# Development and Validation of an Ultra Sensitive UPLC-MS/MS Method for the Determination of Naloxone in Human Plasma

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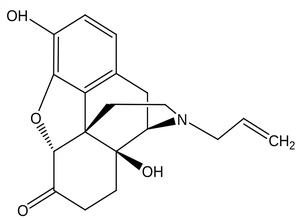
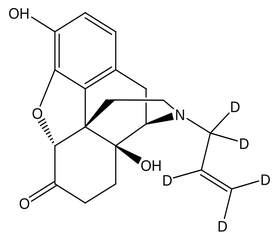
## INTRODUCTION

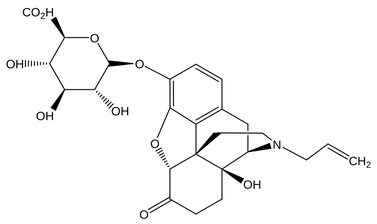
Naloxone(NLX) is a μ-opioid receptor antagonist and commonly used for counteracting opiate overdose. The combination use of buprenorphine and low dose of NLX was approved by the FDA to prevent illicit intravenous use of this formulation. The low dose of NLX requires a corresponding low limit of quantitation of NLX for pharmacokinetic studies (i.e. 5 pg/mL). Naloxone-3-glucoronide(NLX-G) is a major metabolite of NLX. The instability of NLX-G can impact the accurate measurement of NLX at such a low quantitation limit. This poster will summarize development and validation of an ultra sensitive LC-MS/MS assay to quantify NLX in human plasma without the impact from NLX-G. The assay is five times more sensitive than a recently published LC–MS/MS method for NLX.



Naloxone
$C_{19}H_{21}NO_4$
MW.: 327.38



Naloxone-$d_5$
$C_{19}H_{16}D_5NO_4$
MW.: 332.41



Naloxone-3-β-D-glucuronide
$C_{25}H_{29}NO_{10}$
MW: 503.5

## METHODS

### Sample Preparation

**350 µL of human plasma was mixed with internal standard and 350 µL 100 mM ammonium acetate buffer (pH = 5.0) , and then extracted the mixture using Phenomenex X-CW SPE plate.**

**Equilibration:** 1200 µL of ACN & 1200 µL 100 mM ammonium acetate buffer (pH = 5.0)

**Wash 1:** 1200 µL of water:acetic acid at 100:0.1(v:v)

**Wash 2:** 1200 µL of ACN

**Elution:** 800 µL of DCM : IPA : $NH_4OH$ at 80:20:2 (v:v:v)

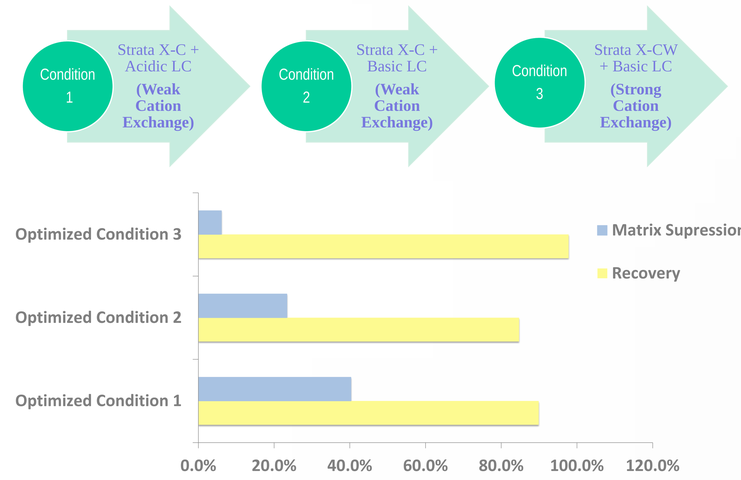
**Reconsititution:** 200 µL Methanol:Water at 5:95 (v:v)

