



Femtogram Quantitation of Naltrexone and 6-β-Naltrexol in Human Plasma by LC/MS/MS

Authors

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Introduction

Diary of a Scientist:

Monday morning, boss wants to talk.

*She tells me she needs an assay for **Naltrexone** and **metabolite 6-β-Naltrexol**.*

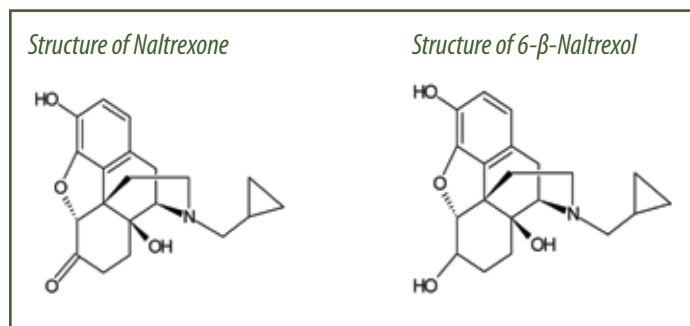
*Apparently **Naltrexone** is an **opioid receptor antagonist** used primarily in the management of **opioid dependence**. Recently Low Dose Naltrexone (LDN) is being used in doses approximately one-tenth those used for drug/alcohol rehabilitation purposes for the treatment of certain immunologically-related disorders, including **HIV/AIDS**.*

*Anyway my mission was to develop a **GLP** assay to **quantitate Naltrexone** in human plasma at low **picogram** levels and its **active metabolite 6-β-Naltrexol** at femtogram levels.*

Oh Yeah, she wants all this to be done by Friday.

Introduction continued

Back at my office. Did she say femtogram? What's this thing look like?



Reaching that level of sensitivity will require:

My most

- ***Sensitive LC/MS, Sciex API 5000***
- ***High Recovery***
- ***Minimal Suppression***
- ***High Selectivity***

Method

- Human plasma EDTA, 1 mL sample volume.
- Extraction by LLE, liquid/liquid extraction (MTBE @ pH=7). Freeze and pour.
- Chromatography on Phenomenex, Luna, C18, 2.0 x 50 mm, 5μ.
- Mobile phase: A:10mM NH₄OAc (aq), B: 10mM NH₄OAc in MeOH , 10% → 40% B at 2.5 min.
- THF wash out to prevent accumulation of high retention species.
- Detection, Sciex API 5000 under positive ion TIS conditions.

Results

Tuesday, crunching the numbers

Recovery looks good

TABLE 1: <i>Recovery > than 80%</i>		
	Naltrexone	6-β Naltrexone
Low QC	91%	82%
Mid QC	100%	92%
High QC	96%	89%

TABLE 2: Matrix Factor (Neat vs. extracted) <i>No significant suppression</i>	
Analyte	Matrix Factor
6-β-Naltrexol	97.9
Naltrexone	98.0

