REPORT

Effect of Thymosin beta 4 on Cloned hERG Potassium Channels Expressed in Human Embryonic Kidney Cells

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1 Signature

Study Director:
Cynthia Wladyka, BS

ChanTest Corporation

22-Feb-2011

Signature Date

2 GLP Compliance Statement

The study complied with the most recent version of the Food and Drug Administration (FDA) Good Laboratory Practices Regulations (21 CFR Part 58), with the following exceptions:

- The positive control article used in this study was a marketed product. In accordance with GLP, information regarding the characterization was taken from the positive control article label provided by the supplier. In addition, there is no documented evidence to support the expiration date of the positive control article.
- The positive control article formulation was not analyzed for stability, homogeneity or concentration (its potency was demonstrated by the comparison of results with the ChanTest historical data presented in Table 5).
- Test article production, testing and the documentation thereof was conducted under GMP standards.

The study complied with ICH Guidance for Industry (July 2001), "S7A Safety Pharmacology Studies for Human Pharmaceuticals" and ICH Guidance for Industry (October 2005), "S7B Nonclinical Evaluation of the Potential for Delayed Repolarization (QT Interval Prolongation) by Human Pharmaceuticals." The study was conducted in accordance with the standard operating procedures (SOPs) of ChanTest Corporation.

Cynthia Wladyka, BS
Study Director
ChanTest Corporation

Signature

22-Feb-2011

Date

3 ChanTest Quality Assurance Statement

Inspection Date	Inspection Type	Date Reported to the Study Director	Date Reported to Management
19-Oct-2010	Protocol Review	19-Oct-2010	19-Oct-2010
20-Oct-2010	Protocol Amendment No. 1	20-Oct-2010	20-Oct-2010
29-Oct-2010	Test Article Preparation	29-Oct-2010	29-Oct-2010
29-Oct-2010	Data Acquisition	29-Oct-2010	29-Oct-2010
03-Nov-2010	Protocol Amendment No. 2	03-Nov-2010	03-Nov-2010
09-Nov-2010	Data	09-Nov-2010	12-Nov-2010
09-Nov-2010	Draft Report	09-Nov-2010	12-Nov-2010
17-Feb-2011	Protocol Amendment No. 3	17-Feb-2011	17-Feb-2011
21-Feb-2011	Final Report	21-Feb-2011	21-Feb-2011

The Quality Assurance Unit has reviewed the report and has determined that the results incorporated into this report accurately reflect the study raw data.

Alicia DePlatchett, BA, RQAP-GLP
Quality Assurance Supervisor
ChanTest Corporation

ADePlatchett 22Feb-2011
Signature Date

4 Summary

The objective of this study was to examine the *in vitro* effects of Thymosin beta 4 on the hERG (human ether-à-go-go-related gene) channel current (a surrogate for I_{Kr}, the rapidly activating, delayed rectifier cardiac potassium current) at near-physiological temperature.

The stability of the test article formulations was confirmed during the method validation portion of the study. Test article concentrations were applied to the test system and dose solution analysis was conducted within the validated stability timeframe.

The sample analysis indicated that all formulations were homogeneous at the beginning of testing. Additionally, samples of the test article formulation solutions collected from the outflow of the perfusion apparatus were analyzed for concentration verification. The results from the sample analysis indicated that the measured concentration of Thymosin beta 4 at the 100 µM test concentration was 15.0% RE, which met the acceptance criteria (% RE ± 15.0%), while the measured concentration for the 600 µM test concentration was slightly outside the acceptable range (% RE = 15.3%). Since the measured concentration results for both formulations were biased high, the nominal concentrations were used when determining the hERG concentration-response relationship as the nominal concentrations were lower and therefore the use of these concentrations is more conservative.

Thymosin beta 4 inhibited hERG current by (Mean ± SEM) 1.4 ± 0.5% at 100 µM (n = 5) and 1.2 ± 0.6% at 600 µM (n = 5) versus 0.6 ± 0.3% (n = 3) in vehicle control. Testing was conducted only at these two concentrations because Thymosin beta 4 was insoluble in the assay vehicle at higher dose concentrations. hERG inhibition at 100 and 600 µM was not statistically significant ($P > 0.05$) when compared to vehicle control values. Due to the solubility limitations, the IC_{50} for the inhibitory effect of Thymosin beta 4 on hERG potassium current was not calculated but was estimated to be greater than 600 µM.

Under similar conditions, the positive control (60 nM terfenadine) inhibited hERG potassium current by (Mean ± SEM; n = 3) 77.6 ± 3.7%. This result confirms the sensitivity of the test system to hERG inhibition.

5 Introduction and Objective

The objective of this study was to examine the *in vitro* effects of Thymosin beta 4 on the hERG (human ether-à-go-go-related gene) channel current (a surrogate for I_{Kr}, the rapidly activating, delayed rectifier cardiac potassium current, Redfern et al., 2003). The concentration-response relationship of the effect of Thymosin beta 4 on the hERG potassium channel current was evaluated at near-physiological temperature in stably transfected mammalian cells that express the hERG gene.

Prior to analysis of the dose formulations, a method validation was conducted to verify that the analytical test method was suitable for determination of the selected Thymosin beta 4 concentrations in hERG assay buffer. The method of analysis used was documented in ChanTest analytical procedure 100805.SFI.00.

5.1 Sponsor Responsibilities

The Sponsor was responsible for the following information:

1. Documentation on the strength, purity, composition, physical properties, stability, and other pertinent information on the bulk test article in the form of a Certificate of Analysis for inclusion in the final report (Appendix B). The Sponsor provided a record of Retest Date Extension to support bulk test article stability (Appendix B).
2. Amount of test article provided.

5.2 Protocol Amendments and Deviations

There were three protocol amendments that did not impact the integrity of the study (Appendix A). There were no deviations from the study protocol.

5.3 Quality Assurance Unit

The Quality Assurance Unit (QAU) at ChanTest monitored the study described in this protocol and conducted appropriate phase inspections of the study according to ChanTest SOP(s). Any observations from QAU inspections were reported to the Study Director and Testing Facility Management. Inspection type and date are documented in Section 3 of this report.

Observations related to the analytical portion of the study were reported to the Dose Analysis Contributing Scientist, the Study Director and Testing Facility Management. Inspection type and date are documented in the QA statement of the analytical report (Appendix C).

6 Test and Control Articles

6.1 Test Article

Test Article ID:	Thymosin beta 4
Alternate Test Article ID:	TB4
Lot Number:	FTHYB40602B (Protocol Amendment No. 1)
Retest Date:	July-2011
Form:	Acetate salt
Molecular Weight:	4963.55 g/mol (free peptide)
Peptide Purity:	98.4% [1]
Peptide Content:	93.1%
Storage Conditions (bulk):	Frozen (-10 to -30 ºC); under argon
Carrier:	Sterile water for injection, USP (sterile water)
Solubility:	At least approximately 66 mM in sterile water. Approximately 660.0 µM in HB-PS + 1.0% sterile water. (Per ChanTest non-GLP study 100805.SFI.PRE.)

[1] Formulations were not corrected for peptide purity (100% was assumed for calculations in this study). Only a correction for peptide content was used in this study.

6.1.1 Rationale for Concentration Selection

Based on the result of initial testing at 100 µM and the solubility limit of Thymosin beta 4 in the hERG assay vehicle, an additional concentration (600 µM) was evaluated.

Per Sponsor: The highest concentration evaluated in this study (600 µM) is approximately 10x the blood levels that will be used in clinical dosing. These PK numbers are based on actual data obtained from a Phase I safety study with the drug administered intravenously.

6.1.2 Test Article Disposition

Bulk test article, stock solutions, and analytical samples remaining at the end of the study will be disposed after issuance of the final report and records of disposition will be filed with the study data.

6.1.3 Test Article Carrier

Name:	Sterile water for injection, USP
Source/Manufacturer:	Moore Medical / Hospira
Lot Number:	86-502-DK
Molecular Weight:	N/A
Storage Conditions (bulk):	Room temperature
Rationale for Selection:	The test article is water-soluble.

6.2 Positive Control Article and Reference Substance Carrier

Name:	DMSO
Source:	Sigma-Aldrich
Lot Number:	76396EM
Retest Date:	31-May-2012
Molecular Weight:	78.13 g/mol
Storage Conditions (bulk):	Room temperature
Rationale for Selection:	Previous results have shown that 0.3% DMSO does not affect channel current (data on file at ChanTest Corporation).

6.3 Vehicle Control

The vehicle consisted of a HEPES-buffered physiological saline (HB-PS) solution (composition in mM): NaCl, 137; KCl, 4.0; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 10; Glucose, 10; pH adjusted to 7.4 with NaOH (refrigerated until used). Chemicals used in vehicle preparation were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted and were of ACS reagent grade purity or higher.

Name:	HB-PS + 1.0% sterile water (for test article) HB-PS + 0.3% DMSO (for positive control article and reference substance)
Source:	ChanTest Corporation
HB-PS Batch Numbers:	Expiration Dates:
101026NEA425	23-Nov-2010
101027NEA427	24-Nov-2010
Storage Conditions (bulk):	Refrigerated
Rationale for Selection:	HB-PS provides the appropriate ionic composition for *in vitro* recording.