### UPLC-MS/MS ANALYSIS

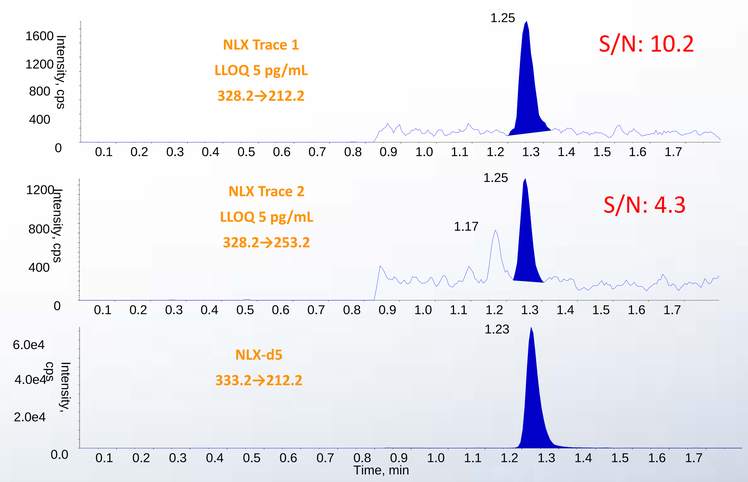
| | |
|---|---|
| **Mass Spectrometry:** | AB-Sciex API 5000. TIS + |
| **UPLC:** | Shimadzu Nexera UPLC |
| **Mobile Phase:** | A: Water:Ammonium Hydroxide at 100:0.1 (v:v)<br>B: Methanol:Ammonium Hydroxide at 100:0.1 (v:v) |
| **Program: Gradient:** | Starting at 40%B and ramping to 65%B |
| **Flow rate:** | **0.6 mL/min** |
| **UPLC column:** | Acquity BEH C18 column, 2.1 x 50 mm, 1.8 µm Waters |

## STRATEGIES TO REACH ULTRA LOW LIMIT

- **Reduce Matrix Effect by the Optimizing Solid Phase Extraction & LC Conditions**




- **Select the MRM Trace with Better S/N and less Interference Peaks**




## RESULTS

- **Inter-day Accuracy and Precision from Validation**

| | Naloxone Concnetration (pg/mL) | | | |
|---|---|---|---|---|
| | LLOQ | LQC | MQC | HQC |
| | 5.00 | 15.0 | 75.0 | 400 |
| Overall Mean | 5.15 | 14.8 | 71.6 | 402 |
| Inter-run CV% | 6.9 | 4.3 | 3.5 | 3.9 |
| Inter-run RE% | 3 | -1.3 | -4.5 | 0.5 |
| n | 18 | 18 | 18 | 18 |

- **Stability of NLX-G in Human Plasma**

| Benchtop Incubation of NLX-G (1 µg/mL) with Human Plasma | | |
|---|---|---|
| | 4◦C (24 hr) | RT (24 hr) |
| Increased NLX Comparing to Time 0 (%) | 0.3% | 23.0% |

- **Other Stability Information of NLX**

- ❖ 2 hours in whole blood at 4 °C
- ❖ 5 freeze/thaw cycles at -20 & -70 °C
- ❖ 97 days frozen at -20 & -70 °C
- ❖ 143 hours for processed sample stability at 4 °C
- ❖ No impact from 2% hemolyzed human plasma
- ❖ No impact from Buprenorphine/Norbuprenorphine/Naltrexone/ 6-β-Naltrexol at 7/5/25/50 ng/mL

## CONCLUSIONS

- The LLOQ level of 5 pg/mL was achieved by the optimization of SPE extraction, LC separation, and MS conditions.
- The conversion from NLX-G to NLX was evaluated and prevented by temperature control.
- The validated method shows adequate selectivity, sensitivity, accuracy, and reproducibility. It has been successfully applied in clinical sample analysis of NLX with ISR passing rate above 95%.