Matrix factor = area of spiked blank / area of neat solution

And blank is clean

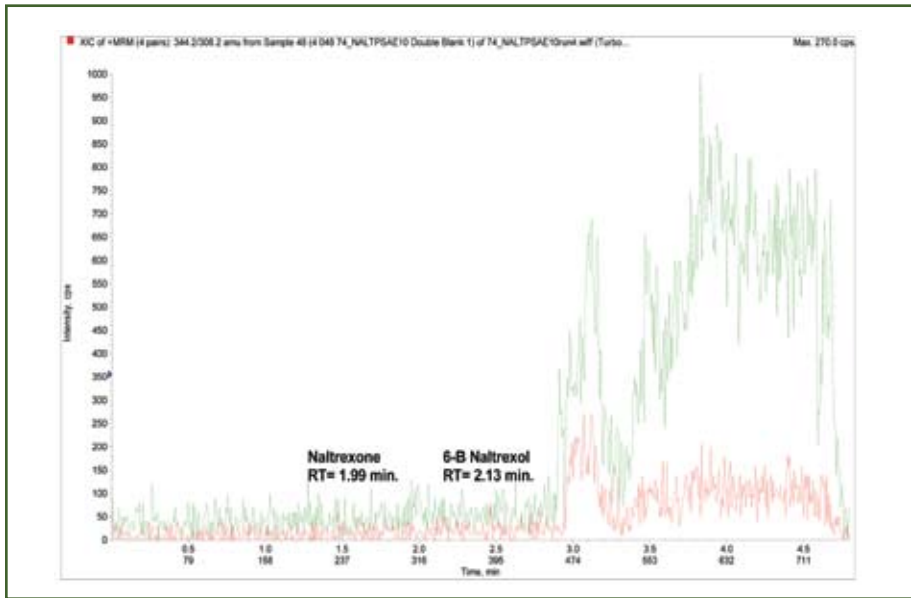
High degree of selectivity



Results continued

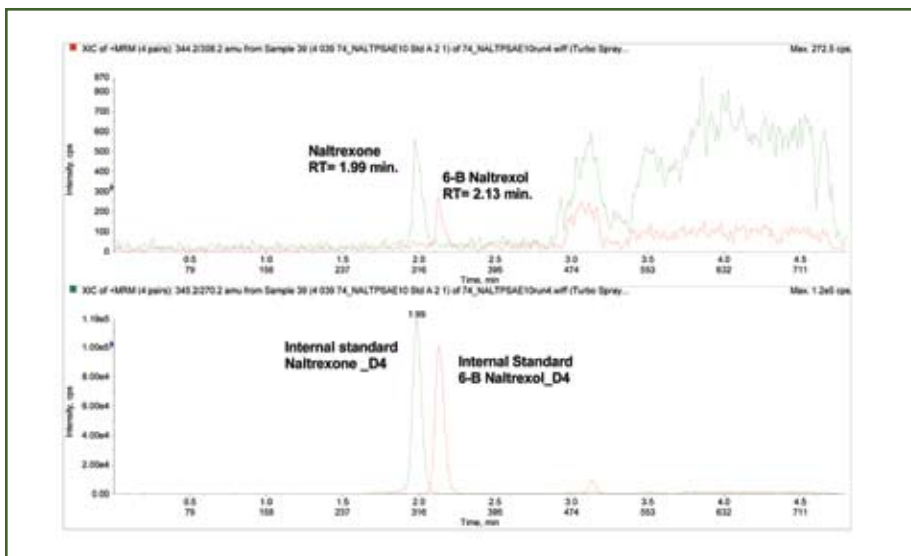
Extracted Human Plasma Blank (Blank following Highest Calibrator)

No significant carry over



Sensitivity is achieved

Extracted Human Plasma LLOQ @ 250 femtogram/mL 6-β-Naltrexol and 4.00 picogram/mL Naltrexone



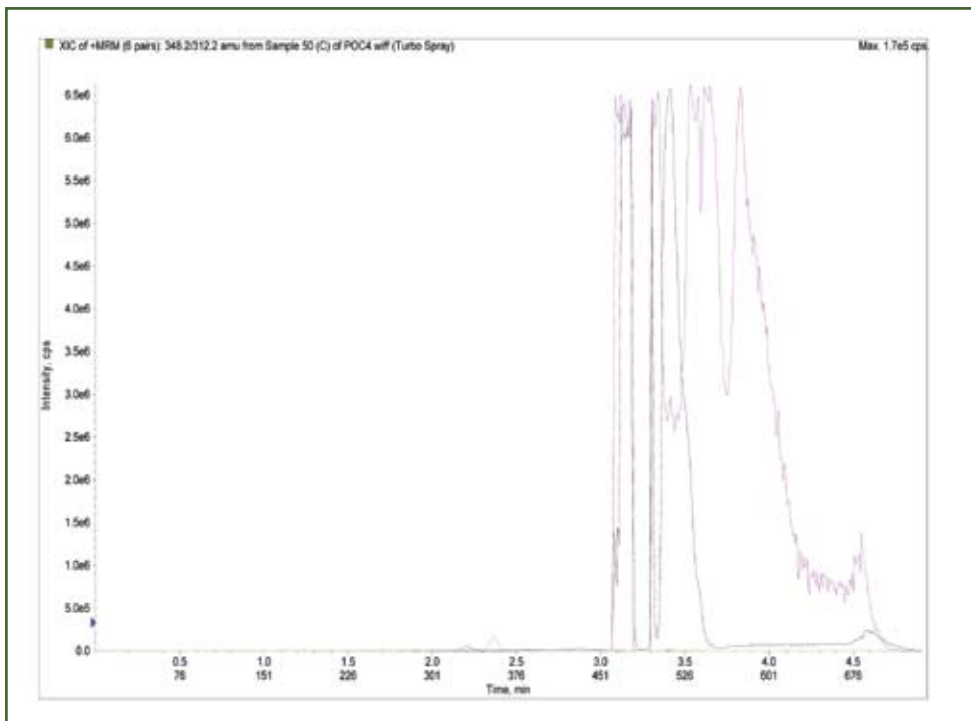
Results continued

Need to wash out high accumulation of lipids and highly retentive compounds

Chromatogram of Extracted Human plasma-Wash out program using THF to remove lipids.

Sample volumes at one mL results in high lipid levels

-Black and purple trace are phospholipid transitions



Wednesday, three days left, avoiding the boss, finds me anyway and asks how's it going, Swimmingly I say (don't know what that means exactly).

My analyst complains about contamination, going from mg stocks to spiking solution at the pg level requires careful preparation.