6.4 Positive Control Article

Name:	Terfenadine
Source:	Sigma-Aldrich
Lot Number:	079K1602
Expiration Date:	31-Dec-2011
Molecular Weight:	471.67 g/mol
Purity:	99.0%
Storage Conditions (bulk):	Refrigerated
Rationale for Selection:	Previous results have shown that 60 nM terfenadine inhibits hERG potassium current by approximately 80% (data on file at ChanTest Corporation).

6.5 Reference Substance

Name:	E-4031
Source:	Sigma-Aldrich
Lot Number:	086K46168
Expiration Date:	31-Aug-2011
Molecular Weight:	474.44 g/mol
Purity:	> 99.0%
Storage Conditions (bulk):	Frozen
Rationale for Selection:	E-4031 selectively inhibits hERG potassium current with IC_{50} = 12 nM (data on file at ChanTest Corporation). A supramaximal inhibiting concentration of 500 nM was used to eliminate the contribution of non-hERG currents.

6.6 Formulations

6.6.1 Positive Control and Reference Substance Formulations

Stock solutions of the positive control and reference substance were prepared in dimethyl sulfoxide (DMSO), aliquoted for individual use, stored frozen, and used within one month. Final positive control and reference substance concentrations were prepared fresh daily by diluting stock solutions in vehicle (final DMSO concentration, 0.3% v/v). Positive control article and reference substance formulations as set forth in this section are solutions and, therefore, homogeneous, by definition. Homogeneity, concentration, and stability analyses were not performed. Their potency for hERG inhibition is demonstrated by the comparison of results with ChanTest historical data.

6.6.2 Test Article Formulations

An approximately 63 mM stock solution of the test article was prepared in sterile water with five minutes of vortex mixing. The stock solution was stored at room temperature. Test article concentrations were prepared fresh on the day of testing by diluting the stock solution in vehicle with one minute of vortex mixing (final sterile water concentration, 1.0% v/v). The pH of the 600 µM test formulation was adjusted from 6.78 to 7.38 with 10 N NaOH.

Test article concentrations used in this study were not corrected for the peptide purity. A peptide purity of 100% was assumed for all calculations. Only a correction for peptide content was used.

6.6.3 Preparation of Vehicle and Formulation Samples

Vehicle control samples (blank, 4 samples, 1 mL each, accurately measured directly into HPLC vials) were taken from the vehicle reservoir at the beginning of testing.

Samples for homogeneity determination (4 samples, 1 mL each, accurately measured directly into HPLC vials) were aliquoted from the top, middle, and bottom of the test

article formulation reservoir for each concentration after preparation on the day of testing.

Representative test article formulation samples for concentration verification were collected on the day of testing. Samples of each test concentration (4 samples, 1 mL each, accurately measured directly into HPLC vials) were aliquoted from the outflow of the perfusion apparatus.

The above formulation samples were stored at room temperature after collection until analytical testing. For analysis, two samples from each condition were transferred to the analytical chemistry unit while the remaining two samples from each condition were retained in storage as backups.

7 Test System

Cells were maintained in tissue culture incubators per ChanTest SOP. Stocks were maintained in cryogenic storage. Cells used for electrophysiology were plated in plastic culture dishes. Each culture dish was identified by a notation of clone identity number, passage number, and date. These data were retained in the study file.

7.1 Rationale for Selection of Ion Channel and Expression Systems

The cardiac potassium channel, hERG, is responsible for a rapid delayed rectifier current (I_{Kr}) in human ventricles. This channel has been selected for evaluation because inhibition of I_{Kr} is the most common cause of cardiac action potential prolongation by non-cardiac drugs (Brown and Rampe, 2000; Weirich and Antoni, 1998; Yap and Camm, 1999). Increased action potential duration causes prolongation of the QT interval and has been associated with a dangerous ventricular arrhythmia, *torsade de pointes* (Brown and Rampe, 2000). In this assay, hERG potassium channels are expressed in a human embryonic kidney (HEK293) cell line that lacks endogenous I_{Kr}.

7.2 HEK/hERG

Organism:	*Homo sapiens*
Designation:	293
Tissue:	Kidney; Transformed with adenovirus 5 DNA; Transfected with human-ether-a-go-go cDNA
Morphology:	Epithelial
Age/Stage:	Embryo
Source-Strain:	ATCC, Manassas, VA
Source-Sub Strain:	ChanTest Corporation, Cleveland, OH

7.3 Cell Culture Procedures

HEK293 cells were stably transfected with hERG cDNA. Stable transfectants have been selected by coexpression with the G418-resistance gene incorporated into the expression plasmid. Selection pressure was maintained by including G418 in the culture medium.

Cells were cultured in Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 µg/mL streptomycin sulfate and 500 µg/mL G418.

Cells used for electrophysiology were plated in 35 mm plastic culture dishes at a low enough density to allow single-cell access (approximately 5 – 15% confluency). Each culture dish was identified by a notation of clone identity number, passage number, and date. These data were retained in the study file. Cells were used 24 – 72 hours after plating.

8 Test Method

8.1 Treatment Groups

All experiments were performed at near-physiological temperature (33 to 35 ºC). Each cell acted as its own control.

8.1.1 Positive Control Group

The positive control was applied to three (3) cells (n = 3).

8.1.2 Concentration-Response Test Group

Two (2) concentrations were selected to evaluate the concentration-response relationship based on the outcome of the initial testing and based on the solubility limit of the test article in the assay vehicle. Each concentration was tested in five (5) cells (n = 5).

8.1.3 Vehicle Control Group

Vehicle control solution was applied to three (3) cells (n = 3). Duration of vehicle application was approximately 5 minutes, which was at least as long as the longest test article application in the study.

8.2 Electrophysiological Procedures

Cells were transferred to the recording chamber and superfused with vehicle control solution. Micropipette solution for whole cell patch clamp recordings was composed of (mM): potassium aspartate, 130; $MgCl_2$, 5; EGTA, 5; ATP, 4; HEPES, 10; pH adjusted to 7.2 with KOH. Micropipette solution was prepared in batches (100824NEA004K; expiration date 30-Jun-2011), aliquoted, stored frozen, and a fresh aliquot thawed each day. The recording was performed at a temperature of 33 to 35 ºC using a combination of in-line solution pre-heater, chamber heater, and feedback temperature controller. Temperature was measured using a thermistor probe in the recording chamber. Micropipettes for patch clamp recording were made from glass capillary tubing using a P-97 micropipette puller (Sutter Instruments, Novato, CA). A commercial patch clamp amplifier was used for whole cell recordings. Before digitization, current records were low-pass filtered at one-fifth of the sampling frequency.

Cells stably expressing hERG were held at -80 mV. Onset and steady state inhibition of hERG potassium current due to Thymosin beta 4 were measured using a pulse pattern with fixed amplitudes (conditioning prepulse: +20 mV for 1 sec; repolarizing test ramp to –80 mV (-0.5 V/s) repeated at 5 s intervals. Each recording ended with a final application of a supramaximal concentration of the reference substance (E-4031, 500 nM) to assess the contribution of endogenous currents. The remaining uninhibited current was subtracted off-line digitally from the data to determine the potency of the test substance for hERG inhibition.

8.3 Experimental Procedures

Thymosin beta 4 at 100 µM was applied to five cells (n = 5). A mean inhibitory effect on hERG potassium current amplitude of 1.4% was observed. Based on this result and the solubility limit of Thymosin beta 4 in the assay vehicle, an additional nominal concentration (600 µM; Protocol Amendment No. 2) was tested.

8.4 Electrophysiological Data Analysis

Data were stored on the ChanTest computer network (and backed-up nightly) for off-line analysis. Data acquisition and analyses were performed using the suite of pCLAMP (Ver. 8.2) programs (MDS-AT, Sunnyvale, CA), and were reviewed by the Study Director. Percent inhibition at each concentration in the test group was compared with the vehicle control group using one-way ANOVA followed by Dunnett's multiple comparison test (JMP Version 5.0.1, SAS Institute, Cary, NC). Significant inhibition was defined at the level of $P < 0.05$.

Steady state was defined by the limiting constant rate of change with time (linear time dependence). The steady state before and after test article application was used to calculate the percentage of current inhibited at each concentration.

9 Results

9.1 Stability of the Bulk Test Article

Thymosin beta 4 was produced, tested and documented under GMP standards. The Certificate of Analysis is presented in Appendix B. The Sponsor provided a record of Retest Date Extension in support of bulk test article stability (Appendix B).

9.2 Analysis of Formulation Samples for Homogeneity, Stability and Concentration Verification

The formulation samples were analyzed at ChanTest by high-performance liquid chromatography using a validated method 100805.SFI.00. The analytical report is presented in Appendix C.

9.2.1 Stability of Test Article Primary Stock and Test Article Formulations

The stability of the test article formulations was confirmed during the method validation study. The data demonstrate that approximately 1.0 and 65.9 mM stock solutions in sterile water are stable for at least approximately 30 hours when stored at room temperature (15 – 30 ºC).

Stability of the Thymosin beta 4 in the formulation matrix (HB-PS + 1.0% sterile water) was evaluated for the following concentrations: 1.0, 10.0 and 660.0 µM. For all concentrations evaluated, Thymosin beta 4 was found to be stable under both room temperature and refrigerated conditions for a period of at least 28 hours.

Test article concentrations were applied to the test system and dose solution analysis was conducted within the validated stability timeframe.

9.2.2 Homogeneity Analysis of Electrophysiology Dose Formulation Samples

Samples for homogeneity determination, aliquoted from the top, middle, and bottom of the dose formulation reservoir at the start of day were analyzed. The % CV values for the dose formulations ranged from 0.3 to 0.7%, demonstrating that the solutions were homogenous.

