SUPPLEMENTARY INFORMATION FOR

14-*O*-Heterocyclic-Substituted Naltrexone Derivatives as Non-Peptide Mu Opioid Receptor Selective Antagonists: Design, Synthesis and Biological Studies

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Content:

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1.Figure 4. Sequence alignment of MOR, KOR, DOR, bovine rhodopsin and β2AR.

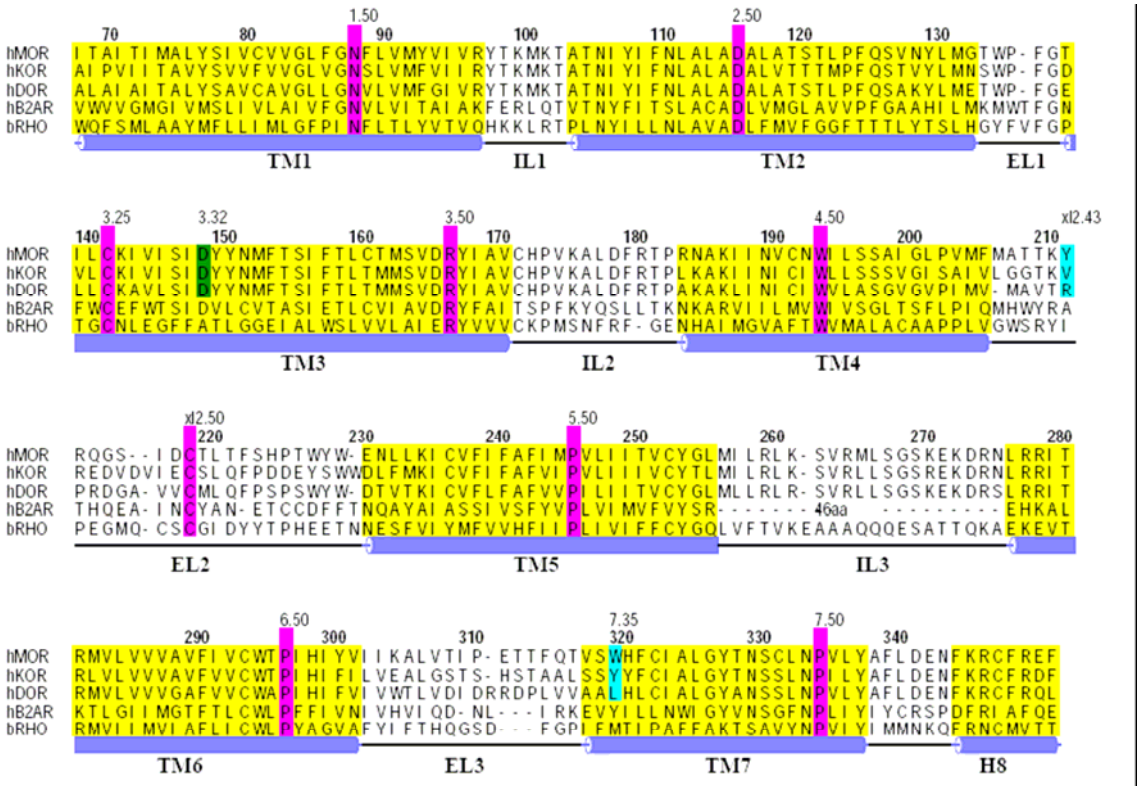
The conserved residues in most of the GPCRs are marked out using Ballesteros-Weinstein numbering system in the top line and colored in pink.

Second line: human Mu opioid receptor sequence number

Seven transmembrane helices are colored in yellow while marked out at the bottom line.

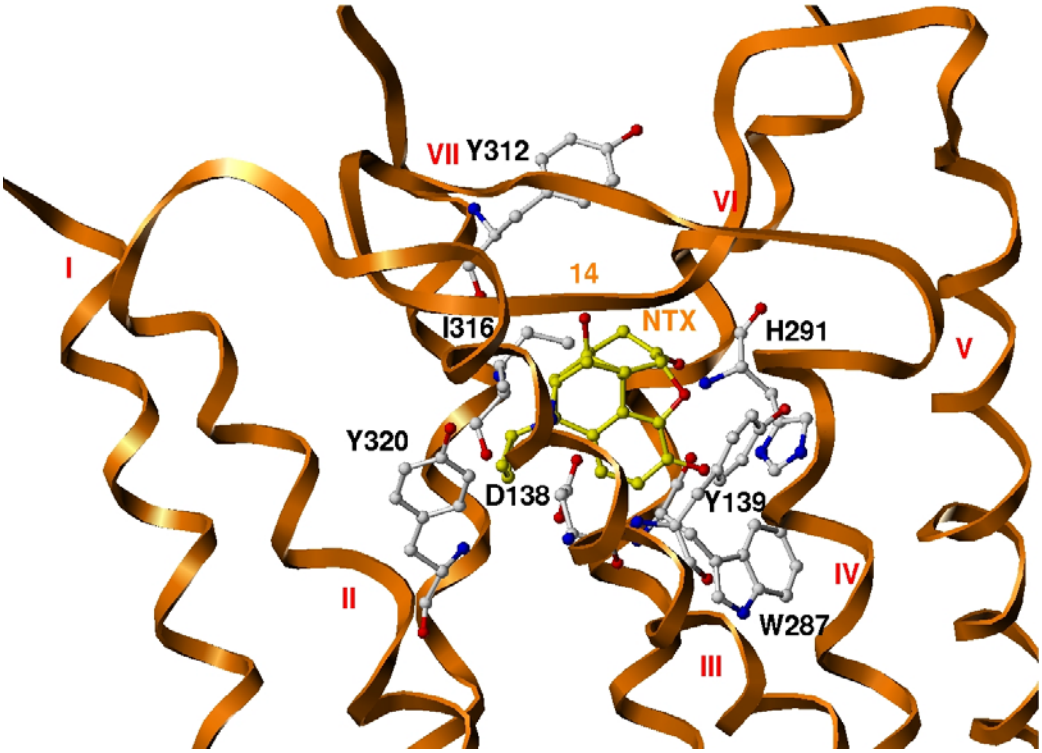
The conserved Asp3.32 residues are colored in green for all three opioid receptors.

Two non-conserved residues xl2.43 and 7.35 are colored in blue for all three opioid receptors.



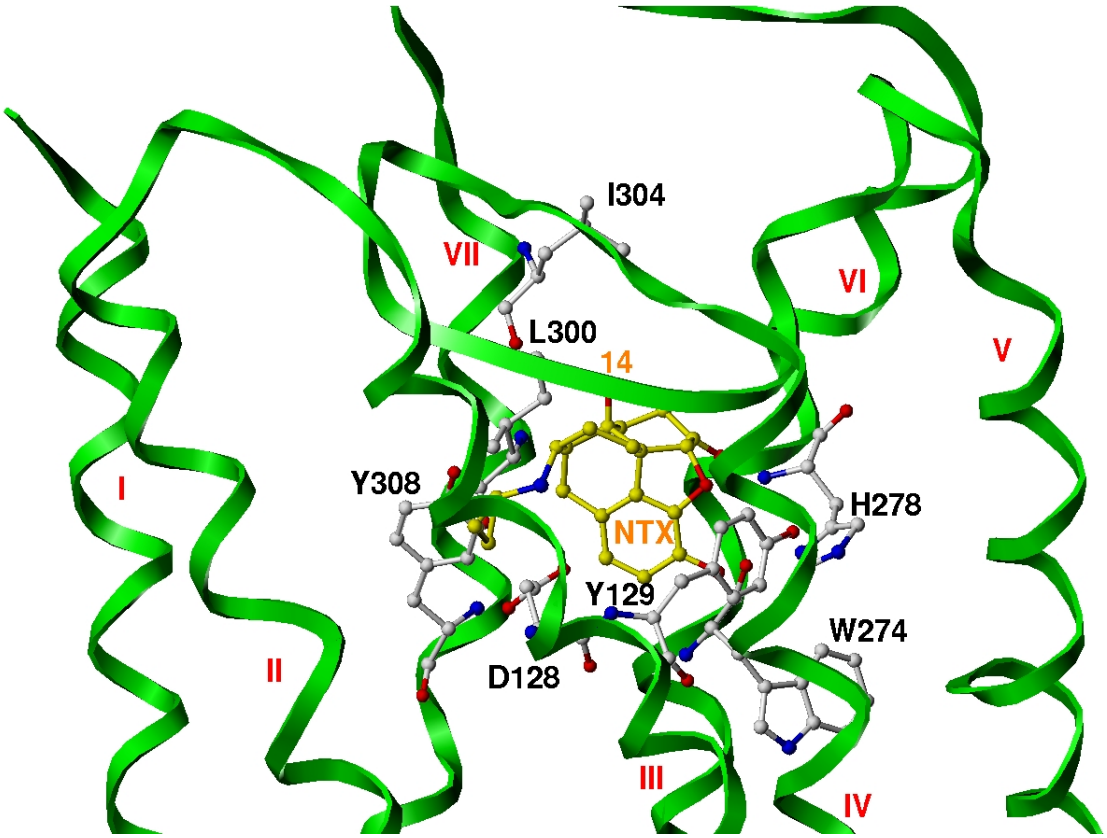
2. Figure 5. Naltrexone in KOR Binding pocket:

Kappa opioid model: ribbon and in orange color; The residues in kappa opioid receptor: ball and stick and in atom color; Naltrexone molecular: ball and stick and in yellow color.



3. Figure 6. Naltrexone in DOR Binding pocket:

Delta opioid model: ribbon and in green color; The residues in delta opioid receptor: ball and stick and in atom color; Naltrexone molecular: ball and stick and in yellow color.



