Overcoming Endogenous Matrix and Analyte-related Interference Effects in the Bioanalysis of Polar Analytes using HILIC-MS/MS

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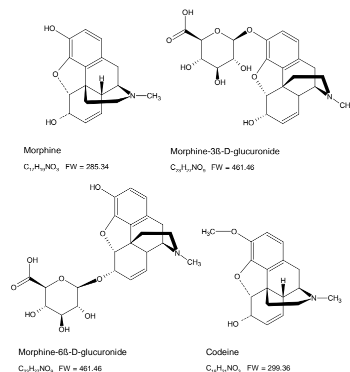
INTRODUCTION

Hydrophilic interaction chromatography (HILIC) using silica columns offers significant advantages for analysis of polar analytes, including improved retention and peak shape, low back pressure, and high sensitivity for MS detection due to the high organic/low aqueous mobile phases. As in reversed-phase HPLC, matrix interference effects, particularly ion suppression from co-extracted phospholipids, can cause assay imprecision, inaccuracy, and loss of sensitivity in HILIC applications. Eliminating these effects may be complicated by the mixed retention mechanisms of silica sorbents, which can include hydrophilic, cation exchange, and hydrophobic interactions. Analyte carryover from the column may also be an issue. Herein we describe several challenges that were overcome during development and validation of a HILIC-MS/MS assay for codeine, morphine, and two glucuronide metabolites in human plasma.

Figure 1. Chemical Structures



ASSAY DEVELOPMENT

SPEs with Oasis MCX and HLB (Waters) and BondElut C18 (Varian) plates were compared. Human plasma sample extracts were analyzed on Thermo Betasil Silica (100 mm x 3 mm, 5 µm) or Varian MonoChrom Si, (100 mm x 4.6 mm, 3 µm) columns, using mobile phases containing 5:95 to 20:80 (v/v) water/acetonitrile with 0.05 to 1% acid modifiers or buffers (ammonium acetate or formate) at various pHs and concentrations.

Analytes were detected using Sciex API4000 with positive TurboIonSpray. MRM transitions corresponding to expected phospholipids (precursor ions: m/z 496, 524, 704, 758 and 806; product ions: m/z 184) were also monitored to evaluate phospholipid retention times.

Figure 2. Retention Time Correlation with pH

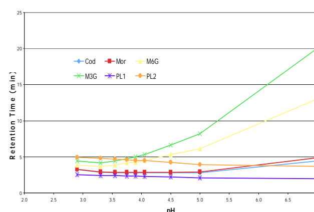
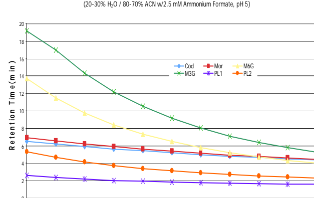


Figure 3. Retention Time vs. Water Content

(20-30% H_2O / 80-70% ACN w/2.5 mM Ammonium Formate, pH 5)



Analyte post-column infusion was used to locate ion suppression regions caused by plasma matrix components. Ion suppression was observed at various retention times in extracts from all SPE sorbents tested. Phospholipid levels may in some cases be reduced with these sorbents but not sufficiently to avoid suppression effects. Positive interferences, originating from matrix-related background and in-source decomposition of glucuronide conjugates, were observed in channels monitored for morphine and codeine. Unlike in reversed-phase HPLC, where phospholipids are strongly retained and more polar monosubstituted lysophospholipids elute before disubstituted phospholipids, these lipids were more readily eluted from silica columns under HILIC conditions, with a reversed elution order.

Figure 4. Retention before Optimization

Phospholipid elution order is from high to low mass on silica column. Phospholipids m/z 806 and 496 bracketed analyte peaks, and phospholipids with intermediates masses (m/z 758, 704, and 524) co-eluted or partially overlapped with analyte peaks.

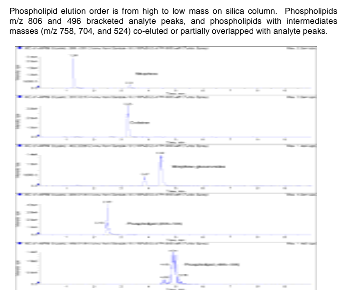


Figure 5. Retention after Optimization

All phospholipids were eluted before the analytes, demonstrating formate concentration and water content can be used effectively to affect elution and achieve complete separation between phospholipid and analyte peaks.

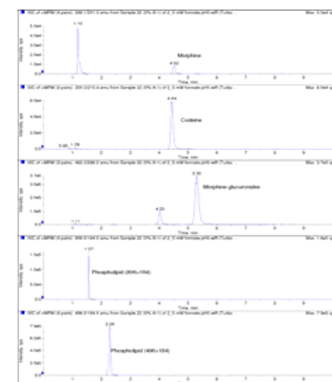
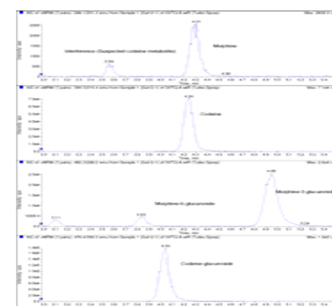


Figure 6. Codeine Glucuronide Final Chromatography

Additional codeine glucuronide peak was revealed in incurred samples and was separated from the analytes chromatographically.



The reversed elution pattern on silica columns is an advantage. To optimize phospholipid separation, other matrix interferences, and glucuronide metabolites from target analytes and ISs, MP modifiers such as TFA, acetic acid, formic acid, and/or ammonium formate were tested. Carryover was reduced using isocratic elution. MP composition tests showed pH and ammonium formate concentration significantly affected glucuronides relative retention, while water content was most important for phospholipids separation. These parameters were optimized to eliminate interferences, and the assay for quantitative determination of codeine, morphine, and two glucuronides in plasma was validated.