9.2.3 Concentration Verification of Electrophysiology Dose Formulation Samples

Samples of the test article formulation solutions collected from the outflow of the perfusion apparatus were analyzed for concentration verification. The results from the sample analysis indicated that the measured concentrations of Thymosin beta 4 at the 100 µM test concentration was 15.0% RE, thereby meeting the acceptance criteria (% RE ± 15.0%), while the measured concentration at 600 µM was outside the acceptable range (%RE = 15.3%; OOS-110110-01).

The reason for the higher than nominal measured concentrations for both the 100 and 600 µM formulations is unknown. The nominal concentrations for both formulations were used for determining the hERG concentration-response relationship. Since the nominal concentrations were lower than the measured concentrations, the use of nominal concentrations is more conservative.

9.3 Electrophysiological Results

Table 1 provides individual data points and Table 2 provides statistics for each concentration of Thymosin beta 4 used to construct the hERG concentration-response relation.

Typical hERG potassium current records acquired during vehicle control, after equilibration with Thymosin beta 4 at 600 µM and after equilibration with the reference substance (0.5 µM E-4031) are superimposed in Figure 1. In the vehicle control trace for this experiment, following activation of hERG potassium channels by the conditioning prepulse (+20 mV), partial repolarization evoked a large, slowly decaying outward

current. In this example, Thymosin beta 4 application reduced the amplitude of the outward tail currents by 0.5% at 600 µM.

Figure 2 exemplifies the time course of the effect of Thymosin beta 4 at 600 µM on the amplitude of hERG tail currents (same experiment as Figure 1). Figure 3 presents concentration-response relationship. Due to solubility limitations, the IC_{50} for the inhibitory effect of Thymosin beta 4 on hERG potassium current was estimated to be greater than 600 µM.

Tables 3-4 summarize the effect of application of terfenadine, the positive control. Terfenadine at 60 nM produced (mean) 77.6% inhibition of hERG potassium current.

10 Conclusions

The *in vitro* effects of Thymosin beta 4 on ionic currents in voltage-clamped human embryonic kidney cells (HEK293) that stably express the human ether-à-go-go-related gene (hERG) were determined. Two concentrations of Thymosin beta 4 (100 and 600 µM) were tested at near-physiological temperature. Thymosin beta 4 inhibited hERG potassium current by (Mean ± SEM) 1.4 ± 0.5% at 100 µM (n = 5) and 1.2 ± 0.6% at 600 µM (n = 5) versus 0.6 ± 0.3% (n = 3) in vehicle control. Testing was conducted only at these two concentrations because Thymosin beta 4 was insoluble in the assay vehicle at higher dose concentrations. Due to the solubility limitations, the IC_{50} for the inhibitory effect of Thymosin beta 4 on hERG potassium current was not calculated but was estimated to be greater than 600 µM. Under similiar conditions, the positive control (60 nM terfenadine) inhibited hERG potassium current by (Mean ± SEM; n = 3) 77.6 ± 3.7%.

Under conditions of the study, one can conclude that Thymosin beta 4 did not inhibit the hERG potassium channel.

11 Archiving of Materials

The protocol (including amendments and deviations), the raw data collected by ChanTest (including but not limited to electronic electrophysiology records, data collection forms, and worksheets), and the final report shall be archived by ChanTest Corporation following ChanTest SOPs for on-site and off-site storage. ChanTest shall be responsible for the retention of such records for five years (or less, upon Sponsor request) following the study completion date, at which time the Sponsor will be contacted for final disposition.

12 References

Brown AM and Rampe D. (2000). Drug-induced long QT syndrome: is HERG the root of all evil? Pharmaceutical News 7, 15-20.

Kirsch GE, Trepakova ES, Brimecombe JC, Sidach SS, Erickson HD, Kochan MC, Shyjka LM, Lacerda AE, and Brown AM. (2004). Variability in the measurement of

hERG potassium channel inhibition: effects of temperature and stimulus pattern. J Pharmacol Toxicol Methods 50, 93-101.

Redfern WS, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Palethorpe S, Siegl PK, Strang I, Sullivan AT, Wallis R, Camm AJ, Hammond TG. (2003). Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. Cardiovascular Research. 58(1):32-45.

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13 Tables and Figures

13.1 Thymosin beta 4 effect on hERG potassium current

Table 1: Percent Inhibition at each Thymosin beta 4 Concentration

Cell ID (filename)	Concentration (μM)		
	0	100	600
B 01 LMS 101029_0000_sub.abf		2.6%	
D 01 LMS 101029_0000_sub.abf		0.5%	
F 01 LMS 101029_0000_sub.abf		1.5%	
E 01 LMS 101029_0000_sub.abf		2.3%	
G 01 LMS 101029_0001_sub.abf		0.2%	
I 01 LMS 101029_0000_sub.abf			1.1%
K 01 LMS 101029_0000_sub.abf			0.5%
M 01 LMS 101029_0002_sub.abf			0.1%
N 01 LMS 101029_0001_sub.abf			3.7%
P 01 LMS 101029_0000_sub.abf			0.8%
Q 01 LMS 101029_0000_sub.abf	0.0%		
S 01 LMS 101029_0000_sub.abf	0.8%		
T 01 LMS 101029_0000_sub.abf	1.1%		

Table 2: Thymosin beta 4 Summary Statistics

Mean percent inhibition at each Thymosin beta 4 concentration (Mean), standard deviation (SD), standard error of the mean (SEM) and number of cells (N).

Concentration (μM)	Mean	SD	SEM	N
0	0.6%	0.6%	0.3%	3
100	1.4%	1.1%	0.5%	5
600	1.2%	1.4%	0.6%	5

No values were statistically different from vehicle alone.

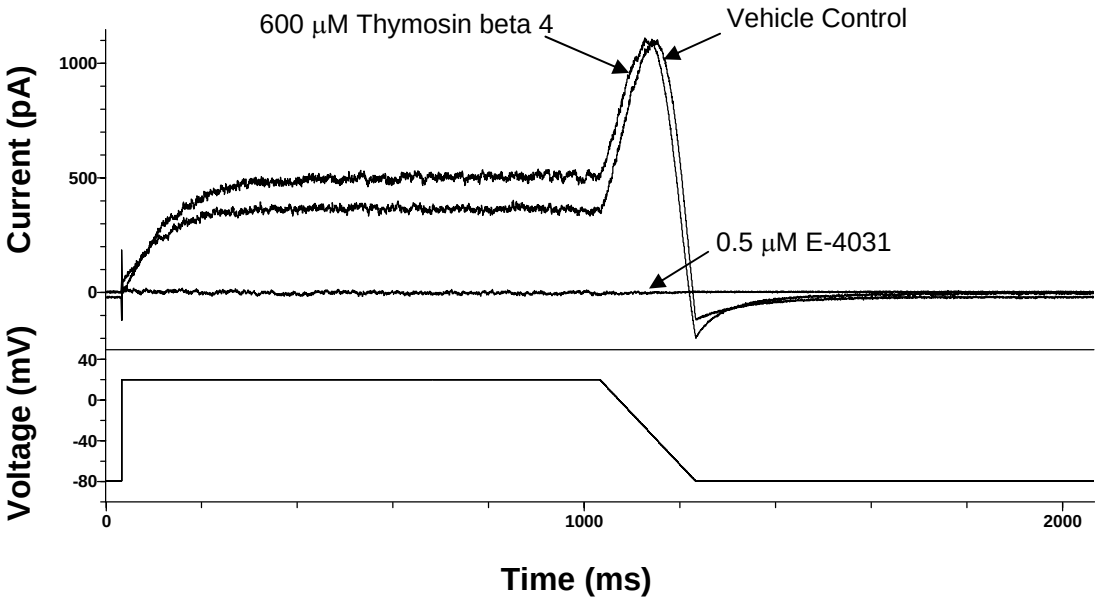

Figure 1: Typical hERG potassium current traces

Upper panel [Current (pA); Time (ms)] shows superimposed, records of hERG potassium currents obtained in a single cell during application of vehicle control, test article and reference substance. hERG potassium currents were evoked by the voltage protocol shown in the lower panel [Voltage (mV)]. Cell ID: K 01 LMS 101029_0000_sub.abf.