3. Experimental Section

3.1 Molecular Modeling:

Homology modeling:

The human mu, kappa, and delta opioid receptor sequences were retrieved from www.expasy.org (Swiss-Prot and TrEMBL). The sequence alignment of mu, kappa, and delta with bovine rhodopsin was assembled using the ClustalX1.83[1] with blosum 30 applied and the gap opening penalty of 15. Based on the dark state bovine rhodopsin crystal structure (PDB code:1U19),[2] the models of the mu, delta and kappa opioid receptor were developed by homology modeling using the SYBYL program (7.3 version, Tripos, Inc.). Because there was no appropriate structural template available, the amino acid residues before helix 1 in the N-terminal and the amino acid residues after the helix 8 in the C-terminal were removed from the models. Transmembrane helices 1-7 and helix 8 in the C-terminal were directly generated from mutation according to the rhodopsin template. The intercellular (I1, I2, I3) and extracellular loops (EL1, EL2, EL3) were created using loop search function in the SYBYL program.

The resulting structures were renumbered and the side chains were added using the program SCWRL3.0.[3] Then the energy of obtained structures were minimized using SYBYL7.3 with default parameters except the following: using Gasteigen-Hückel charges, Dielectric Constant in 4.0; and maximum Iterations in 100,000.

The analysis of φ, ψ, χ_1, χ_2 angles of the resulting protein conformations was conducted with Procheck 4.1. All amino acid residues have reasonable bond lengths and bond

angles. All the residues are in the most favored regions, the additional allowed regions, or the generously allowed regions and no one in the disallowed regions.

Small molecule naltrexone:

Naltrexone (NTX) was built using SYBYL. Minimizations with steepest descent followed by conjugate gradient were performed to generate the lowest energy conformation for the ligand (Tripos force field and default termination values were adopted). Then a molecular dynamics simulation was performed (an equilibration phase of 1,000 fs at 300 K, followed by a collection phase of 5,000 fs at the same temperature) to further study the small molecule conformation. We used the lowest energy conformation of the small molecule from the 5 ps molecular dynamics calculations, as the initial configuration for docking into the proposed binding site of the opioid receptors. The ligand was modeled in its nitrogen-protonated form.

Docking Studies:

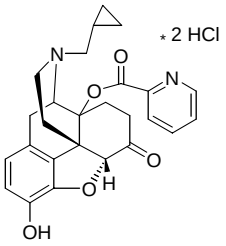
The program GOLD3.1[4] was used to perform the docking study with default parameters. Based on the fitness scores and binding orientation of NTX, the best solution of GOLD binding results was selected and merged into the structure of the corresponding receptor. The combined structure ligand and receptor was minimized together in order to allow the receptor to adopt the presence of NTX. Subsequently, the structure of NTX was removed from the receptor complex. The resulting structure was checked by Procheck again.

3.2 Chemistry:

All reagents were purchased from Sigma-Aldrich or as otherwise states. Melting points were determined by Fischer Scientific melting point apparatus and were uncorrected. IR spectra were obtained using a Nicolet Avatar 360 FT-IR Instruments. Proton (300 MHz)

and carbon-13 (75M Hz) nuclear magnetic resonance (NMR) spectra were obtained on a Varian Gemini spectrometer and tetramethylsilane was used as the internal standard. LC-MS was performed on a Waters Micromass QTOF-2 instrument using ESI source.

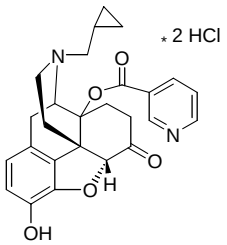
General procedure: The mixture of naltrexone (1 equivalent), acyl chloride (3 equivalent), and triethylamine (6 equivalent) was heated to 100 °C for 6 hours under N_2 protection in dry DMF. After cooling, the reaction mixture was concentrated under vacuum to remove DMF. The resulting crude intermediate was dissolved in MeOH and 4% H_2SO_4 aqueous (potassium carbonate aqueous, pH≈10, for compound **4**, **8**) and stirred overnight at room temperature. After concentrating, the residue was partitioned between water and CH_2Cl_2. The water layer was extracted with CH_2Cl_2 three times. The combined CH_2Cl_2 solution was washed with brine, and dried over Na_2SO_4. After concentrating to remove solvent under vacuum, the resulting residue was purified using a silica gel column with a CH_2Cl_2/ MeOH (150:1→100:1) (1 % NH_3H_2O) solvent system as eluent to give the target product. The final target compound's purity was tested by Varian ProStar HPLC System using Microsorb-MV 100-5 C18 column (250 x 4.6mm) with injection volume at 10 μL and sample concentrations at 1 – 2 mg/0.5mL in 100% acetonitrile; The sample was detected at single wavelength of 210 nm with eluent system of acetonitrile: water (70:30) at 1 mL/min over 50 min. The free base in foam or oil was transformed into hydrochloride salt using 1.25 M HCl in MeOH solution at 0 °C.



MW= 519.42

$C_{26}H_{26}N_2O_5$ * 2 HCl

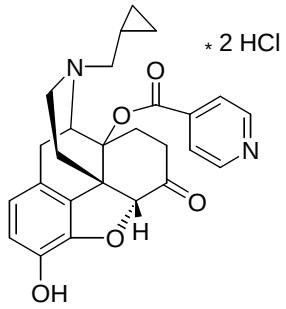
17-cyclopropylmethyl-4,5α-epoxy-3-hydroxy-14β-*O*-(pyridinyl-2'-carboxy)morphinan-6-one (compound 1): m.p. 250°C (decomposed); **IR** ν_{max}(KBr, cm^{-1}) : 3411, 1660, 1259, 794; **^{1}H-NMR (300 MHz, CD_3OD) (hydrochloride salt)** δ 8.96 (m, 1 H, Ar-H), 8.70 (m, 1 H, Ar-H), 8.18 (m, 1 H, Ar-H), 8.26 (m, 1 H, Ar-H), 6.75 and 6.73(2 d, 1 H each, *J= 8.1Hz,* C_1-H, C_2-H), 4.11(m, 1 H, C_5-H); **^{13}C-NMR (75 MHz, CD_3OD)** δ 207.59, 161.31, 146.38, 142.36, 139.87, 138.89, 129.90, 127.39, 125.40, 119.98, 117.95, 117.50, 88.54, 61.69, 56.92, 52.99, 37.51, 34.17, 30.23, 29.30, 27.89, 26.90, 22.49, 4.97, 4.34, 1.56; **MS (ESI) *m/z*:** 447$(M+H)^+$, 342.



MW= 519.42

$C_{26}H_{26}N_2O_5$ * 2 HCl

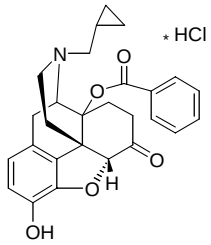
17-cyclopropylmethyl-4,5α-epoxy-3-hydroxy-14β-*O*-(pyridinyl-3'-carboxy)morphinan-6-one (compound 2): m.p. 202°C (decomposed); **IR** ν_{max}(KBr, cm^{-1}) : 2946, 1716, 1282, 1108, 737; **^{1}H-NMR (300 MHz, CD_3OD) (hydrochloride salt)** δ 9.63 (m, 1 H, Ar-H), 9.28 (m, 1 H, Ar-H), 9.14 (m, 1 H, Ar-H), 8.28 (m, 1 H, Ar-H), 6.75 and 6.65(2 d, 1 H each, *J= 7.8Hz,* C_1-H, C_2-H), 4.82(m, 1 H, C_5-H); **^{13}C-NMR (75 MHz, $CDCl_3$)** δ 207.43, 163.37, 152.17, 149.96, 143.14, 139.31, 137.56, 137.42, 127.42, 123.75, 123.37, 119.76, 118.21, 93.97, 89.35, 58.86, 55.33, 50.85, 43.47, 35.29, 30.30, 26.56, 22.87, 9.00, 3.46, 3.30; **MS (ESI) *m/z:*** 447$(M+H)^+$, 342.



MW= 519.42

$C_{26}H_{26}N_2O_5$ * 2 HCl

17-cyclopropylmethyl-4,5α-epoxy-3-hydroxy-14β-*O*-(pyrodinyl-4'-carboxy)morphinan-6-one (compound 3): m.p. 190-195°C; **IR** ν_{max}(KBr, cm^{-1}): 3385, 1755, 1724, 1270, 1241, 749; **^{1}H-NMR (300 MHz, CD_3OD) (hydrochloride salt)** δ 9.18 (m, 2 H, Ar-H), 8.74 (m, 2H, Ar-H), 7.27 and 7.05(2 d, 1 H each, *J= 8.4Hz,* C_1-H, C_2-H), 4.20(m, 1 H, C_5-H); **^{13}C-NMR (75 MHz, CD_3OD)** δ 207.27, 160.13, 147.69, 145.32, 143.50, 132.56, 129.71, 129.13, 127.53, 127.02, 123.80, 121.02, 120.88, 90.43, 70.16, 69.69, 62.11, 57.76, 49.17, 34.68, 30.97, 27.21, 23.81, 5.80, 5.23, 2.46; **MS (ESI) *m/z:*** 447$(M+H)^+$, 342, 224.

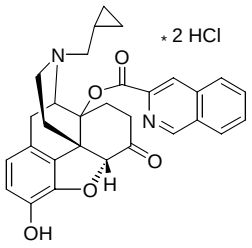


MW= 481.97

$C_{27}H_{27}NO_5$ * HCl

17-cyclopropylmethyl-4,5α-epoxy-3-hydroxy-14β-*O*-(benzoylcarboxy)morphinan-6-one (compound 4): m.p. 161-165°C; **IR** ν_{max}(KBr, cm^{-1}): 3398, 1730, 1239, 1055, 710; **^{1}H-NMR (300 MHz, CD_3OD) (hydrochloride salt)** δ 8.21 (m, 2 H, Ar-H), 7.73 (m, 1H, Ar-H), 7.61 (m, 2H, Ar-H) 7.14 and 7.00(2 d, 1 H each, *J= 7.8Hz,* C_1-H, C_2-H), 4.88(m, 1 H, C_5-H); **^{13}C-NMR (75 MHz, CD_3OD)** δ 205.92, 163.75, 147.54, 133.52, 132.66,

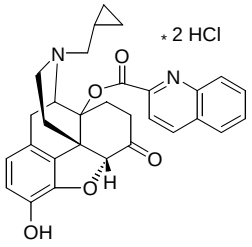
132.18, 129.39, 129.31, 123.44, 122.50, 119.74, 96.46, 93.10, 89.12, 69.35, 69.12, 62.11, 61.64, 58.04, 56.98, 33.76, 30.03, 26.89, 22.87, 4.91, 4.34, 1.56; **MS (ESI)** ***m/z:*** 446$(M+H)^+$, 342.