METHOD SUMMARY

Validation Samples

Calibrators were made at 8 concentration levels spanning the quantitation ranges of 1.00 to 100 ng/mL for codeine, 0.200 to 20.0 ng/mL for morphine, 0.500 to 50.0 ng/mL for morphine-6ß-glucuronide, and 2.00 to 200 ng/mL for morphine-3ß-glucuronide. QC pools were prepared at concentrations spanning the quantitation ranges for the evaluation of assay performance, stability, and recovery.

Extraction Method

A 250-µL sample aliquot was fortified with 25 µL of internal standard working solution. Analytes were isolated through SPE using Varian Bond Elut C18, 50-mg, 96-well SPE plates and eluted with 800 µL of 50:50 methanol / water, v/v. The eluate was evaporated under a nitrogen stream at approximately 50 °C, and the remaining residue was reconstituted with 200 µL of 10:90 ethanol / acetonitrile, v/v.

Chromatographic and MS Conditions

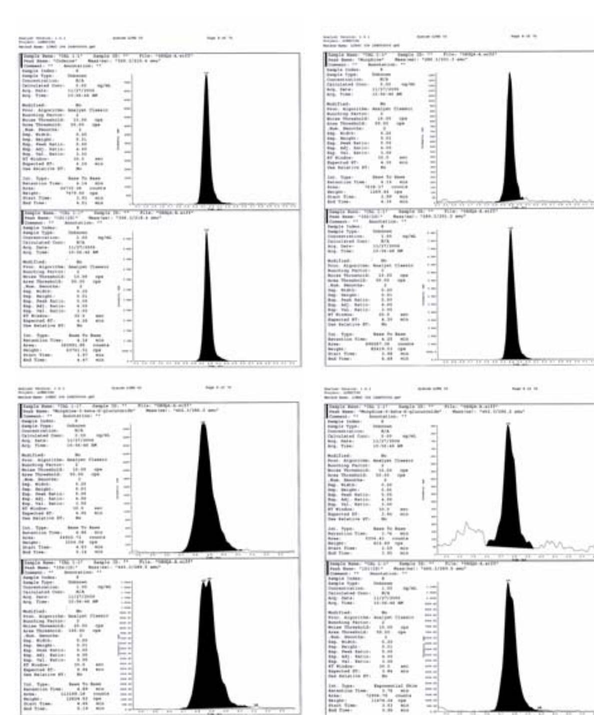
LC separation was achieved with a mobile phase of 2.5 mM ammonium formate in 70:30 acetonitrile/water, v/v flowing at 1 mL/min on a Varian MonoChrom Si column (4.6 mm x 100 mm, 3 µm). The flow from the analytical column was diverted to waste with a switching valve during the first 3 min, then to the MS for 2.5 min, and to waste again at the end of the run. A second pump was used to generate make-up flow with the same mobile phase during valve switching. The total run time was 5.5 min.

Inter-assay Precision and Accuracy

SAMPLE	Codeine			
	Concn (ng/mL)	%CV	%Bias	n
LLOQ	1.00	7.85	0.564	18
QC 1	2.00	5.48	-1.62	21
QC 2	4.00	5.27	-0.638	21
QC 3	10.0	4.28	-2.49	21
QC 4	25.0	5.24	-2.11	21
QC 5	75.0	6.63	-1.94	21
Sample	**Morphine**			
	Concn (ng/mL)	%CV	%Bias	n
LLOQ	0.200	12.0	-9.18	18
QC 1	0.400	5.08	-1.12	21
QC 2	0.800	18.6	10.2	21
QC 3	2.00	4.02	-1.46	21
QC 4	5.00	4.50	0.122	21
QC 5	15.0	4.08	-0.650	21
Sample	**Morphine-3ß-glucuronide**			
	Concn (ng/mL)	%CV	%Bias	n
LLOQ	2.00	8.50	-5.58	18
QC 1	4.00	5.33	-2.16	21
QC 2	8.00	5.61	-0.843	21
QC 3	20.0	6.66	1.45	21
QC 4	50.0	4.90	4.84	21
QC 5	150	5.39	0.712	21
Sample	**Morphine-6ß-glucuronide**			
	Concn (ng/mL)	%CV	%Bias	n
LLOQ	0.500	13.4	-0.331	18
QC 1	1.00	9.56	-3.66	24
QC 2	2.00	9.51	-2.32	24
QC 3	5.00	5.25	-4.51	24
QC 4	12.5	5.82	-0.690	24
QC 5	37.5	3.60	-1.44	24

Figure 7.

LLOQ for Codeine, Morphine, Morphine-3ß-glucuronide, and Morphine-6ß-glucuronide



RESULTS

Calibration curves were constructed using the chromatographic peak area ratios of the analyte and internal standard from the calibration samples by applying a linear, 1/concentration (1/c) weighted regression algorithm. Analyte concentrations in unknown samples were then calculated from their peak area ratios versus the calibration curve. Analyte stability in matrix has been demonstrated for seven freeze/thaw cycles and up to 25 hr at room temperature. Extract stability has been demonstrated for up to 132 hr at ~ 15 °C.

CONCLUSIONS

An LC/MS/MS assay has been successfully developed and validated for the determination of codeine, morphine, morphine-3ß-glucuronide, and morphine-6ß-glucuronide in human plasma containing sodium heparin. The assay is suitable for the analysis of clinical study samples as demonstrated by its specificity, precision, accuracy, recovery, and stability characteristics.

ACKNOWLEDGMENTS

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