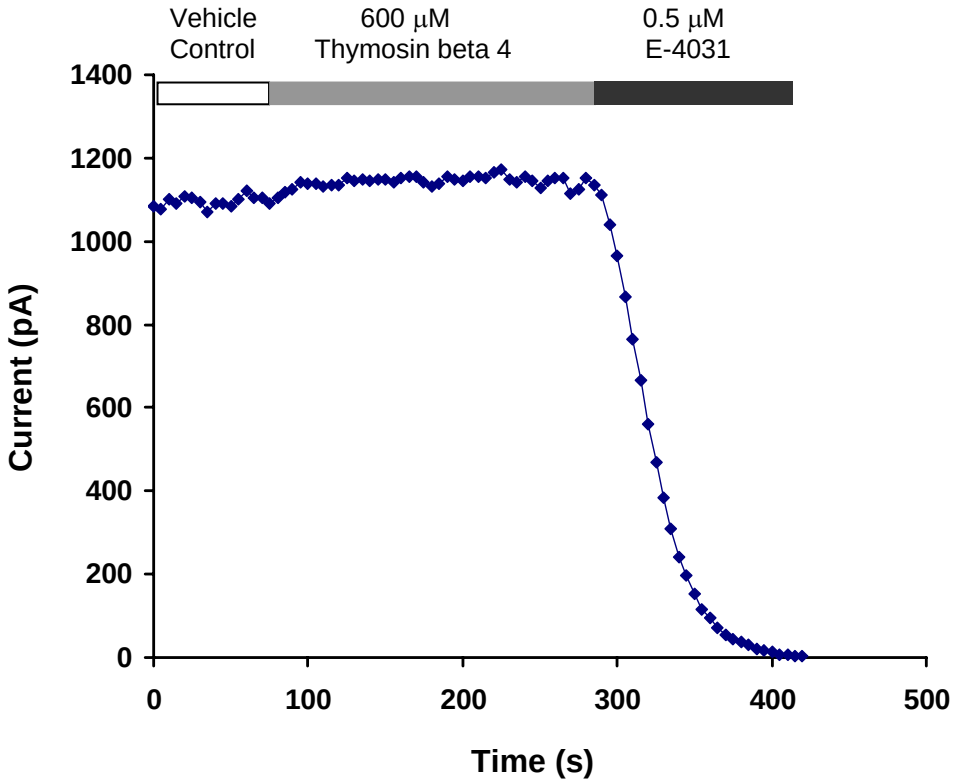

Figure 2: Typical time course of the effect of Thymosin beta 4 on the hERG current

Peak current amplitude during application of vehicle control, test article, and reference substance. The horizontal bars indicate the vehicle control, test article concentration and reference substance. Cell ID: K 01 LMS 101029_0000_sub.abf.

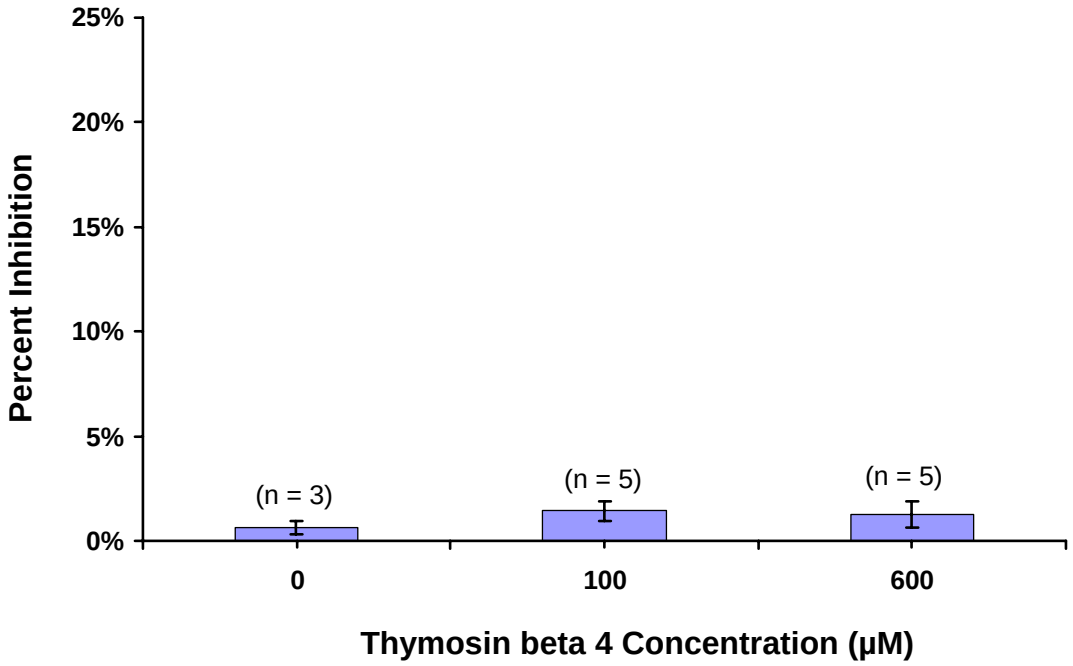

Figure 3: Concentration-response relationship

Percent inhibition of hERG potassium current after application of each concentration of Thymosin beta 4 (Mean ± SEM). The IC_{50} for the inhibitory effect of Thymosin beta 4 on hERG potassium current was estimated to be greater than 600 µM. The number of cells at each concentration is given in parentheses.

13.2 Terfenadine effect on hERG potassium current

Table 3: Percent Inhibition at 60 nM Terfenadine

Cell ID (filename)	Terfenadine Concentration (nM)
	60
K 01 LMS 101028_0000_sub.abf	84.9%
M 01 LMS 101028_0000_sub.abf	75.1%
O 01 LMS 101028_0001_sub.abf	72.8%

Table 4: Terfenadine Summary Statistics

Mean percent inhibition after application of 60 nM terfenadine (Mean), standard error of the mean (SEM) and number of cells (N).

Terfenadine concentration (nM)	Mean	SEM	N
60	77.6%	3.7%	3

Table 5: ChanTest Historical Positive Control Data for Terfenadine (2002 – 2009)

Mean percent inhibition after application of 60 nM terfenadine (Mean), standard deviation (SD), standard error of the mean (SEM) and number of cells (N).

Terfenadine concentration (nM)	Mean	SD	SEM	N
60	80.6%	5.6%	0.1%	2110

14 Appendix A – Protocol and Protocol Amendments

PROTOCOL

Effect of Thymosin beta 4 on Cloned hERG Potassium Channels Expressed in Human Embryonic Kidney Cells

ChanTest Study Number:	100805.SFI
Testing Facility:	ChanTest Corporation 14656 Neo Parkway Cleveland, OH 44128
Sponsor:	RegeneRx Biopharmaceuticals, Inc. 15245 Shady Grove Road Rockvile, MD 20850
Estimated Experiment Start Date:	Week of 18-Oct-2010
Estimated Experiment End Date:	Week of 25-Oct-2010
Estimated draft report to Sponsor Date:	Week of 08-Nov-2010

Note: Due to the nature of the experiments, conditions may arise to delay study completion. These conditions may include, but are not limited to: 1) cell health, 2) toxicity of test article, 3) solubility of test article, and 4) the inhibiting properties of test article. Every effort will be made to stay within the expected timeframe; however, the Sponsor will be informed if any difficulties arise.

Personnel

Testing Facility Study Director	Cynthia Wladyka, BS ChanTest Corporation 14656 Neo Parkway Cleveland, OH 44128 Tel. 216-332-1665 Fax 216-332-1706 Email: cwladyka@chantest.com
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Sponsor Study Monitor	Christian B. Allan, PhD RegeneRx Biopharmaceuticals, Inc. 15245 Shady Grove Road Rockville, MD 20850 Tel. 301-208-9191 Email: callan@regenerx.com

1 Introduction and Objective

The objective of this study is to examine the *in vitro* effects of Thymosin beta 4 on the hERG (human ether-à-go-go-related gene) channel current (a surrogate for I_{Kr}, the rapidly activating, delayed rectifier cardiac potassium current, Redfern et al., 2003). The concentration-response relationship of the effect of Thymosin beta 4 on the hERG potassium channel current will be evaluated at near-physiological temperature in stably transfected mammalian cells that express the hERG gene.

Prior to analysis of the dose formulations, a method validation will be conducted to verify that the analytical test method is suitable for determination of the selected Thymosin beta 4 concentrations in hERG assay buffer. The method of analysis to be used will be documented in ChanTest analytical procedure 100805.SFI.XX (where "XX" denotes the version number).

2 Regulatory Compliance

The study will be conducted in compliance with the most recent version of the Food and Drug Administration (FDA) Good Laboratory Practices Regulations (21 CFR Part 58), with the following exceptions:

- The positive control article is a marketed product that shall be characterized according to its label. However, the characterization is considered non-GLP and stability of the control article will not be demonstrated.
- The positive control article formulation will not be analyzed for stability, homogeneity, or concentration (its potency will be demonstrated by the comparison of results with ChanTest historical data).

The study will comply with the ICH Guidance for Industry (July 2001), "S7A Safety Pharmacology Studies for Human Pharmaceuticals" and the ICH Guidance for Industry (October 2005), "S7B Nonclinical Evaluation of the Potential for Delayed Repolarization (QT Interval Prolongation) by Human Pharmaceuticals." The study will be conducted in accordance with published procedures (Kirsch et al., 2004) and with the Standard Operating Procedures (SOPs) of ChanTest Corporation.

2.1 Sponsor Responsibilities

The Sponsor will provide documentation on the strength, purity, composition, physical properties, stability, and other pertinent information on the bulk test article in the form of a Certificate or Record of Analysis for inclusion in the final report. The Sponsor will provide a Certificate of Stability or other documentation for the bulk test article for inclusion in the final report. The sponsor is responsible for the amount of test article provided.

2.2 Protocol Amendments and Deviations

Any modifications to or deviations from the protocol will be documented, authorized and evaluated by the Study Director, and included in the final report as an appendix.

2.3 Quality Assurance Unit

The Quality Assurance Unit (QAU) at ChanTest will monitor the study described in this protocol and conduct appropriate phase inspections of the study according to ChanTest SOP(s). Any observations from QAU inspections will be reported to the Study Director and Testing Facility Management. Inspection type and date will be documented in the QA statement of the final report.

Observations related to the analytical portion of the study will be reported to the Dose Analysis Contributing Scientist, the Study Director and Testing Facility Management. Inspection type and date will be documented in the QA statement of the analytical report.