*Suggest using filtered pipette tips and emphasize preparing the working solutions in a **HOT ZONE** that is removed from the sample preparation area.*

Results continued

Filtered Pipette tips-minimize contamination

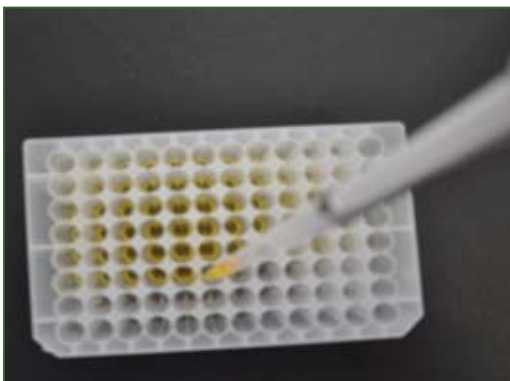


Photo courtesy of Wentzel Productions

Analyst also complains that the large sample volume requires a “manual” tube extraction as the volume is too large for our present automation systems to handle, and aliquoting reconstituted samples into a 96 well block from tubes is difficult as you can’t always tell where you have already aliquoted.

*Suggest we **add dye** to recon (**Bromothymol Blue**). Analyst is happy (see picture). She thinks I’m brilliant now...but that won’t last.*

Indicator Dye in Recon Simplifies Aliquoting



Analyst Marta X





Results continued

Thursday, start doing analytical runs, all goes well, analyst feels overworked but not bored.

Accuracy and Precision- 3 Analytical Runs- Naltrexone

TABLE 3: Accuracy and Precision- 3 Analytical Runs- Naltrexone						
		LLOQ QC	QC 1	QC 2	QC 3	QC 4
		pg/mL				
	Theor. Conc.	4.00	12.0	160	1600	3200
RUN 1	Found Conc.					
	Mean	3.76	12.2	172	1610	3312
	%CV	10.7	4.9	2.6	2.4	1.8
	%Theoretical	94	101	108	101	103
	n	6				
RUN 2	Found Conc.					
	Mean	3.67	12.2	170	1612	3335
	%CV	12.0	9.5	2.2	4.2	3.8
	%Theoretical	92	102	106	101	104
	n	6				
RUN 3	Found Conc.					
	Mean	3.67	12.1	173	1583	3138
	%CV	5.2	3.0	4.0	1.9	3.8
	%Theoretical	92	101	108	99	98
	n	6				
INTER-RUN	Found Conc.					
	Mean	3.70	12.1	172	1602	3262
	%CV	9.1	6.0	2.8	3.0	4.1
	%Theoretical	93	101	107	100	102

Results continued

TABLE 4: Accuracy and Precision-3 Analytical Runs- 6-β-Naltrexol						
		LLOQ QC	QC 1	QC 2	QC 3	QC 4
		pg/mL				
	Theor. Conc.	0.250	0.750	10.0	100	200
RUN 1	Found Conc.					
	Mean	0.249	0.792	10.8	100	209
	%CV	7	5	1	1	2
	%Theoretical	100	106	108	100	105
	n	6				
RUN 2	Found Conc.					
	Mean	0.217	0.738	10.6	97.3	204
	%CV	8	8	3	3	3
	%Theoretical	87	98	106	97	102
	n	6				
RUN 3	Found Conc.					
	Mean	0.253	0.83	10.8	98.3	205
	%CV	8	5	4	3	4
	%Theoretical	101	111	108	98	103
	n	6				
INTER-RUN	Found Conc.					
	Mean	0.239	0.787	10.7	98.6	206
	%CV	10	7	3	3	3
	%Theoretical	96	105	107	99	103

Results continued

TABLE 5:
Room Temperature Stability and Freeze Thaw Stability—6-β-Naltrexol

	QC 1 Room Temp 24 Hrs	QC 4 Room Temp 24 Hrs	QC 1 4 Freeze-Thaw cycles	QC 4 4 Freeze-Thaw cycles
	pg/mL			
Theor. Conc.	0.750	200	0.750	200
Found Conc.				
Mean	0.722	200	0.785	210
%CV	11.1	2	5.9	2.4
%Theoretical	96.3	100	104.7	105
n	6	6	6	6