MW= 569.57

$C_{30}H_{28}N_2O_5$ * 2 HCl

17-cyclopropylmethyl-4,5α-epoxy-3-hydroxy-14β-*O*-(isoquinolinyl-3'-carboxy)morphinan-6-one (compound 5): m.p. 201-204°C; **IR** ν_{max}(KBr, cm^{-1}): 3392, 2921, 1725, 1182, 781; **^{1}H-NMR (300 MHz, CD_3OD) (hydrochloride salt)** δ 8.87 (m, 1 H, Ar-H), 8.50 (m, 1 H, Ar-H), 8.25 (m, 1 H, Ar-H), 8.01 (m, 3 H, Ar-H), 7.21 and 7.00(2 d, 1 H each, *J= 7.8Hz,* C_1-H, C_2-H), 4.04(m, 1 H, C_5-H); **^{13}C-NMR (75 MHz, $CDCl_3$)** δ 207.23, 161.70, 147.36, 138.94, 136.64, 132.23, 131.66, 130.75, 130.19, 129.85, 128.34, 128.16, 126.34, 125.01, 122.62, 119.05, 90.27, 69.63, 61.48, 58.79, 53.68, 50.25, 43.05, 33.68, 30.79, 30.38, 22.61, 8.97, 3.62, 3.45; **MS (ESI)** ***m/z****:* 497 $(M+H)^+$.

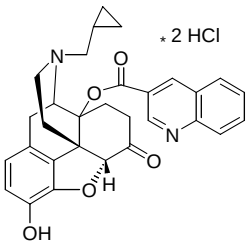


MW = 569.57

$C_{30}H_{28}N_2O_5$ * 2HCl

17-cyclopropylmethyl-4,5α-epoxy-3-hydroxy-14β-*O*-(quinolinyl-2'-carboxy)morphinan-6-one (compound 6): m.p. 85-88°C; **IR** ν_{max}(KBr, cm^{-1}): 3179,

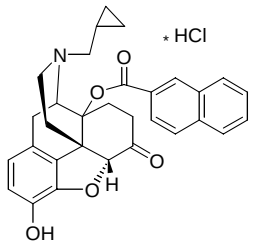
1731, 1660, 1453, 1240, 730; **^{1}H-NMR (300 MHz, CD_3OD) (hydrochloride salt)** δ 8.99 (m, 1 H, Ar-H), 8.50 (m, 1 H, Ar-H), 8.39 (m, 1 H, Ar-H), 8.25 (m, 1 H, Ar-H), 8.09 (m, 1 H, Ar-H), 7.93 (m, 1 H, Ar-H), 7.26 and 7.03(2 d, 1 H each, *J= 8.1Hz,* C_1-H, C_2-H), 3.40(m, 1 H, C_5-H); **^{13}C-NMR (75 MHz, $CDCl_3$)** δ 209.92, 163.00, 143.76, 143.11, 140.65, 138.64, 133.72, 131.41, 130.28, 128.51, 127.38, 123.52, 122.34, 121.09, 119.50, 117.66, 115.70, 90.06, 69.90, 61.56, 58.76, 50.60, 43.20, 35.79, 30.94, 30.18, 22.20, 8.96, 3.62, 3.39; **MS (ESI) *m/z:*** 497 $(M+H)^+$, 342.



MW= 569.57

$C_{30}H_{28}N_2O_5$ * 2HCl

17-cyclopropylmethyl-4,5α-epoxy-3-hydroxy-14β-*O*-(quinolinyl-3'-carboxy)morphinan-6-one (compound 7): m.p. 187°C (decomposed); **IR** ν_{max}(KBr, cm^{-1}): 3386, 1725, 1189, 762; **^{1}H-NMR (300 MHz, CD_3OD) (hydrochloride salt)** δ 9.64 (m, 1 H, Ar-H), 9.56 (m, 1 H, Ar-H), 8.32 (m, 3 H, Ar-H), 7.98(m, 1 H, Ar-H), 7.27 and 7.08(2 d, 1 H each, *J= 8.1Hz,* C_1-H, C_2-H), 3.49(m, 1 H, C_5-H); **^{13}C-NMR (75 MHz, CD_3OD)** δ 206.38, 160.69, 147.10, 146.82, 144.14, 143.32, 134.82, 132.07, 129.70, 129.02, 128.44, 128.19, 127.06, 123.67, 123.29, 122.14, 120.07, 89.52, 69.37, 64.98, 61.44, 56.97, 33.87, 30.16, 26.93, 22.96, 13.57, 4.97, 4.42, 1.63; MS (ESI) *m/z:* 497 $(M+H)^+$, 342.



MW= 532.03

$C_{31}H_{29}NO_5$ * HCl

17-cyclopropylmethyl-4,5α-epoxy-3-hydroxy-14β-*O*-(pyrodinyl-4'-carboxy)morphinan-6-one (compound 8): m.p. 137-140°C; **IR** ν_{max}(KBr, cm^{-1}): 3386, 1732, 1189, 1056, 776; **^{1}H-NMR (300 MHz, CD_3OD) (hydrochloride salt)** δ 8.97 (m, 1 H, Ar-H), 8.10 (m, 4 H, Ar-H),7.70 (m, 2 H, Ar-H), 7.21 and 7.02(2 d, 1 H each, *J= 8.1Hz,* C_1-H, C_2-H), 4.93(m, 1 H, C_5-H); **^{13}C-NMR (75 MHz, CD_3OD)** δ 206.04, 164.81, 135.49, 132.78, 132.02, 131.25, 128.70, 128.15, 127.72, 127.36, 127.04, 126.62, 124.37, 123.53, 122.32, 121.53, 119.80, 119.17, 96.52, 93.17, 89.18, 76.69, 69.37, 61.65, 56.96, 33.79, 26.90, 22.86, 4.92, 4.39, 1.50; MS (ESI) *m/z:* 496 $(M+H)^+$, 342.

3.3 HPLC methods and purity test results:

HPLC System: Varian ProStar;
Column: Microsorb-MV 100-5 C18 column (250 x 4.6mm);
Injection volume: 10 μL;
Sample concentrations: 1 – 2 mg/0.5mL in 100% acetonitrile;
Single wavelength: 210 nm;
Eluent: acetonitrile: water(70: 30) at 1 mL/min over 50 min.

Table. HPLC purity assessment of target compounds

Compounds	Compounds code in spectra	Time (min)	Purity (%)
1	VZMN031	25.31	99.30
2	VZMN020	26.61	98.49
3	VZMN022	24.79	98.36
4	VZMN024	41.76	98.25
5	VZMN021	3.55	98.83
6	VZMN016	9.84	98.86
7	VZMN017	4.12	99.76
8	VZMN023	28.80	96.03

vzmn031

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Base rate 20.0000 Hz Bunch 1 Effective rate 20.00000 Hz. T = 0.000833 min.
43254 points from 0.000 to 36.045 minutes
Sampling every 4 point(s)
Printing points from 0.000 to 36.045 min, point 0 to 43253
Channels 1 to 1
Results for Channel 1 = 210 nm
Title: Report Title
Run File: c:\star\data\guo li\naltrexamine derivatives\vzmn031,7-30-2007,11;09;17 am.run

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Injection Info

Method: C:\star\data\Guo li\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Sample: 1.4-17CH3CN75_3ul
Injection Date: Mon Jul 30 11:09:17 2007
Manual injection
Operator: gl
Instrument: Varian Star #1
Notes:

Misc Info

Workstation: STARWORKSTATIOÿ HÚ é
Bus Address: 44
Run Time: 0.000 to 36.045 min.

Recalc Info

Recalc Method: C:\star\data\Guo li\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Recalc Date: Mon Jul 30 11:45:20 2007
Operator: gl
Instrument: Varian Star #1
Notes:
Sample Rate: 20.000 Hz.
Measurement Type: 1 = Area
Calculation Type: 1 = %
Normalize Results: No