3 Test and Control Articles

3.1 Test Article

Test Article ID:	Thymosin beta 4
Alternate Test Article ID:	TB4
Lot Number:	FTHYB40602A
Form:	Acetate salt
Molecular Weight:	4963.55 g/mol (free peptide)
Peptide Purity:	98.4% [1]
Peptide Content:	93.1%
Storage Conditions (bulk):	Frozen (set to maintain -10 to -30 °C); under argon
Carrier:	Sterile water for injection, USP (sterile water)

[1] Formulations will not be corrected for peptide purity. Only a correction for peptide content will be used.

3.1.1 Rationale for Concentration Selection

Concentrations will be chosen to achieve a range of approximately 10% to 90% inhibition of hERG current, within the specifications of Section 6.3 (Experimental Procedures) of this protocol and the limits imposed by the physiochemical effects of the test article (e.g. solubility or cytotoxicity).

3.1.2 Test Article Disposition

Bulk test article, stock solutions and analytical samples remaining at the end of the study will be disposed after issuance of the final report and records of disposition will be filed with the study data. These items may be disposed prior to issuance of the final report upon Sponsor request.

3.1.3 Test Article Carrier

Name:	Sterile water for injection, USP
Source:	To be documented in the final report
Lot Number:	To be documented in the final report
Molecular Weight:	N/A
Storage Conditions:	To be documented in the final report
Rationale for Selection:	The test article is water-soluble.

3.2 Positive Control Article and Reference Substance Carrier

Name:	DMSO
Source:	Sigma-Aldrich
Lot Number:	To be documented in the final report
Molecular Weight:	To be documented in the final report
Storage Conditions:	To be documented in the final report
Rationale for Selection:	Previous results have shown that 0.3% DMSO does not affect channel current (data on file at ChanTest Corporation).

3.3 Vehicle Control

The vehicle will consist of a HEPES-buffered physiological saline (HB-PS) solution (composition in mM): NaCl, 137; KCl, 4.0; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 10; Glucose, 10; pH adjusted to 7.4 with NaOH (refrigerated until use). Chemicals used in vehicle preparation will be purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted and will be of ACS reagent grade purity or higher.

Name:	HB-PS + 1.0% sterile water (for test article) HB-PS + 0.3% DMSO (for positive control article and reference substance)
Source:	ChanTest Corporation
HB-PS Batch Number:	To be documented in the final report
Storage Conditions:	To be documented in the final report
Rationale for Selection:	HB-PS provides the appropriate ionic composition for *in vitro* recording.

3.4 Positive Control Article

Name:	Terfenadine
Source:	Sigma-Aldrich
Lot Number:	To be documented in the final report
Molecular Weight:	To be documented in the final report
Purity (%):	To be documented in the final report
Storage Conditions (bulk	To be documented in the final report

compound):

Rationale for Selection:	Previous results have shown that 60 nM terfenadine inhibits hERG potassium current by approximately 80% (data on file at ChanTest Corporation).

3.5 Reference Substance

Name:	E-4031
Source:	Sigma-Aldrich
Lot Number:	To be documented in the final report
Molecular Weight:	To be documented in the final report
Purity (%):	To be documented in the final report
Storage Conditions:	To be documented in the final report
Rationale for Selection:	E-4031 selectively inhibits hERG potassium current with IC_{50} = 12 nM (data on file at ChanTest Corporation). A supramaximal inhibiting concentration of 500 nM will be used to eliminate the contribution of non-hERG currents.

3.6 hERG Assay Formulations

3.6.1 Positive Control and Reference Substance Formulations

Stock solutions of the positive control and reference substance will be prepared in dimethyl sulfoxide (DMSO), aliquoted for individual use, stored frozen, and used within one month. Final positive control and reference substance concentrations will be prepared fresh daily by diluting stock solutions in vehicle (final DMSO concentration, 0.3% v/v). Positive control article and reference substance formulations are solutions and, therefore, homogeneous, by definition. Homogeneity, concentration, and stability analyses will not be performed. Their potency for hERG inhibition will be demonstrated by the comparison of results with ChanTest historical data.

3.6.2 Test Article Formulations

Stock solutions of the test article will be prepared in sterile water and stored at the recommended conditions based on stock stability determined during method validation. Test article concentrations will be prepared fresh daily by diluting the stock solutions in vehicle (final sterile water concentration, 1.0% v/v). To avoid precipitation near the solubility limit, test article formulations will be prepared at room temperature (15 - 30° C) with sonication if necessary (the time required will be noted in the raw data). A description of the method for preparing test article formulations will be included in the final report.

3.6.3 Preparation of Vehicle and Formulation Samples

Vehicle control samples (blank, 4 samples, 1 mL each, accurately measured directly into HPLC vials) will be taken from the vehicle reservoir at the beginning of testing.

Samples for homogeneity determination (4 samples, 1 mL each, accurately measured directly into HPLC vials) will be aliquoted from the top, middle, and bottom of the test article formulation reservoir after preparation on the first day of testing each concentration.

Representative test article formulation samples for concentration verification will be collected on each day of testing. Samples of each test concentration (4 samples, 1 mL each, accurately measured directly into HPLC vials) will be aliquoted from the outflow of the perfusion apparatus.

Additional samples may be taken at the discretion of the study director.

The above formulation samples will be stored after collection at recommended conditions based upon test article stability determined during method validation and will be stored at these conditions until analytical testing. For analysis, two samples from each condition will be transferred to the analytical chemistry unit while the remaining two samples from each condition will be retained in storage as backups.

3.7 Analysis of Formulation Samples

Formulation samples will be analyzed by a validated ChanTest Analytical Test Method. Concentration verification samples within ± 15.0% RE will be considered acceptable. Homogeneity samples that are ≤ 15.0% CV will be considered acceptable. Re-testing of out-of-specification samples will be conducted in compliance with ChanTest SOP.

An analytical report assessing concentration verification and homogeneity of the test article dose formulation samples will be written and included in the final report.

3.8 Analytical Solutions

3.8.1 Preparation of Matrix Blank (Control)

Matrix blank solution(s) consisting of HB-PS + 1.0% sterile water will be prepared daily and stored at room temperature during use.

3.8.2 Preparation of Test Article Primary Stock Solutions

Two primary stocks (independent weighings, prepared in to separate vials) of the test article will be prepared in sterile water. One stock will be used for the preparation of the calibration standards; the second primary stock will be used for the preparation of the QC samples.

If test article supply permits, a third primary stock will be made to determine formulation stability during method validation. If test article supply is not sufficient for preparation of three primary stocks then the primary stock used to prepare QC samples will be used for preparation of formulation stability samples.

Unless stability has been demonstrated, all other stock solutions will be disposed of at the end of the day of use. Disposition of stock solutions will be documented and filed in the study file.

3.8.3 Preparation of Test Article Working Stock Solutions

Working stock solutions will be prepared from stock solutions by serial dilution. All working stock solutions will be disposed of at the end of the day of use. Disposition of stock solutions will be documented and filed in the study file.

3.8.4 Preparation of Calibration Standards

Appropriate calibration standards (to be documented in the final report), as determined in the method assessment pre-study Quantitation, will be prepared fresh daily by diluting the calibration working stock solution with HB-PS and sterile water. The final matrix of all calibration standards will be HB-PS + 1.0% sterile water.

3.8.5 Preparation of QC Samples for Accuracy and Precision

Replicates of each QC sample concentration level will be prepared fresh daily by diluting the QC working stock solutions with HB-PS and sterile water. The final matrix of all QC samples will be HB-PS + 1.0% sterile water. The ULOQ QC will be prepared directly from the QC primary stock solution.

QC samples will be prepared at concentrations determined in the pre-study:

- Low QC – concentration within 3 times the lowest calibration standard (LLOQ),
- Mid QC – concentration near the middle calibration standard,
- High QC – concentration near the upper boundary of the calibration curve,
- ULOQ QC - If the concentration of actual test article formulations is expected to be greater than the High QC sample, prepare 3 to 5 replicate ULOQ QC samples at a concentration sufficient to cover the range of the anticipated test article formulations (for example at 110 to 120% of the nominal concentration of the highest test article formulation.). Dilute each ULOQ QC sample with HB-PS + 0.3% DMSO to fit within the range of the calibration curve.

3.8.6 Preparation of Samples for Formulation Stability Evaluation

Formulation stability will be determined at room temperature (set to maintain 15 – 30 °C) and under refrigerated conditions (set to maintain 2 – 8 °C). Samples will be analyzed at

time intervals of approximately 6 and 28 hours (at room temperature) and 28 hours (refrigerated).

Low and High formulation stability QC samples will be prepared as described in Section 3.8.5. Three (3) replicates of each QC stability sample concentration level for each time period/storage condition combination to be tested, including Time 0, will be prepared by diluting the QC stability working stock solutions with HB-PS + 1.0% sterile water. The final matrix of all formulation stability QC samples will be HB-PS + 1.0% sterile water. The ULOQ stability samples (if appropriate) will be prepared directly from the formulation stock / stability primary stock solution.

3.8.7 Preparation of Samples for Stock Stability Evaluation

The stability of the test article prepared in sterile water will be evaluated during Method Validation. The samples will be stored at room temperature (15 – 30 °C) for approximately 7 hours followed by approximately 23 hours. After each storage interval, the stocks will be diluted to a concentration within the calibration curve (in triplicate).

