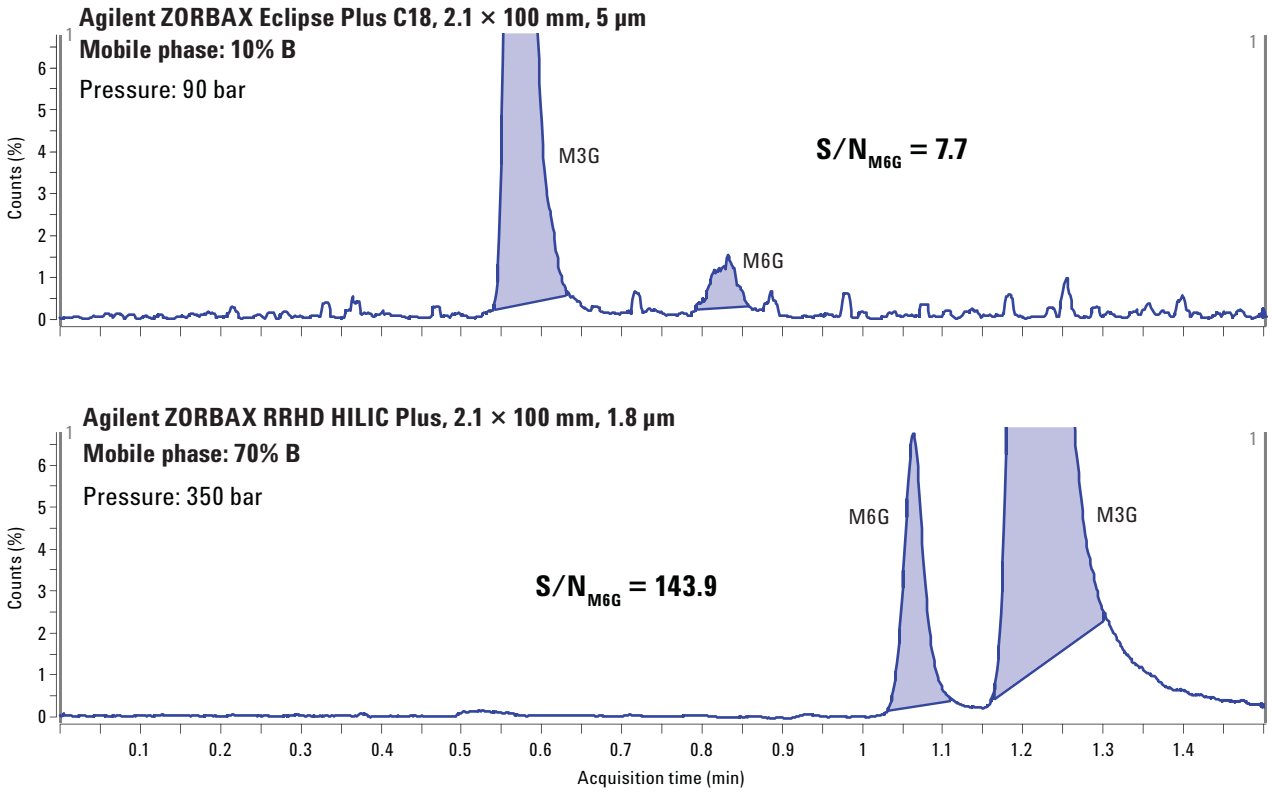



Figure 7. HILIC mode with UHPLC columns improves MS sensitivity by a factor of 20, compared to traditional LC columns in RPLC mode with MS detection; LC/MS method parameters are detailed in the Experimental section.

The high 1200 bar pressure limits of both the UHPLC column and LC system can be exploited by increasing the mobile phase flow rate. This shows that not only is the HILIC method more sensitive than a comparable RPLC method with traditional columns, but it can be accomplished in less than half the time while still substantially improving sensitivity by more than a factor of 10 (Figure 8). The sensitivity of this UHPLC analysis in HILIC mode is slightly less than the previous HILIC analysis, because the flow rate is increased to 1 mL/min from the optimal 0.4 mL/min, and so some column efficiency is likely to be lost.