Peak Info for Channel 1

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8 Peaks (tR	Timeoffset		RRT	Sepcode	Width	Counts	Result	Name)
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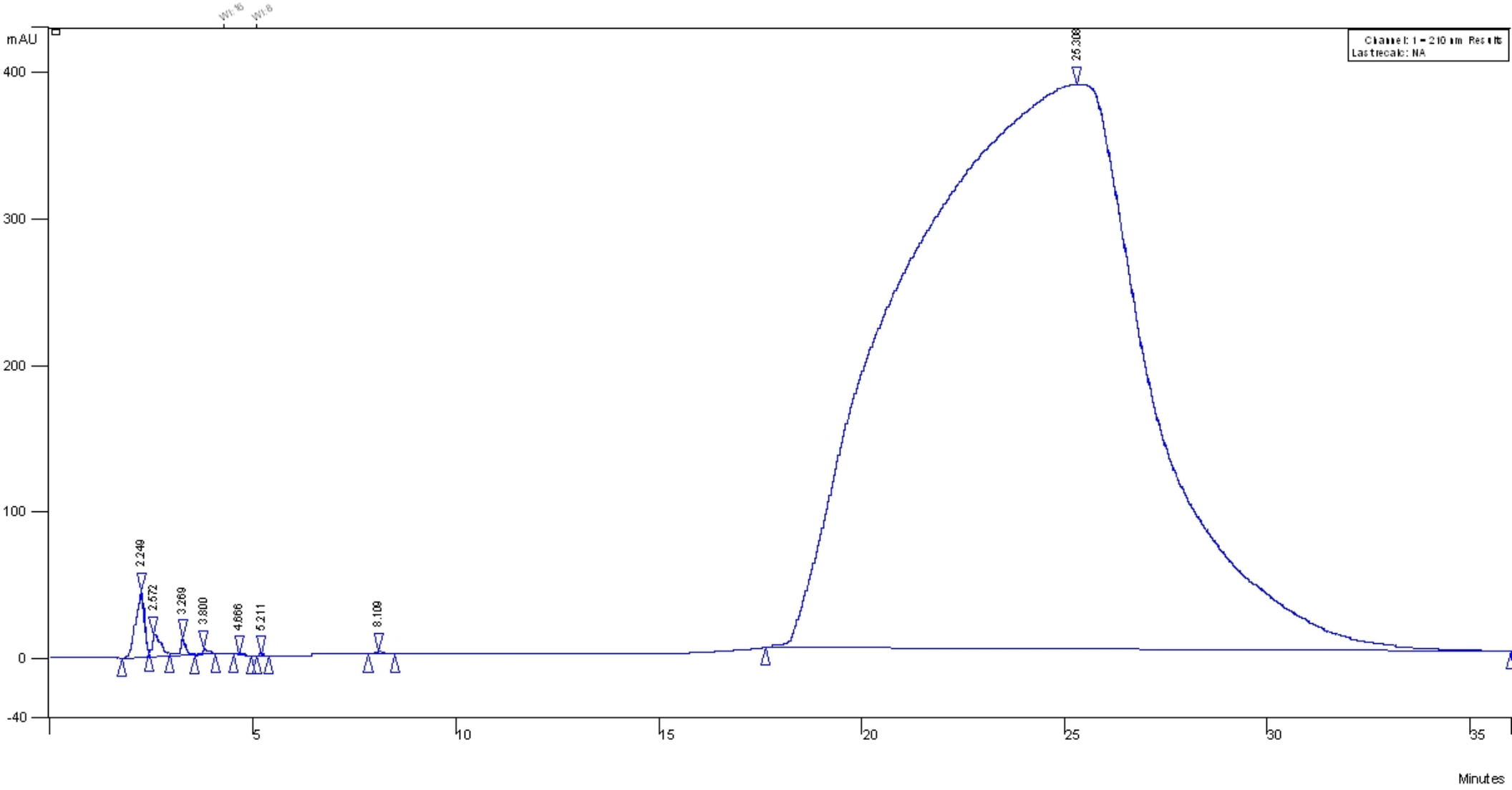
2.572	0.000	0.0000	VV	17.06	2493388	0.153300		
3.269	0.000	0.0000	VV	7.91	1193394	0.073373		
3.800	0.000	0.0000	VB	17.18	474120	0.029150		
4.666	0.000	0.0000	BB	6.76	56573	0.003478		
5.211	0.000	0.0000	BB	6.03	37059	0.002278		
8.109	0.000	0.0000	BB	8.40	151787	0.009332		
25.308	0.000	0.0000	BB	198.57	1615148800		99.303383	


mAU
400
300
200
100
0
-40
2.249
2.572
3.269
3.800
4.666
5.211
8.109
25.306
5
10
15
20
25
30
35
Minutes

File c:\star\data\guo li\naltrexamine derivatives\vzmn020.run Filetype 325
WTOASCII Parameters: C:\star\WTOASCII.TXT
1 channels
Channel 1 210 nm in AU
Base rate 20.0000 Hz Bunch 1 Effective rate 20.00000 Hz. T = 0.000833 min.
50469 points from 0.000 to 42.057 minutes
Sampling every 4 point(s)
Printing points from 0.000 to 42.057 min, point 0 to 50468
Channels 1 to 1
Results for Channel 1 = 210 nm
Title: Report Title
Run File: c:\star\data\guo li\naltrexamine derivatives\vzmn020.run

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Injection Info

Method: C:\star\data\Guo li\Naltrexamine derivatives\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Sample: 1,6-78+78CH3CN75
Injection Date: Tue Jul 31 13:05:05 2007
Manual injection
Operator: gl
Instrument: Varian Star #1
Notes:

Misc Info

Workstation: STARWORKSTATIOÿ HÚ é
Bus Address: 44
Run Time: 0.000 to 42.057 min.

Recalc Info

Recalc Method: C:\star\data\Guo li\Naltrexamine derivatives\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Recalc Date: Tue Jul 31 13:47:10 2007
Operator: gl
Instrument: Varian Star #1
Notes:
Sample Rate: 20.000 Hz.
Measurement Type: 1 = Area
Calculation Type: 1 = %
Normalize Results: No

Peak Info for Channel 1

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10 Peaks (tR Timeoffset RRT Sepcode Width Counts Result Name)

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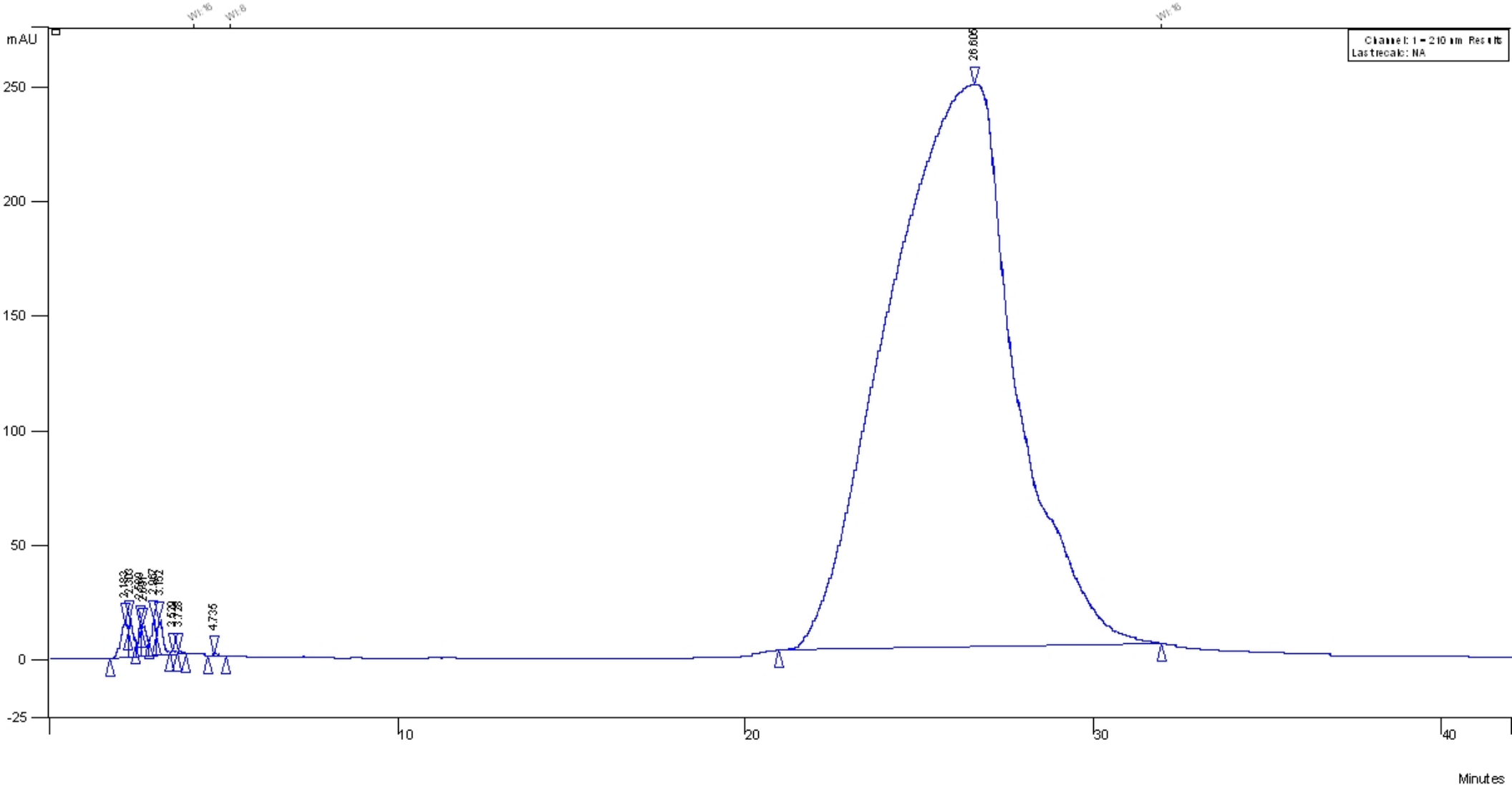
2.303	0.000	0.0000	VV	13.62	1642716	0.259194	
2.589	0.000	0.0000	VV	8.12	1029495	0.162437	
2.691	0.000	0.0000	VV	0.00	1271082	0.200556	
2.967	0.000	0.0000	VV	9.21	1465132	0.231174	
3.152	0.000	0.0000	VV	11.40	1618189	0.255324	
3.529	0.000	0.0000	VV	0.00	184044	0.029039	
3.728	0.000	0.0000	VB	0.00	142952	0.022555	
4.735	0.000	0.0000	BB	7.26	58309	0.009200	
26.605	0.000	0.0000	BB	184.95	624212992	98.490662	


mAU
250
200
150
100
50
0
-25
26.605
4.735
10
20
30
40
Minutes

vzmn022

File c:\star\data\guo li\naltrexamine derivatives\vzmn022.run Filetype 325
WTOASCII Parameters: C:\star\WTOASCII.TXT
1 channels
Channel 1 210 nm in AU
Base rate 20.0000 Hz Bunch 1 Effective rate 20.00000 Hz. T = 0.000833 min.
46343 points from 0.000 to 38.619 minutes
Sampling every 4 point(s)
Printing points from 0.000 to 38.619 min, point 0 to 46342
Channels 1 to 1
Results for Channel 1 = 210 nm
Title: Report Title
Run File: c:\star\data\guo li\naltrexamine derivatives\vzmn022.run

---

Injection Info

Method: C:\star\data\Guo li\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Sample: 1.6-77CH3CN75_3ul
Injection Date: Mon Jul 30 15:12:58 2007
Manual injection
Operator: gl
Instrument: Varian Star #1
Notes:

Misc Info

Workstation: STARWORKSTATIOÿ HÚ é
Bus Address: 44
Run Time: 0.000 to 38.619 min.