4 Method Validation

4.1 Overview

The objective of the method validation portion of this study is to verify that ChanTest Analytical Test Method 100805.SFI.XX (where 'XX" denotes the version number) is suitable for determination of Thymosin beta 4 concentration in hERG assay buffer by high performance liquid chromatography (HPLC) with UV detection. The method of analysis to be used will be documented in ChanTest analytical procedure 100805.SFI.XX. The analytical test method will be developed based on test article information supplied by the Sponsor and other information gathered by the analysts at ChanTest Corporation. The method will be conducted by high performance liquid chromatography (HPLC) and will be validated prior to use for determination of concentration verification and homogeneity of the hERG dose formulation samples.

The analytical method will be validated to establish stability, linearity, accuracy and intra-assay precision (repeatability), quantifiable range, and lack of interference in the chromatographic region of interest. Additional tests may be performed at the discretion of the Study Director or upon sponsor request and will be documented in the study file and, if appropriate, included in the final report.

The following will be analyzed during the method validation:

- Two matrix blank controls to establish chromatographic non-interference.
- Six to eight non-zero calibration standards to establish calibration range linearity, and back-calculated accuracy of calibration standards.

- Quality Control (QC) samples (Day 0) – five replicates at three concentrations to establish accuracy and repeatability (Intra-assay Precision). Five ULOQ QC samples (if appropriate) diluted to fit within the range of the calibration curve.
- Quality Control (QC) samples for ~28 hour formulation and ~30 hour stock stability analysis – three to five replicates at three concentrations to establish accuracy and repeatability. Three to five ULOQ QC samples (if appropriate) diluted to fit within the range of the calibration curve.
- Formulation Stability samples – tested to establish stability of test article in the matrix solution. Three replicates at two concentrations equivalent to the Low and High concentrations will be prepared for each non-zero time period and storage condition. Three ULOQ samples (if appropriate) will be prepared and aliquoted un-diluted for each non-zero time period and storage condition.
- Stability of stock solution sample(s) – for each non-zero time period and storage condition(s) tested to establish stability of test article in sterile water.

4.2 Calculations

Peak area of Thymosin beta 4 will be measured using Empower Software manufactured by Waters Corp. The equation of the line and the coefficient of determination (R^2) will be calculated by the software.

Descriptive statistics including arithmetic mean (average) and standard deviation, % CV, % RE and % Change will be calculated using Microsoft Excel, version 2003.

% Relative Error (% RE) = 100 * (measured concentration – nominal concentration)/nominal concentration

% Coefficient of Variance (% CV) = 100 * (std. dev. of concentration of replicates/mean concentration of replicates)

% Change = 100 * (mean concentration at T*n* – mean concentration at T0)/mean concentration at T0

Where T*n* = time of the non-zero time point

4.3 Acceptance Criteria

Test	Acceptance Criteria
Chromatographic Non-Interference	There are no interfering peaks in the matrix blank within the retention time window of the analyte.
Linearity	Coefficient of Determination, $R^2 \geq 0.990$
Accuracy (Calibration Standards)	% RE ± 15.0%
Accuracy (QC Samples)	% RE ± 15.0%
Repeatability (QC Samples)	% CV ≤ 15.0%
Range	The range will be determined based on the results from the linearity, accuracy, and repeatability sections.
Accuracy/Precision at T0 (QC Samples)	% RE ± 15.0%; % CV ≤ 15.0%
Stability (QC Samples)	% Change ± 15.0% of Time 0 Stability QC samples % CV ≤ 15.0%
Stability (Stock Solution)	% RE ± 15.0%; % CV ≤ 15.0%

4.4 System Suitability

The middle calibration standard will be reinjected periodically during analysis to demonstrate system suitability. The % RE for the back-calculated concentration of the standard checks should be ± 15.0% of the back calculated value of the middle calibration standards throughout the analysis run.

4.5 Linearity

Calibration standards will be used to determine the linear relationship between the analyte concentration and instrument response, as well as the range over which the response is linear. The linearity of the method will be determined based on the acceptable R^2 value of the calibration curve and accuracy of the back-calculated concentrations of the calibration standards. Linearity will be evaluated as follows:

1. The calibration standards will be injected according to the test method.
2. The equation of the line and the coefficient of determination, R^2 will be calculated for the resulting calibration curve.
3. The resulting coefficient of determination will be compared to that stated in Section 4.3 (Acceptance Criteria).

4.6 Chromatographic Non-Interference

Chromatography of the blank matrix control(s) will be compared to that of the lowest calibration standard to determine if the sample matrix produces any interfering peaks in the window of interest (retention time window) for the analyte.

4.7 Accuracy

The accuracy, or closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found, will be evaluated by determination of the accuracy of the calibration standards and the accuracy of the QC samples.

4.7.1 Accuracy of Calibration Standards

The accuracy of the calibration standards will be determined based on the % RE values attained for each of the back-calculated measured concentrations. For calibration standard concentration, the % RE will be determined and compared to the acceptance criteria.

4.7.2 Accuracy of QC Samples

The accuracy of the Quality Control samples will be determined based on the % RE values of the QC samples. For each QC sample concentration, the % RE of the mean value of the replicate samples will be determined and compared to the acceptance criteria.

4.8 Repeatability (Intra-assay Precision)

The intra-assay precision is the repeatability demonstrated within a given analytical run. For each concentration level, the % CV of the replicate QC samples used for the accuracy will be calculated and compared to the acceptance criteria.

4.9 Range

The range over which the analytical method is considered acceptable is determined based on the results from the linearity, accuracy and repeatability sections.

4.10 Test Article Formulation Stability

Formulation stability is determined based on the % Change between the mean of the replicate for each concentration/time period/storage condition combination and the mean of the Time 0 stability samples for that concentration.

Additional backup stability samples will be prepared for each storage condition and maintained for a period greater than 28 hours (to be analyzed in the event that dose formulation for the hERG assay is conducted more than 28 hours after analytical sample collection).

Formulation stability samples and backups will be placed in the appropriate storage conditions and the time, date, and location of storage will be documented. The temperature ranges for each storage condition will be documented in the study file.

1. At the beginning of Day 0, triplicate solutions of each of the low and high concentration QC samples and three ULOQ samples (un-diluted) if appropriate, will be prepared and aliquoted for each non-zero time period/storage condition combination to be tested.
2. The formulation stability samples for each time interval will be placed in the appropriate storage conditions and the time, date, and location of storage will be documented.
3. The triplicate Time 0 aliquots of each of the low and high concentration QC samples will be analyzed. The ULOQ samples, if appropriate, will be diluted to fall within the range of the calibration curve for the baseline Time 0 analysis. The Time 0 QC samples will be analyzed according to the test method.
4. After the appropriate storage intervals, the designated samples will be removed from storage (the ULOQ samples, if appropriate, will be diluted to fit with the range of the calibration curve) and will be analyzed with freshly prepared calibration standards and QC samples according to the test method. If stock solution stability has not been established, calibration standards and QC samples must be prepared from a freshly prepared primary stock. The time and date that the samples were removed from storage will be documented.

   Note: Day 0, time period formulation stability samples may be added to the Day 0 formulation stability baseline sample set if the HPLC run is still in progress, thus not requiring preparation of fresh calibration standards and QC samples.
5. At each non-zero time interval/storage condition combination, the mean concentration measured for replicate samples of each QC sample concentration will be determined and the % Change of the mean of the non-zero time point from the mean concentration at T0 will be calculated.
6. The % CV and % Change will be compared with the acceptance criteria above.

4.11 Test Article Stock Solution Stability

Stability of the test article stock solution(s) in sterile water will be determined at approximately 7 hours, followed by an additional approximately 23 hours of room temperature storage (set to maintain 15 - 30° C).

1. The Day 0 stock solution will be allowed to stand at the appropriate temperature for approximately 7 hours.
2. After approximately 7 hours, the stock solution will be removed from storage. The time and date that the stock(s) were removed from storage will be documented.

3. Triplicate dilutions of the primary stock will be prepared to achieve the working stock concentration resulting in a final matrix of HB-PS + 1.0% sterile water. From each working stock, a concentration within the calibration curve will be diluted in singlet.
4. The triplicate stock solution dilutions will be analyzed with the formulation stability QC sample run which should still be in progress.
5. The stock solution(s) will be placed in to storage and the time, date, and location of storage will be documented.
6. The concentrations of the samples will be compared to that of the nominal concentration of the stock solution. The % CV for each sample and the % RE of the mean will be calculated.
7. After approximately 30 hours the Day 0 stock solution(s) will be removed from storage. The time and date that the stock(s) were removed from storage will be documented.
8. Triplicate dilutions of the working stock concentration will be prepared to result in a final matrix of HB-PS + 1.0% sterile water. Each working stock will be diluted to a concentration within the calibration curve.
9. The stock solution(s) will be placed back in storage and the time, date, and location of storage will be documented.
10. The triplicate stock solution dilutions will be analyzed with the 28-hour formulation stability QC sample run which should still be in progress.
11. The concentrations of the samples will be compared to that of the stock solution's nominal concentration. The % CV of each sample and the % RE of the mean for each sample will be calculated.
12. The % CV and % RE will be compared with the acceptance criteria above.
13. If information on longer term stability of the stock solution(s) is desirable, the stock solution(s) may be removed from storage after extended storage and analyzed versus freshly prepared stock solution(s) in a similar manner as that described above.

5 hERG Test System

Cells will be maintained in tissue culture incubators per ChanTest SOP. Stocks will be maintained in cryogenic storage. Cells used for electrophysiology will be plated in plastic culture dishes. Each culture dish will be identified by a notation of clone identity number, passage number, and date. These data will be retained in the study file.