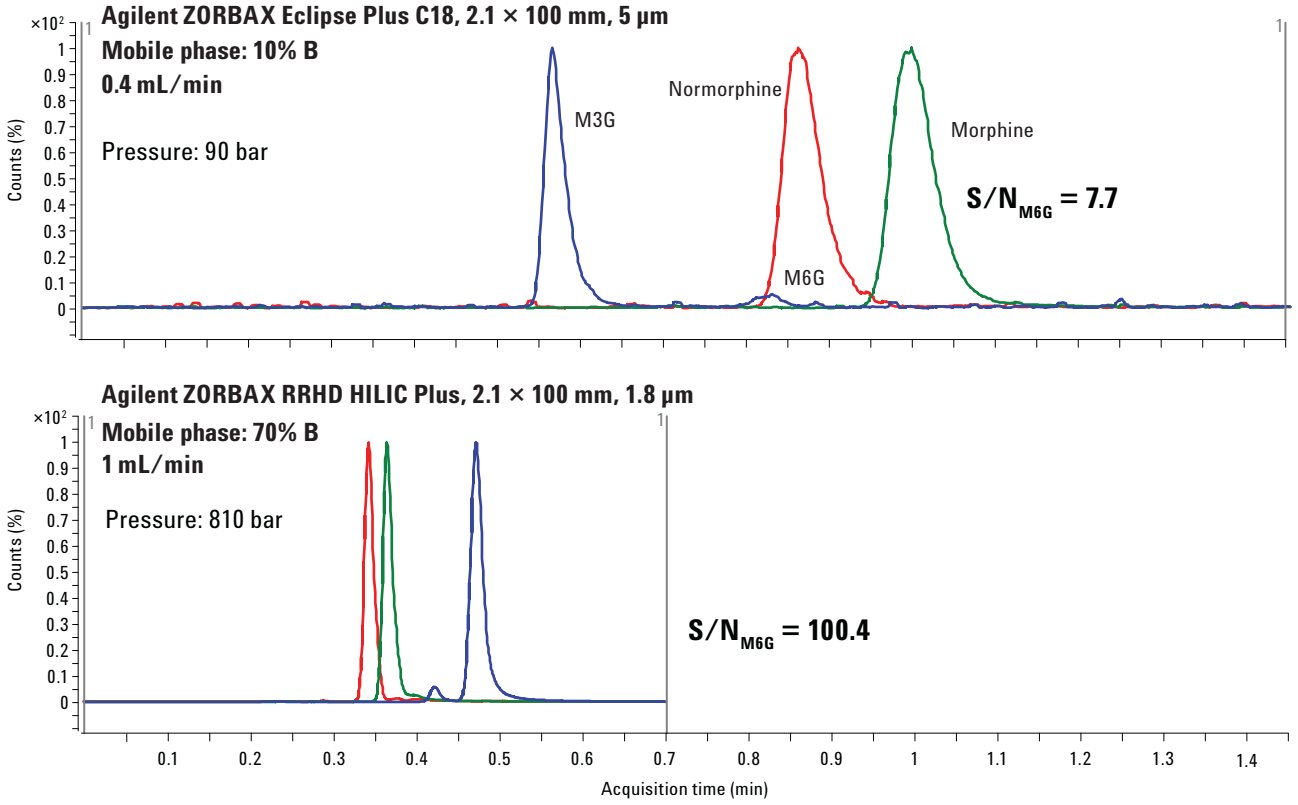



Figure 8. HILIC mode with UHPLC columns cuts analysis time in half, while improving sensitivity by more than a factor of 10, compared to traditional LC columns in RPLC mode with MS detection; LC/MS method parameters are detailed in the Experimental section.

## Conclusions

Using these analyses for morphine and its metabolites, it is demonstrated that high efficiency sub-2 µm UHPLC columns can improve sensitivity by 5x when compared to traditional 5 µm columns. Also, HILIC mode can improve ESI-MS efficiency due to its more volatile highly organic mobile phase by 4x, compared to a similar UHPLC analysis in RPLC mode. Combining the improvements possible with UHPLC columns and HILIC mode with MS detection can yield as much as a 20x improvement in sensitivity, in contrast to a traditional 5 µm column in RPLC mode. Furthermore, the UHPLC column and LC system can be exploited with high flow rates to accomplish the analysis in less time, while still improving sensitivity, in this case by more than 10x.

## References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