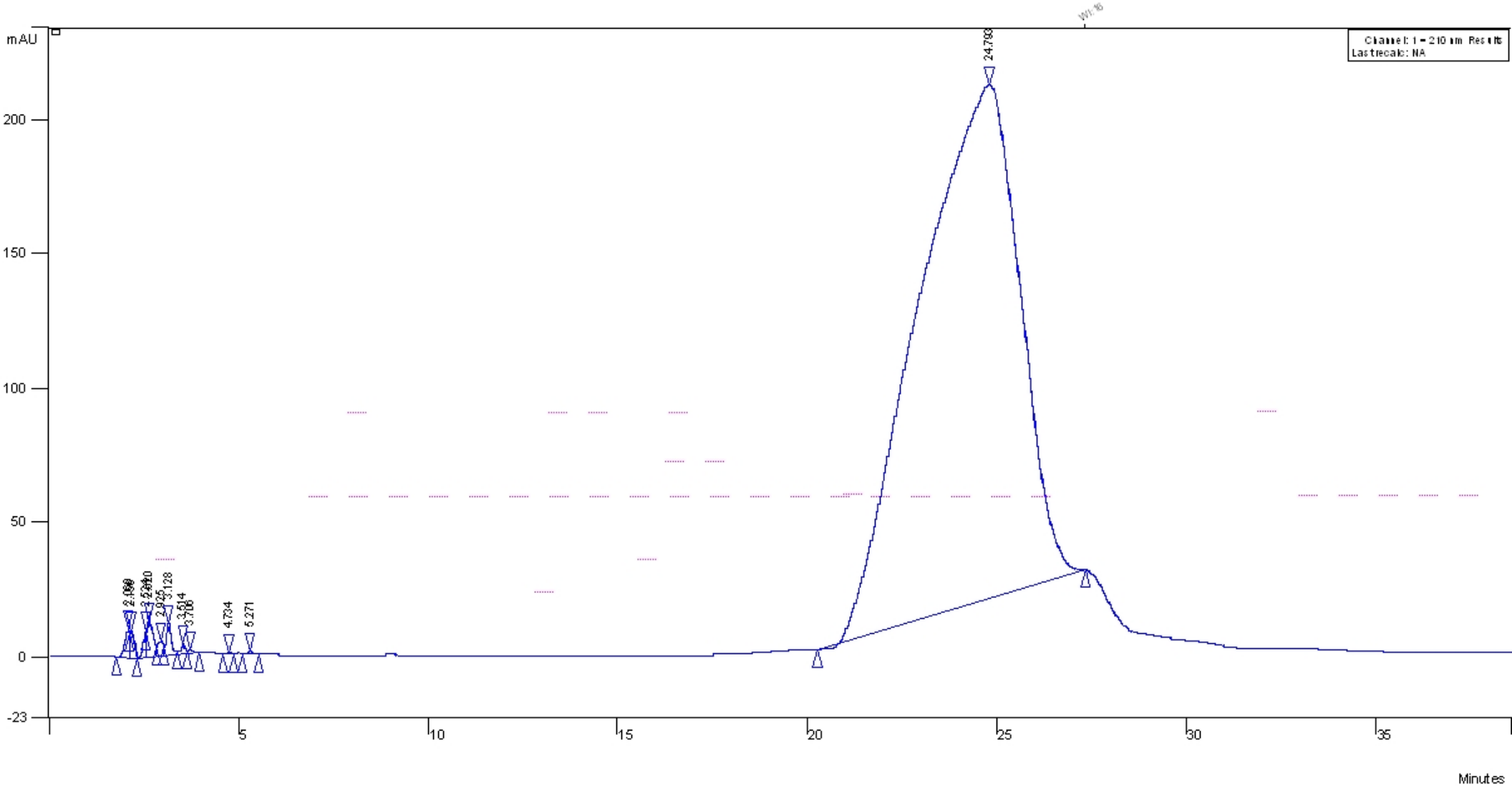
Recalc Info

Recalc Method: C:\star\data\Guo li\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Recalc Date: Mon Jul 30 15:51:36 2007
Operator: gl
Instrument: Varian Star #1
Notes:
Sample Rate: 20.000 Hz.
Measurement Type: 1 = Area
Calculation Type: 1 = %
Normalize Results: No

Peak Info for Channel 1

---

11 Peaks (tR	Timeoffset		RRT	Sepcode	Width	Counts	Result	Name)
2.060	0.000	0.0000	BV	10.22	926342	0.255423		
2.156	0.000	0.0000	VP	10.83	722926	0.199334		
2.524	0.000	0.0000	PV	7.42	646502	0.178262		
2.620	0.000	0.0000	VV	14.65	1402210	0.386635		
2.925	0.000	0.0000	VV	0.00	645531	0.177994		
3.128	0.000	0.0000	VV	7.09	1027411	0.283291		
3.514	0.000	0.0000	VV	8.47	345431	0.095247		
3.706	0.000	0.0000	VB	0.00	129574	0.035728		
4.734	0.000	0.0000	BB	6.04	29687	0.008186		
5.271	0.000	0.0000	BB	5.68	61518	0.016963		
24.793	0.000	0.0000	BB	164.24	356733120		98.362946	


mAU
200
150
100
50
0
-23
24.793
4.734
5.271
Channel: 1 = 210 nm Results
Last recalc: NA
5
10
15
20
25
30
35
Minutes

File c:\star\data\guo li\naltrexamine derivatives\vzmn024.run Filetype 325
WTOASCII Parameters: C:\star\WTOASCII.TXT
1 channels
Channel 1 210 nm in AU
Base rate 20.0000 Hz Bunch 1 Effective rate 20.00000 Hz. T = 0.000833 min.
134638 points from 0.000 to 112.198 minutes
Sampling every 4 point(s)
Printing points from 0.000 to 112.198 min, point 0 to 134637
Channels 1 to 1
Results for Channel 1 = 210 nm
Title: Report Title
Run File: c:\star\data\guo li\naltrexamine derivatives\vzmn024.run

---

Injection Info

Method: C:\star\data\Guo li\Naltrexamine derivatives\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Sample: 4-21CH3cn75_20ULNEW
Injection Date: Wed Aug 15 12:58:45 2007
Manual injection
Operator: gl
Instrument: Varian Star #1
Notes:

Misc Info

Workstation: STARWORKSTATIOÿ HÚ éu
Bus Address: 44
Run Time: 0.000 to 112.198 min.

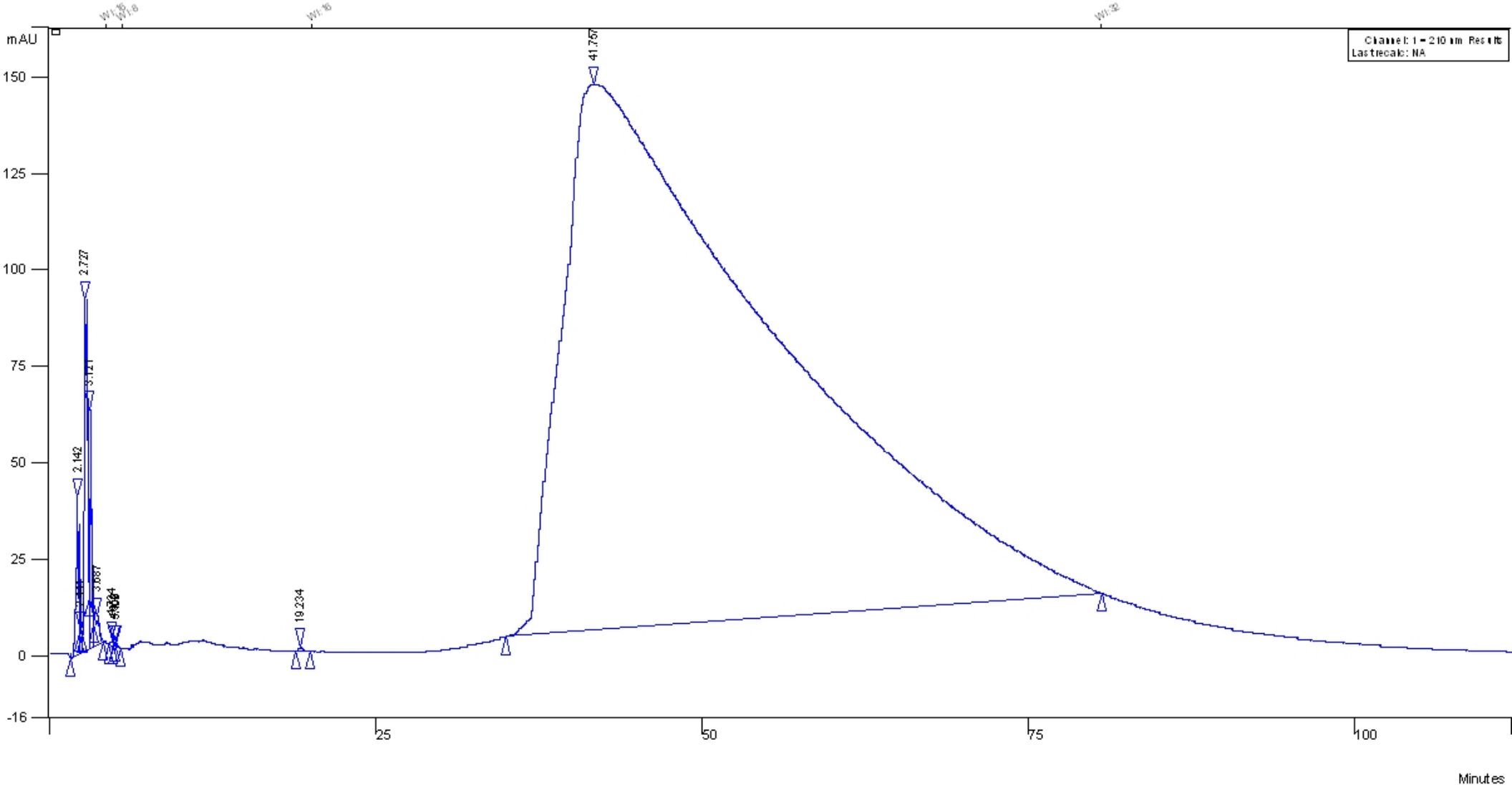
Recalc Info

Recalc Method: C:\star\data\Guo li\Naltrexamine derivatives\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Recalc Date: Wed Aug 15 14:50:58 2007
Operator: gl
Instrument: Varian Star #1
Notes:
Sample Rate: 20.000 Hz.
Measurement Type: 1 = Area
Calculation Type: 1 = %
Normalize Results: No