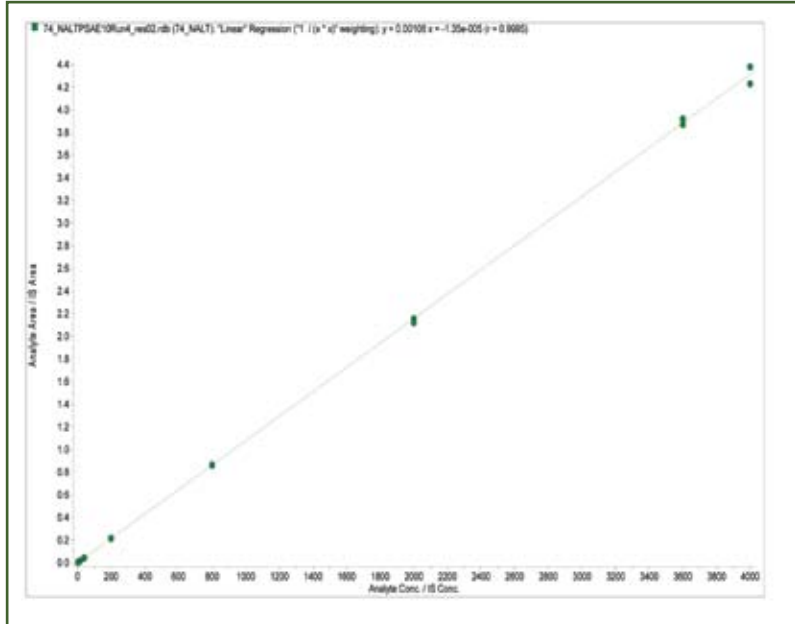
TABLE 6:
Room Temperature Stability and Freeze Thaw Stability—Naltrexone

	QC 1 Room Temp 24 Hrs	QC 4 Room Temp 24 Hrs	QC 1 4 Freeze-Thaw cycles	QC 4 4 Freeze-Thaw cycles
	pg/mL			
Theor. Conc.	12.0	3200	12.0	3200
Found Conc.				
Mean	12.1	3220	11.9	3280
%CV	1.7	1.6	11.9	2.9
%Theoretical	100.8	100.6	99.2	102.5
n	6	6	6	6

Results continued

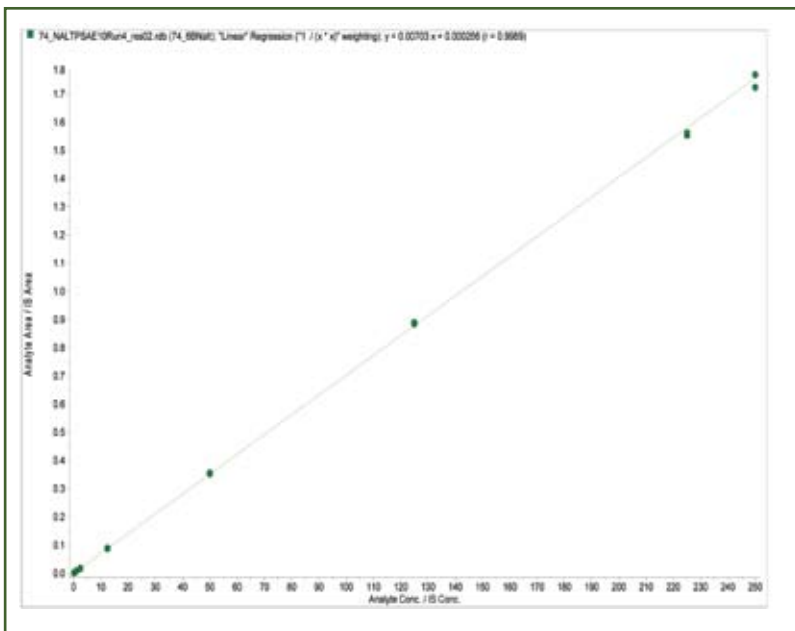
Calibration Curve Naltrexone

Linear over three orders of magnitude, $r=0.9995$



Calibration Curve 6-β-Naltrexol

Linear over three orders of magnitude, $r=0.9989$



Discussion

Several problems:

- **Sensitivity**

Needed femtogram sensitivity. Achieved by;

- USE OF API 5000
- HIGH RECOVERY
- CLEAN EXTRACTS
- OPTIMIZATION OF CHROMATOGRAPHY/SELECTIVE CHROMATOGRAPHY
- NO SIGNIFICANT SUPPRESSION

- **Sample Volume Size**

- Relatively large sample volume required a manual tube extraction over the normal semi-automated (TOMTEC) extraction methods.
- SPE cation exchange (MCX) was evaluated which could be automated but was not specific enough, peaks in blank as well as more costly.
- Freeze and pour and with indicator in the reconstitution solvent was chosen.

- **Contamination**

Contamination was a concern as there was a 1 million fold concentration difference from stocks to working solutions.

- Overcome by stock preparation in **hot zone** only, an area removed from the sample processing area.
- Use of filtered pipettes.
- Use of disposable supplies only.

Conclusion

Friday, waiting for boss to ask how it went, I tell her,

A GLP Assay was Validated for Naltrexone and Metabolite in Human Plasma at the pico/femtogram level.

And it's the best assay I'VE ever done. Mumbles something about how I say that every time.

I'm going home.