Determination of Free (Unbound) Anamorelin in Human Plasma by Ultrafiltration

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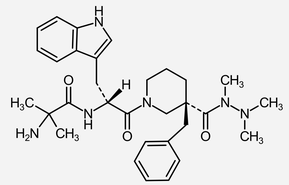
INTRODUCTION

Anamorelin is under investigation for cancer cachexia, a devastating, often late-stage complication of an underlying malignancy. Despite the significant importance of cancer-related cachexia, treatments are lacking and no products are approved for this indication. Anamorelin, by virtue of its ghrelin agonist activity, may serve a role in the treatment of cancer cachexia.

To evaluate the extent of free (unbound) Anamorelin in human plasma, the appropriate procedures for processing samples were investigated. Ultrafiltration was found to be valid technique with limited non-specific binding (NSB) observed to the device. LC-MS/MS methods were then validated for the analysis of free and total Anamorelin.



Chemical Structure

METHOD

Protein Binding Assessments

- Non-Specific Binding
- Concentration Dependency
- Affect of pH
- Affect of Freeze/Thaw
- Anticoagulant (Heparin *vs.* EDTA)
- Binding to Selected Proteins

Ultrafiltration

Device: Amicon Centrifree®, YM-30
Centrifugation: 1000 x *g,* Fixed Angle Rotor
Maximum Filtration: ⅓ Sample Volume
Temperature: 37 ± 2 ºC

Sample Extraction

Plasma (50 mcL) or Ultrafiltrate (150 mcL) were combined with 400 mcL internal standard (Piroxicam in MeOH/ACN, 50:50). Samples were centrifuged 5 min at 15,000 rpm and the resulting supernatants combined 1:1 with 10 mM ammonium formate buffer, pH 3.2, and analyzed by LC-MS/MS.

METHOD (Cont.)

Extracts were analyzed using fully validated methods for
Human Plasma (5 to 1280 ng/mL)
Human Plasma Ultrafiltrate (1 to 100 ng/mL)

Liquid Chromatography

HPLC : Shimadzu 10AD
Autosampler : Perkin Elmer 200
Column : Pursuit C18 (50 x 2 mm) 5µ
Temperature: 30 ºC
MP A : ACN/Ammonium Formate Buffer, pH 3.2 (10/90)
MP B : ACN/MeOH (40/60)
Flow Rate: 0.35 mL/min
Rinse : ACN/DI H_2O (90/10)

Mass Spectrometry

AB Sciex API 3000 with Turbo-ion Spray interface
Polarity : Positive
Scan type : Multiple Reaction Monitoring

Anamorelin: 547.5 → 276.3 *m/z*
Piroxicam (IS): 332.0 → 164.0 *m/z*

RESULTS

Non-Specific Binding (NSB)

Ultrafiltrate Conc. *ng/mL*	Recovery %	NSB %
3	92.4	7.6
15	93.9	6.1

Concentration Dependency

Plasma Conc. *ng/mL*	Free Fraction (Mean) %
100	3.57 ± 0.27
200	3.59 ± 0.39
300	3.91 ± 0.35
375	3.67 ± 0.40
750	4.05 ± 0.21
1000	4.43 ± 0.27
1500	4.66 ± 0.12

Li Heparin, n = 5 replicates, pH 7.40 at 37 ºC

RESULTS (Cont.)

Affect of pH

pH	Free Fraction (Mean) %
7.00	6.91± 0.11
7.40	6.54 ± 0.34
8.20	5.80 ± 0.13

Li Heparin, n = 5 replicates, c = 375 ng/mL at 37 ºC.
Plasma pH was adjusted using carbon dioxide gas.

Affect of Freeze/Thaw

Anamorelin Conc. *ng/mL*	Plasma	Free Fraction (Mean) %
200	Fresh (Non-Frozen)	4.63 ± 0.14
375		5.23 ± 0.01
200	3 Cycles Freeze/Thaw	4.86 ± 0.12
375		4.93 ± 0.07

Li Heparin, n = 4 replicates at pH 7.40 at 37 ºC.

Affect of Anticoagulant

Slightly lower free fractions were observed for Anamorelin in plasma containing Li heparin (3.57 – 4.66%) compared to plasma containing K_2EDTA anticoagulant (4.06 – 5.15%).

Binding to Selected Plasma Proteins

Anamorelin Conc. *ng/mL*	Plasma Protein	Free Fraction (Mean) %
200	α_1–Acid Glycoprotein (AGP)	2.79
375		3.05
200	Human Albumin	62.8
375		63.7

AGP, 1.25 mg/mL and Albumin, 40 mg/mL in PBS, pH 7.40; (n = 4 replicates) at 37 ºC.

RESULTS (Cont.)

Clinical Trial Results

Protein binding assessment for Anamorelin have been conducted in three separate clinical trails.

ST-ANAM-110 entitled “An Open-Label, Single and Multiple-Dose, Pharmacokinetic, Safety and Tolerability Study of Anamorelin HCl in Healthy Normal Volunteers”.

RC-1291-203 entitled “A Two-Part Phase II Study of the Effects of RC-1291HCl in Patients with Cancer Anorexia/Cachexia: An In-patient Crossover Study of the Appetite-Enhancing and Biochemical Effects of RC-1291 HCl followed by an Out-patient Parallel Group Study of the Effects of RC-1291 HCl on Body Weight, Body Composition and Functional Performance”.

ST-ANAM-207 entitled “A Randomized, Double-Blind, Placebo-Controlled, Multicenter Phase II Anamorelin HCl Dose Range Study to Evaluate the Safety and Efficacy of Anamorelin HCl in Patients with NSCLC”.

Subject Status	Protocol	Free Fraction (%) Mean	Low	High
Healthy	ST-ANAM-110	6.31	3.86	10.6
Cancer	RC-1291-203	4.07	1.94	7.07
Cancer	ST-ANAM-207	4.53	2.09	8.61

CONCLUSION

An accurate procedure was developed and validated for the determination of free Anamorelin in human plasma samples using ultrafiltration. Of significance was the high percentage to which Anamorelin binds to α_1-acid glycoprotein (≥ 97%) with nominal binding to albumin (~36.5%).

The results from clinical sample analyses suggests higher extent of plasma protein binding in subjects with cancer (4.1- 4.5% free) compared to healthy normal subjects (6.3% free). The upcoming investigation will now explore the correlation between the extent of Anamorelin protein binding and α_1-acid glycoprotein levels.