Peak Info for Channel 1

---

10 Peaks (tR	Timeoffset		RRT	Sepcode	Width	Counts	Result	Name)
2.142	0.000	0.0000	BV	9.57	4583288	0.279313		
2.441	0.000	0.0000	VV	0.00	815798	0.049716		
2.727	0.000	0.0000	VV	15.30	15264348		0.930235	
3.121	0.000	0.0000	VV	7.93	6140359	0.374204		
3.687	0.000	0.0000	VB	50.93	1412135	0.086058		
4.724	0.000	0.0000	BV	7.26	123844	0.007547		
4.952	0.000	0.0000	VV	6.60	72950	0.004446		
5.100	0.000	0.0000	VB	0.00	68008	0.004145		
19.234	0.000	0.0000	BB	18.74	266128	0.016218		
41.757	0.000	0.0000	BB	1600.11	1612166144		98.248116	


mAU
150
125
100
75
50
25
0
-16
2.727
3.121
2.142
3.687
19.234
41.757
Channel: 1 = 210 nm Results
Last recalc: NA
25
50
75
100
Minutes

File c:\star\data\lindsey\vzmn021,2-11-2008,12;35;32 pm.run Filetype 325
WTOASCII Parameters: C:\star\WTOASCII.TXT
1 channels
Channel 1 210 nm in AU
Base rate 20.0000 Hz Bunch 1 Effective rate 20.00000 Hz. T = 0.000833 min.
18360 points from 0.000 to 15.300 minutes
Sampling every 4 point(s)
Printing points from 0.000 to 15.300 min, point 0 to 18359
Channels 1 to 1
Results for Channel 1 = 210 nm
Title: Report Title
Run File: c:\star\data\lindsey\vzmn021,2-11-2008,12;35;32 pm.run

---

Injection Info

Method: C:\star\data\Guo li\Naltrexamine derivatives\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Sample: NTX freebase
Injection Date: Mon Feb 11 12:35:32 2008
Manual injection
Operator: gl
Instrument: Varian Star #1
Notes:

Misc Info

Workstation: STARWORKSTATIOÿ HÚ é
Bus Address: 44
Run Time: 0.000 to 15.300 min.

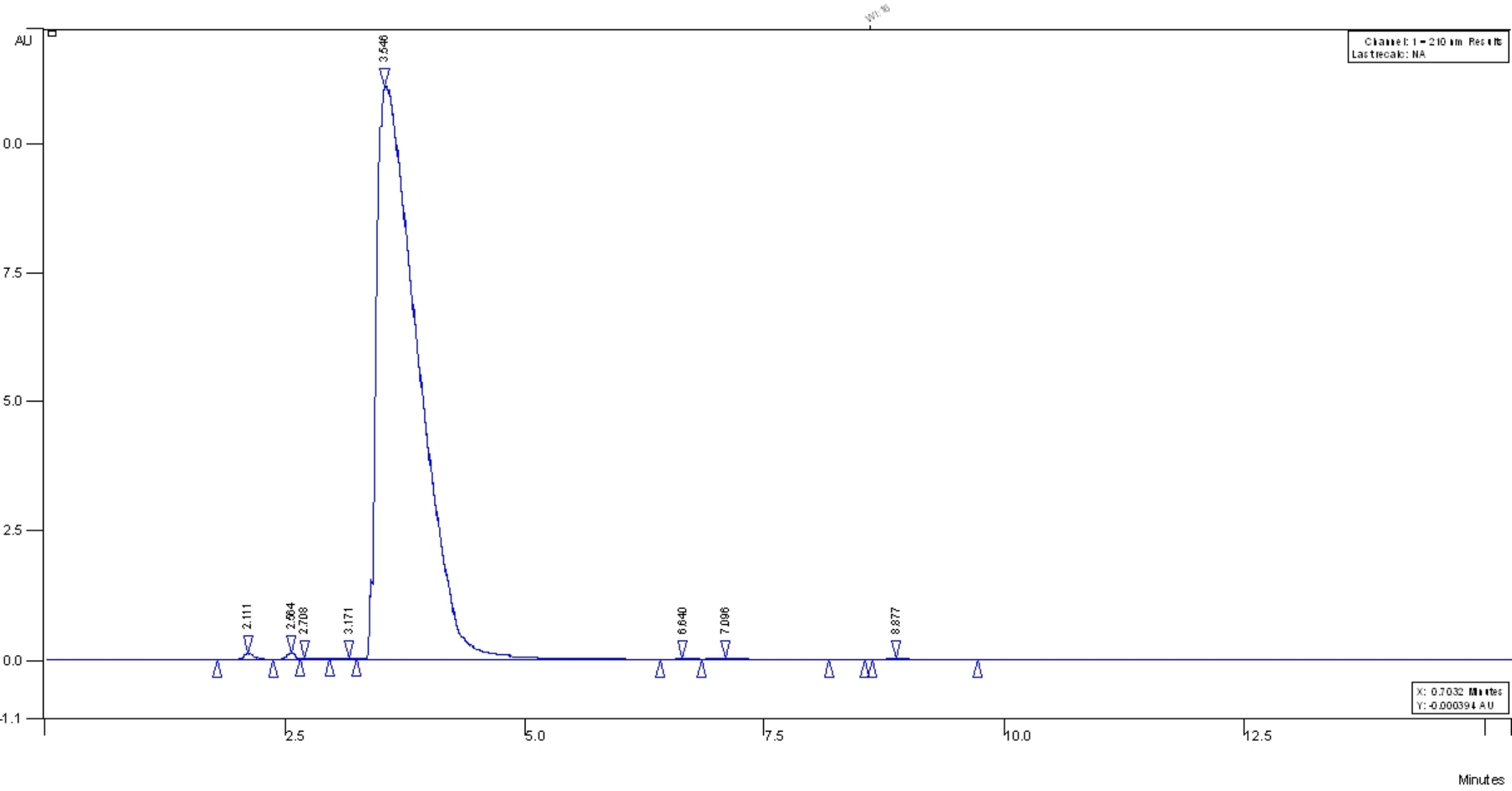
Recalc Info

Recalc Method: C:\star\data\Guo li\Naltrexamine derivatives\GL-4-09\H2O 25 CH3CN 75 1.0 210.mth
Recalc Date: Mon Feb 11 12:50:51 2008
Operator: gl
Instrument: Varian Star #1
Notes:
Sample Rate: 20.000 Hz.
Measurement Type: 1 = Area
Calculation Type: 1 = %
Normalize Results: No

Peak Info for Channel 1

---

8 Peaks (tR	Timeoffset		RRT	Sepcode Width	Counts	Result	Name)
2.111	0.000	0.0000	BP	7.76	10447155	0.309794	
2.564	0.000	0.0000	PV	6.30	9812229	0.290967	
2.708	0.000	0.0000	VV	22.02	7226687	0.214296	
3.171	0.000	0.0000	VV	0.00	5152396	0.152786	
3.546	0.000	0.0000	VB	27.92	3332947200	98.833466	
6.640	0.000	0.0000	TF	0.00	598189	0.017738	
7.096	0.000	0.0000	TF	0.00	2758845	0.081809	
8.877	0.000	0.0000	BB	10.74	3343466	0.099145	


AU
Channel: 1 = 210 nm Results
Last recalc: NA
3.546
2.111
2.664
2.708
3.171
6.640
7.096
8.877
0.0
7.5
5.0
2.5
0.0
-1.1
X: 0.7032 Minutes
Y: -0.000394 AU
2.5
5.0
7.5
10.0
12.5
Minutes

File c:\star\data\guo li\naltrexamine derivatives\vzmn01610ul,3-20-2008,12;39;35 pm.run Filetype 325
WTOASCII Parameters: C:\star\WTOASCII.TXT
1 channels
Channel 1 254 nm in AU
Base rate 20.0000 Hz Bunch 1 Effective rate 20.00000 Hz. T = 0.000833 min.
31786 points from 0.000 to 26.488 minutes
Sampling every 4 point(s)
Printing points from 0.000 to 26.488 min, point 0 to 31785
Channels 1 to 1
Results for Channel 1 = 254 nm
Title: Report Title
Run File: c:\star\data\guo li\naltrexamine derivatives\vzmn01610ul,3-20-2008,12;39;35 pm.run

---

Injection Info

Method: C:\star\data\Lindsey\Methods\H2O85_CH3CN15_1.0 _254.mth
Sample: ntx2uL+Gl-3-7110uL
Injection Date: Thu Mar 20 12:39:35 2008
Manual injection
Operator: gl
Instrument: Varian Star #1
Notes:

Misc Info

Workstation: STARWORKSTATIOÿ HÚ én
Bus Address: 44
Run Time: 0.000 to 26.488 min.