5.1 Rationale for Selection of Ion Channel and Expression Systems

The cardiac potassium channel, hERG, is responsible for a rapid delayed rectifier current (I_{Kr}) in human ventricles. This channel has been selected for evaluation because inhibition of I_{Kr} is the most common cause of cardiac action potential prolongation by

non-cardiac drugs (Brown and Rampe, 2000; Weirich and Antoni, 1998; Yap and Camm, 1999). Increased action potential duration causes prolongation of the QT interval and has been associated with a dangerous ventricular arrhythmia, *torsade de pointes* (Brown and Rampe, 2000). In this assay, hERG potassium channels are expressed in a human embryonic kidney (HEK293) cell line that lacks endogenous I_{Kr}.

5.2 HEK/hERG

Organism:	*Homo sapiens*
Designation:	293
Tissue:	Kidney; Transformed with adenovirus 5 DNA; Transfected with human-ether-a-go-go cDNA
Morphology:	Epithelial
Age/Stage:	Embryo
Source-Strain:	ATCC, Manassas, VA
Source-Sub Strain:	ChanTest Corporation, Cleveland, OH

5.3 Cell Culture Procedures

HEK293 cells have been stably transfected with hERG cDNA. Stable transfectants have been selected by coexpression with the G418-resistance gene incorporated into the expression plasmid. Selection pressure will be maintained by including G418 in the culture medium. Cells will be cultured in Dulbecco's Modified Eagle Medium / Nutrient Mixture F-12 (D-MEM/F-12) supplemented with 10% fetal bovine serum, 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate and 500 μg/mL G418.

6 Test Method

6.1 Treatment Groups

All experiments will be performed at near-physiological temperature (33 to 35 °C). Each cell will act as its own control.

6.1.1 Positive Control Group

The positive control will be applied to at least two (2) cells ($n \geq 2$). The performance of the test system will be considered acceptable if application of the positive control elicits a response within ± 2 standard deviations from the historic average response obtained by ChanTest. Results outside this range will require an out-of-specification investigation before proceeding with the concentration-response experiments.

6.1.2 Concentration-Response Group

Experiments will be performed in two phases as outlined in Section 6.3 (Experimental Procedures). Four (4) concentrations will be selected to evaluate the concentration-response relationship based on the outcome of the initial concentration range

determination. If appropriate, fewer than four (4) concentrations may be selected. The concentrations used to evaluate the concentration-response relationship will be documented in a protocol amendment. Each concentration will be tested in at least three (3) cells ($n \geq 3$).

6.1.3 Vehicle Control Group

Vehicle control solution will be applied to at least three (3) cells ($n \geq 3$). Duration of vehicle application will be at least as long as the longest test article application in the study ($\leq$ 12 minutes).

6.2 Electrophysiological Procedures

Cells will be superfused with vehicle control solution. Micropipette solution for whole cell patch clamp recordings will be composed of (mM): potassium aspartate, 130; $MgCl_2$, 5; EGTA, 5; ATP, 4; HEPES, 10; pH adjusted to 7.2 with KOH. Micropipette solution will be prepared in batches, aliquoted, stored frozen, and a fresh aliquot thawed each day. The recording will be performed at a temperature of 33 to 35 °C using a combination of in-line solution pre-heater, chamber heater, and feedback temperature controller. Temperature will be measured using a thermistor probe in the recording chamber. Micropipettes for patch clamp recording will be made from glass capillary tubing using a P-97 micropipette puller (Sutter Instruments, Novato, CA). A commercial patch clamp amplifier will be used for whole cell recordings. Before digitization, current records will be low-pass filtered at one-fifth of the sampling frequency.

Cells stably expressing hERG will be held at -80 mV. Onset and steady state inhibition of hERG potassium current due to Thymosin beta 4 will be measured using a pulse pattern with fixed amplitudes (conditioning prepulse: +20 mV for 1 s; repolarizing test ramp to -80 mV (-0.5 V/s) repeated at 5 s intervals. Each recording will end with a final application of a supramaximal concentration of the reference substance (E-4031, 500 nM), to assess the contribution of endogenous currents. The remaining uninhibited current will be subtracted off-line digitally from the data to determine the potency of the test substance for hERG inhibition.

6.3 Experimental Procedures

As outlined below, Phase I of the study will identify the approximate concentration range, and Phase II will determine the concentration-response relationship. All experiments will be conducted within the limits imposed by the physiochemical effects of the test article (e.g. solubility or cytotoxicity).

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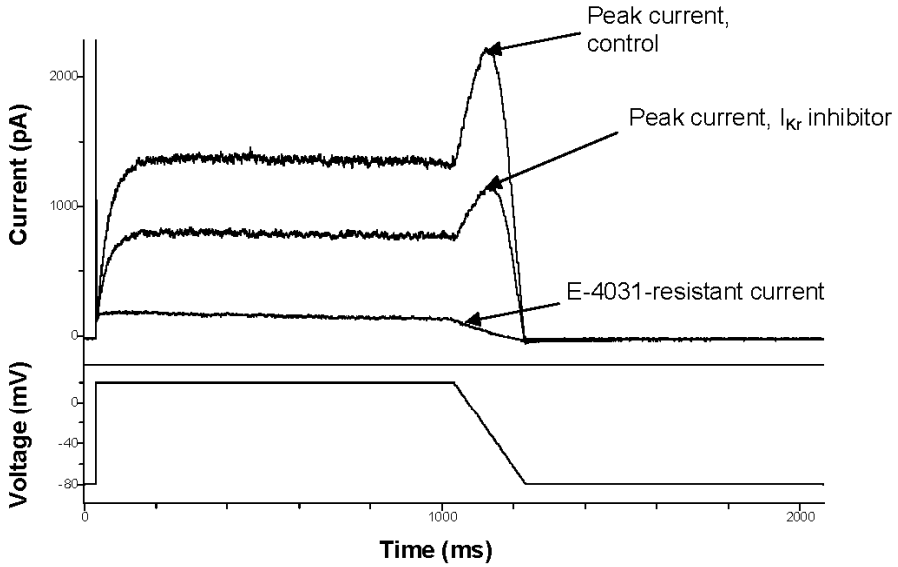

Figure 1: Typical hERG potassium current tracings recorded at 33 °C.
Superimposed records in control after application of an inhibitor, and after 500 nM E-4031 application. Lower panel shows voltage stimulus (depolarizing prepulse, +20 mV; repolarizing test ramp, +20 mV to –80 mV at –0.5 V/s) repeated at 5 s intervals from a holding potential of –80 mV.

6.3.1 Phase I Concentration Range

Thymosin beta 4 at 100 μM (or highest achievable concentration) will be applied to at least 1 to 3 cells and the effect on hERG potassium current amplitude will be monitored. If a steady state inhibitory response ≥ 20% is observed, the IC_{50} will be estimated and concentrations will be selected to span the range of approximately 10-90% inhibition in half-log increments to evaluate the concentration-response relationship (Phase II). If the concentration-response relationship is not well-defined by half-log increment concentrations of Thymosin beta 4, other (not half-log) concentrations may be tested at the discretion of the Study Director. If a steady state cannot be reached within 12 minutes, the response at 12 minutes will be substituted for the steady state value and a notation made in the final report. If the inhibitory response is less than 20%, the process will be repeated (n ≥ 3 cells) at 600 μM or at a concentration selected by the Study Director. If the inhibitory response remains below 20%, then the study is complete. Otherwise, the concentration response relationship will be determined (Phase II).

6.3.2 Phase II Concentration-Response

One or more test article concentrations will be applied sequentially (without washout between test article concentrations) in ascending order, to each cell (n ≥ 3 where

n = number of observations). Peak current will be measured during the test ramp. A steady state will be maintained for at least 20 s before applying test article or positive control. Peak current will be measured until a new steady state is achieved or 12 minutes of exposure time have elapsed.

6.4 Electrophysiological Data Analysis

Data will be stored on the ChanTest computer network (and backed-up nightly) for off-line analysis. Data acquisition and analyses will be performed using the suite of pCLAMP (Version 8.2) programs (MDS-AT, Sunnyvale, CA), and will be reviewed by the Study Director. Percent inhibition at each concentration in the test group will be compared with the vehicle control group using one-way ANOVA followed by Dunnett's multiple comparison test (JMP Version 5.0.1, SAS Institute, Cary, NC). Significant inhibition will be defined at the level of $P<0.05$.

Steady state is defined by the limiting constant rate of change with time (linear time dependence). The steady state before and after test article application will be used to calculate the percentage of current inhibited at each concentration. Concentration-response data will be fit to an equation of the form:

$$\% \text{ Inhibition} = \{1-1/[1+([\text{Test}]/IC_{50})^{N}]\}*100$$

Where [Test] is the test article concentration, IC_{50} is the test article concentration at half-maximal inhibition, N is the Hill coefficient, and % Inhibition is the percentage of current inhibited at each test article concentration. Nonlinear least squares fits will be solved with the Solver add-in for Excel 2000, or later (Microsoft, WA). The IC_{50} will be calculated if the test article produces greater than 50% inhibition at the highest concentration. If the inhibitory response is less than 50% the Sponsor Study Monitor will be notified, and IC_{50} will not be determined pending consultation with the Study Monitor.

7 Reporting

During the course of the study, brief progress reports will be made available to the Sponsor upon request.