Recalc Info

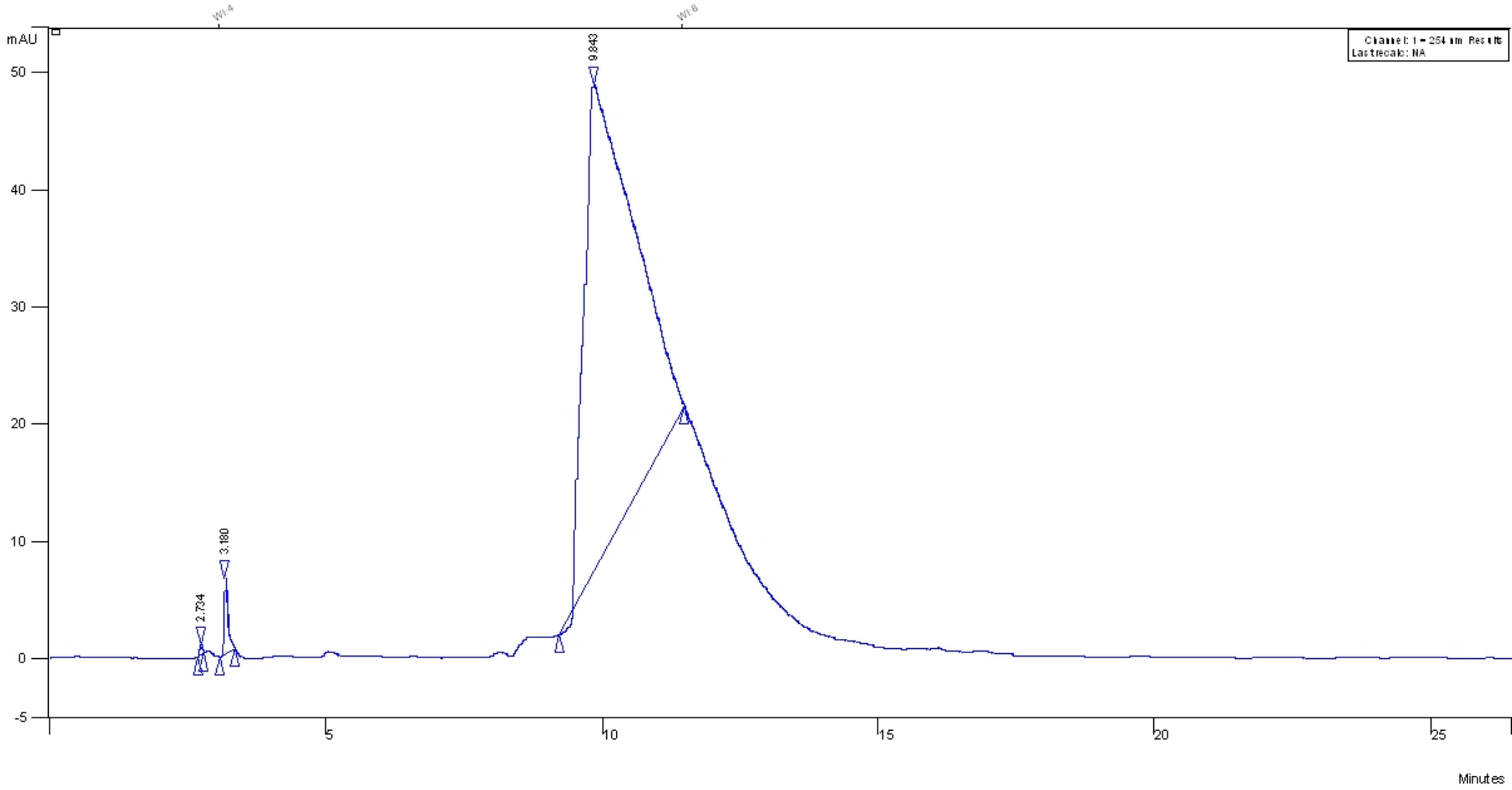
Recalc Method: C:\star\data\Lindsey\Methods\H2O85_CH3CN15_1.0 _254.mth
Recalc Date: Thu Mar 20 13:06:05 2008
Operator: gl
Instrument: Varian Star #1
Notes:
Sample Rate: 20.000 Hz.
Measurement Type: 1 = Area
Calculation Type: 1 = %
Normalize Results: No

Peak Info for Channel 1

---

3 Peaks (tR Timeoffset RRT Sepcode Width Counts Result Name)

2.734	0.000	0.0000	BB	3.55	25281	0.097331		
3.180	0.000	0.0000	BB	3.37	271394	1.044880		
9.843	0.000	0.0000	BB	93.82	25677054		98.857796	


mAU
50
40
30
20
10
0
-5
9.843
3.180
2.734
5
10
15
20
25
Minutes
Channel: 1 - 254 nm Results
Last recalc: NA

File c:\star\data\guo li\naltrexamine derivatives\vzmn017,3-20-2008,11;20;47 am.run Filetype 325
WTOASCII Parameters: C:\star\WTOASCII.TXT
1 channels
Channel 1 254 nm in AU
Base rate 20.0000 Hz Bunch 1 Effective rate 20.00000 Hz. T = 0.000833 min.
7958 points from 0.000 to 6.632 minutes
Sampling every 4 point(s)
Printing points from 0.000 to 6.632 min, point 0 to 7957
Channels 1 to 1
Results for Channel 1 = 254 nm
Title: Report Title
Run File: c:\star\data\guo li\naltrexamine derivatives\vzmn017,3-20-2008,11;20;47 am.run

---

Injection Info

Method: C:\star\data\Lindsey\Methods\H2O75_CH3CN25_1.0 _254.mth
Sample: ntx
Injection Date: Thu Mar 20 11:20:47 2008
Manual injection
Operator: gl
Instrument: Varian Star #1
Notes:

Misc Info

Workstation: STARWORKSTATIOÿ HÚ é
Bus Address: 44
Run Time: 0.000 to 6.632 min.

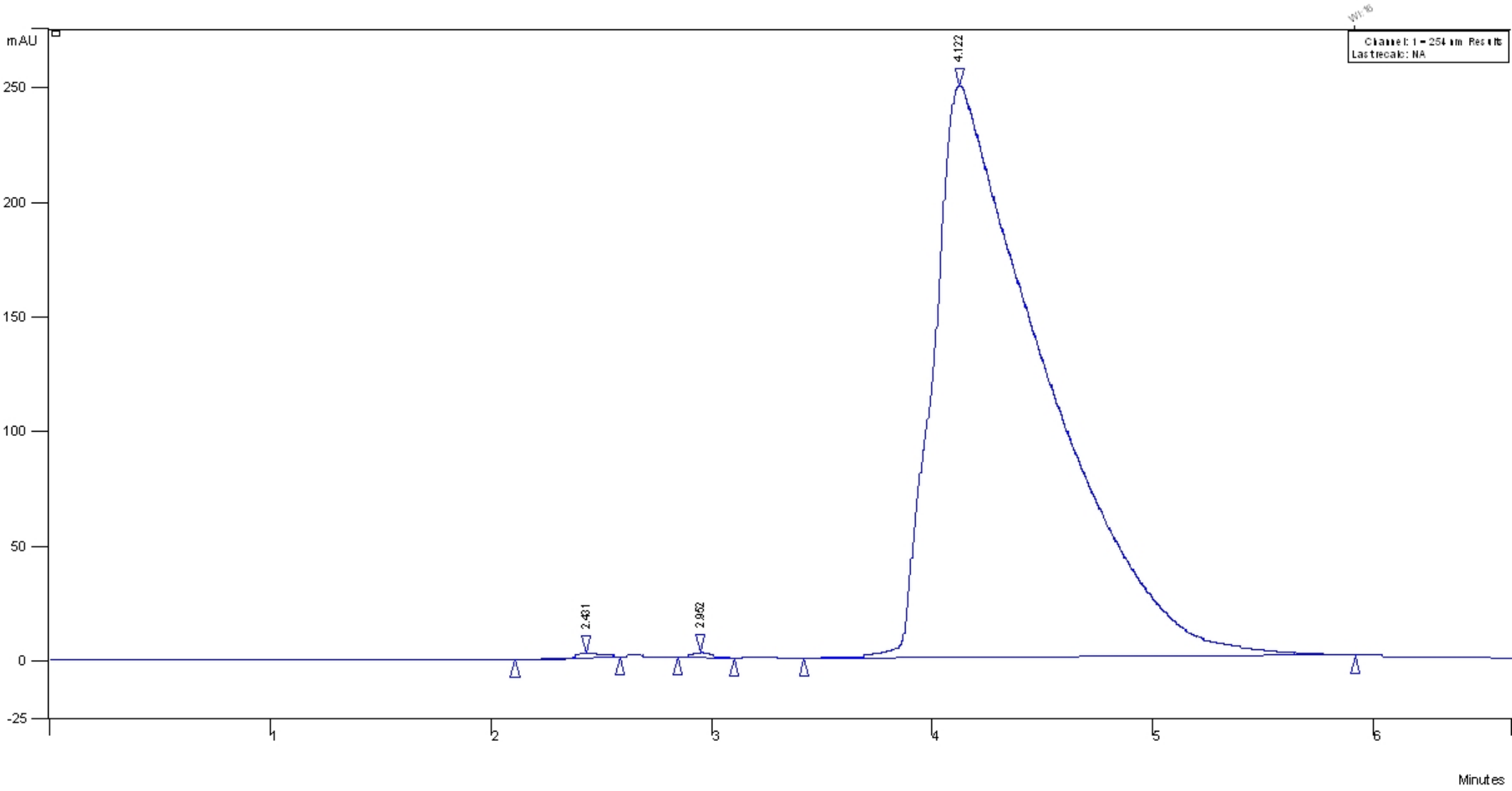
Recalc Info

Recalc Method: C:\star\data\Lindsey\Methods\H2O75_CH3CN25_1.0 _254.mth
Recalc Date: Thu Mar 20 11:27:26 2008
Operator: gl
Instrument: Varian Star #1
Notes:
Sample Rate: 20.000 Hz.
Measurement Type: 1 = Area
Calculation Type: 1 = %
Normalize Results: No

Peak Info for Channel 1

---

3 Peaks (tR	Timeoffset		RRT	Sepcode	Width	Counts	Result	Name)
2.431	0.000	0.0000	BB	0.00	90234	0.103173		
2.952	0.000	0.0000	BB	5.76	119253	0.136353		
4.122	0.000	0.0000	BB	30.70	87249416		99.760475	


mAU
250
200
150
100
50
0
-25
2.481
2.962
4.122
1
2
3
4
5
6
Minutes
Channel: 1 = 254 nm Results
Last recalc: NA

File c:\star\data\guo li\naltrexamine derivatives\vzmn023_2ul,7-27-2007,9;31;31 am.run Filetype 325
WTOASCII Parameters: C:\star\WTOASCII.TXT
1 channels
Channel 1 210 nm in AU
Base rate 20.0000 Hz Bunch 1 Effective rate 20.00000 Hz. T = 0.000833 min.
98348 points from 0.000 to 81.957 minutes
Sampling every 4 point(s)
Printing points from 0.000 to 81.957 min, point 0 to 98347
Channels 1 to 1
Results for Channel 1 = 210 nm
Title: Report Title
Run File: c:\star\data\guo li\naltrexamine derivatives\vzmn023_2ul,7-27-2007,9;31;31 am.run

---

Injection Info

Method: C:\star\data\Guo li\GL-4-09\H2O 30 CH3CN 70 1.0 210.mth
Sample: 1.4-12CH3CN70_2ul
Injection Date: Fri Jul 27 09:31:31 2007
Manual injection
Operator: gl
Instrument: Varian Star #1
Notes:

Misc Info

Workstation: STARWORKSTATIOÿ HÚ é
Bus Address: 44
Run Time: 0.000 to 81.957 min.

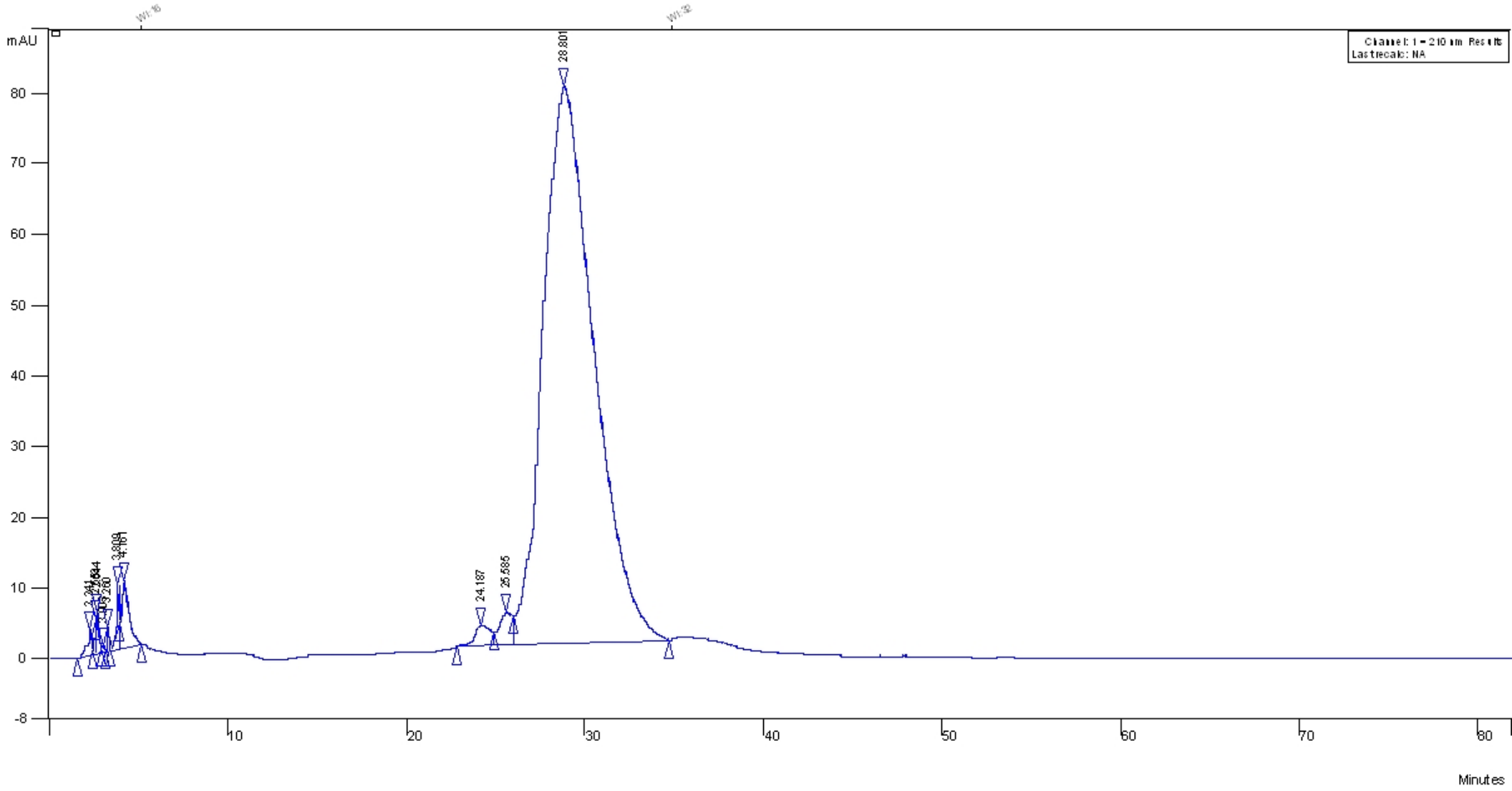
Recalc Info

Recalc Method: C:\star\data\Guo li\GL-4-09\H2O 30 CH3CN 70 1.0 210.mth
Recalc Date: Fri Jul 27 10:53:30 2007
Operator: gl
Instrument: Varian Star #1
Notes:
Sample Rate: 20.000 Hz.
Measurement Type: 1 = Area
Calculation Type: 1 = %
Normalize Results: No

Peak Info for Channel 1

---

10 Peaks (tR	Timeoffset		RRT	Sepcode	Width	Counts	Result	Name)
2.241	0.000	0.0000	BV	14.34	706942	0.429533		
2.553	0.000	0.0000	VV	7.12	283257	0.172105		
2.644	0.000	0.0000	VV	12.23	585994	0.356046		
3.003	0.000	0.0000	VV	14.65	63879	0.038812		
3.260	0.000	0.0000	VV	8.85	280643	0.170517		
3.809	0.000	0.0000	VV	10.20	1027035	0.624019		
4.161	0.000	0.0000	VB	29.44	2650553	1.610458		
24.187	0.000	0.0000	BV	46.76	1805442	1.096974		
25.585	0.000	0.0000	VV	59.93	2417250	1.468704		
28.801	0.000	0.0000	VB	196.98	154762800		96.032721	


mAU
80
70
60
50
40
30
20
10
0
-8
28.801
24.187
25.585
4.161
3.809
10
20
30
40
50
60
70
80
Minutes

3.4 In vitro competitive radioligand and functional binding assays: Binding assays were conducted to study the selectivity of the ligands using mono-cloned opioid receptors expressed in CHO cell lines as described previously, with slight modifications.[5,6] Briefly, [^{3}H]naloxone, [^{3}H]NTI and [^{3}H]norBNI (obtained from NIH/NIDA Drug Supply Program) were used to label the mu, delta and kappa opioid receptors, respectively. Isolated membranes (50 µg) were incubated with the radioligands in the presence of varying concentrations of the drug under investigation at 30 ºC for 90 min. Specific binding was determined as the difference in binding obtained in the absence and presence of 10 µM naltrexone. The potencies of the drugs in competing for the specific binding of the radioligand were determined using linear regression analysis of Hill plots. The IC_{50} values were then determined and corrected to K_i values using the Cheng-Prusoff equation. ^{35}S-GTP[γS] binding assays were conducted in the same cell membranes used for the receptor binding assays, as previously described with slight modifications.[6] Briefly, membranes (10 µg) were incubated with 10 µM GDP, 0.1 nM ^{35}S-GTP[γS] and varying concentrations of the drug under investigation for 30min at 90°C. Non-specific binding was determined with 10 µM unlabeled GTP[γS]. A maximally effective concentration (3 µM) of DAMGO was included for comparison in each assay as a full agonist for the mu opioid receptor. Stimulation of ^{35}S-GTP[γS] binding was calculated as net stimulation: stimulated – basal ^{35}S-GTP[γS] binding, measured in fmol/mg. These data were normalized to the stimulation produced by 3µ M DAMGO, which was set to 100%, as follows: (net stimulation by each concentration of each ligand / net stimulation by 3µM DAMGO) x 100%. Relative E_{max} and EC_{50} values were then determined by nonlinear regression analysis of the concentration-effect curves, performed using JMP

(SAS for Macintosh). An E_{max} value given as "0" means that no significant concentration-dependent stimulation of ^{35}S-GTP[γS] binding was obtained with that ligand.

4. Refenences:

1. Thompson, J. D.; Higgins, D. G.; Gibson, T. J. *Nucleic Acids Res*. **1994,** *22*, 4673-4680.

2. Okada, T.; Sugihara, M.; Bondar, A. N.; Elstner, M.; Entel, P.; Buss, V. *J. Mol. Biol.* **2004**, *342*, 571-583.

3. Dunbrack, R. L. Jr. *Proteins*, **1999**, *Suppl 3*, 81-7.

4. Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. *J. Mol. Biol*. 1997, *267*, 727-748.

5. Selley, D. E.; Sim, L. J.; Xiao, R.; Liu, Q.; Childers, S. R. *Mol. Pharmacol.* **1997**, *51(1*), 87-96.

6. Selley, D. E.; Cao, C. C.; Liu, Q.; Childers, S. R. *J. Pharmacol. Exp. Ther*. **1998**, *285*, 496-505.