7.1 Analytical Report

The Dose Analysis Contributing Scientist shall submit to the Study Director an audited draft report containing a summary of the methods used during the conduct of the validation, the results of validation testing to provide assurance that the Test Method is suitable for its intended use, and a summary of the results of the formulation analysis.

The final analytical report will be signed by the Dose Analysis Contributing Scientist and shall include a statement signed by the QAU. This report will be appended to the hERG assay report.

7.2 Draft Report

An audited draft report of this study will be submitted to the Sponsor after review by the Study Director and QAU. The Sponsor shall submit comments on the draft report to the Study Director in writing. ChanTest will issue a final report within thirty (30) days following receipt of the Sponsor's comments. If no response to the draft report is received from the Sponsor within thirty (30) days, then ChanTest may issue the final report.

7.3 Final Report

The final report will include the effects of the test article on hERG potassium channel current and a summary of the analytical testing results. The Analytical Report will be included as an appendix. The report also will include a compliance statement and a statement of quality assurance. The signed, final report shall be submitted electronically (PDF file). An unbound hardcopy and/or an electronic version on CD-ROM will be submitted upon request.

The Study Director will issue a final report within 60 days of receiving the signed analytical report and the bulk test article characterization and stability documentation. A final report must be issued no later than two years after the study initiation date. If necessary, the final report will be issued with any necessary compliance exceptions without sponsor approval.

8 Archiving of Materials

The protocol (including amendments and deviations), the raw data collected by ChanTest (including but not limited to electronic electrophysiology records, data collection forms, and worksheets), and the final report shall be archived by ChanTest Corporation following ChanTest SOPs for on-site and off-site storage. ChanTest shall be responsible for the retention of such records for five years (or less, upon Sponsor request) following the study completion date, at which time the Sponsor will be contacted for final disposition.

9 Protocol Distribution

ChanTest

1. Original – Study File
2. Copy – QAU
3. Copy – Study Director
4. Copy – Dose Analysis Contributing Scientist
5. Copy – Staff Scientist

Additional signed copies of the final protocol will be provided to employees as required to complete the experimental and report writing phases of the study.

Sponsor

Copy

10 References

Brown AM and Rampe D. (2000). Drug-induced long QT syndrome: is HERG the root of all evil? Pharmaceutical News *7*, 15-20.

Kirsch GE, Trepakova ES, Brimecombe JC, Sidach SS, Erickson HD, Kochan MC, Shyjka LM, Lacerda AE, Brown AM. (2004). Variability in the measurement of hERG potassium channel inhibition: effects of temperature and stimulus pattern. J Pharmacol Toxicol Methods *50*, 93-101.

Redfern WS, Carlsson L, Davis AS, Lynch WG, MacKenzie I, Palethorpe S, Siegl PK, Strang I, Sullivan AT, Wallis R, Camm AJ, Hammond TG. (2003). Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. Cardiovascular Research. 58(1):32-45.

Weirich J and Antoni H. (1998). Rate-dependence of antiarrhythmic and proarrhythmic properties of class I and class III antiarrhythmic drugs. Basic Res Cardiol *93 Suppl 1*, 125-132.

Yap YG and Camm AJ. (1999). Arrhythmogenic mechanisms of non-sedating antihistamines. Clin Exp Allergy *29 Suppl 3*, 174-181.

11 Protocol Approval

On behalf of RegeneRx Biopharmaceuticals, Inc.,

Christian B. Allan, PhD
Study Monitor

15 Oct 2010
Date

On behalf of ChanTest Corporation,

Jessica Brimecombe, PhD
Testing Facility Management

15-Oct-2010
Date

William Giroski, BS
Dose Analysis Contributing Scientist

15-Oct-2010
Date

Cynthia Wladyka, BS
Study Director

15-Oct-2010
Date

Protocol Amendment No. 1

Study Title: Effect of Thymosin beta 4 on Cloned hERG Potassium Channels Expressed in Human Embryonic Kidney Cells

Modification #1

Modification to the Final Protocol
Original: Reference: Page 4, Section 3.1 Test Article
Lot Number: FTHYB40602A

Change:
Lot Number: FTHYB40602B

Reason for Change
Due to a typographical error, the last character in the lot number was incorrect.

Impact Statement
This change will not impact the integrity of the study. The lot number in the protocol is now updated to be cohesive with the test article vial and the other test article documents.

Accepted for ChanTest Corporation:

18-Oct-2010

Cynthia Wladyka, BS — Date
Study Director

Protocol Amendment No. 2

Study Title: Effect of Thymosin beta 4 on Cloned hERG Potassium Channels Expressed in Human Embryonic Kidney Cells

Modification #1

Modification to the Final Protocol

Original: Reference: Page 15-16, Section 6.1.2 Concentration-Response Group

The concentrations used to evaluate the concentration-response relationship will be documented in a protocol amendment.

Change:

Per the study protocol, initial testing was conducted at a nominal concentration of 100 µM. Based on the minimal hERG inhibition observed at this concentration, an additional nominal concentration, 600 µM (also per the study protocol), was tested to evaluate the concentration-response relationship.

Reason for Change

This change is to document the test concentrations.

Impact Statement

This change will not impact the integrity of the study. The test concentrations are now documented.

Accepted for ChanTest Corporation:

02-Nov-2010

Cynthia Wladyka, BS — Date
Study Director

Protocol Amendment No. 3

Study Title: Effect of Thymosin beta 4 on Cloned hERG Potassium Channels Expressed in Human Embryonic Kidney Cells

Modification #1

Modification to the Final Protocol
Original: Reference: Page 1, Sponsor

RegeneRx Biopharmaceuticals, Inc.
15245 Shady Grove Road
Rockvile, MD 20850

Change:

RegeneRx Biopharmaceuticals, Inc.
15245 Shady Grove Road
Rockville, MD 20850

Reason for Change
This change is to correct a typographical error in the city of location for the Sponsor.

Impact Statement
This change will not impact the integrity of the study.

Accepted for ChanTest Corporation:

17-Feb-2011

Cynthia Wladyka, BS — Date
Study Director

15 Appendix B – Test Article Certificate of Analysis and Retest Date Extension

BACHEM OVER 30 YEARS OF COMMITMENT TO THE SCIENTIFIC COMMUNITY

RETEST DATE EXTENSION

PRODUCT:	Thymosin β4
LOT NUMBER:	FTHYB40602B
NEW RETEST DATE:	July 2011
PEPTIDE PURITY:	98.4% (See attached HPLC trace)
PURITY RETESTED:	July 2010

Bassem A. Zeid — 07/22/10
QUALITY CONTROL DEPARTMENT — DATE

Karen Laureco — 07/22/10
QUALITY ASSURANCE DEPARTMENT — DATE

Retest date extensions apply only to bulk dry powder materials stored at recommended storage conditions. Extensions are based upon results from tests performed on materials stored at Bachem, Inc. under conditions specifically established for in-house stability studies.

BACHEM, Inc.
3132 Kashiwa Street
Torrance, CA 90505
Telephone (310) 517-1858
Toll Free (888) 422-2436
Telefax (310) 530-2426

BACHEM

Certificate of Analysis

PRODUCT: Thymosin β4

LOT NUMBER: FTHYB40602B

RETEST DATE: March 2008
(May be extended on the basis of stability data.)

PRODUCT NUMBER: FBC0098

DATE OF MANUFACTURE: March 2007

MOLECULAR WEIGHT: 4963.55

MOLECULAR FORMULA: $C_{212}H_{350}N_{56}O_{78}S$

STRUCTURE: Ac-Ser-Asp-Lys-Pro-Asp-Met-Ala-Glu-Ile-Glu-Lys-Phe-Asp-Lys-Ser-Lys-Leu-Lys-Lys-Thr-Glu-Thr-Gln-Glu-Lys-Asn-Pro-Leu-Pro-Ser-Lys-Glu-Thr-Ile-Glu-Gln-Glu-Lys-Gln-Ala-Gly-Glu-Ser-OH

TEST	SPECIFICATION	RESULTS
APPEARANCE:	White to off-white powder; no visible extraneous materials	White powder; no visible extraneous materials
SOLUBILITY:	1 mg/mL in water yields a clear and colorless solution	Clear and colorless solution at 1 mg/mL in water
pH (1 mg/mL in water):	Report	4.48
MASS SPECTRAL ANALYSIS:	4963.6 ± 2.0 Da (average mass)	4963.3 Da
SPECIFIC OPTICAL ROTATION: $[\alpha]_D^{-25}$ (c = 0.1, water)	Report (corrected for anhydrous, acetic acid-free substance)	-102.4°

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TEST	SPECIFICATION	RESULTS
AMINO ACID ANALYSIS:	Ala: 1.8 to 2.2 Asx: 3.6 to 4.4 Glx: 9.9 to 12.1 Gly: 0.9 to 1.1 Ile: 1.8 to 2.2 Leu: 1.8 to 2.2 Lys: 8.1 to 9.9 Met: 0.9 to 1.1 Phe: 0.9 to 1.1 Pro: 2.7 to 3.3 Ser: 2.8 to 4.4 Thr: 2.7 to 3.3	Ala: 1.9 Asx: 4.0 Glx: 11.2 Gly: 0.9 Ile: 1.9 Leu: 2.0 Lys: 9.1 Met: 1.0 Phe: 1.0 Pro: 3.1 Ser: 3.5 Thr: 2.8
IDENTITY BY BY RP-HPLC:	Coelution with reference material	Complies in both systems
PEPTIDE PURITY BY RP-HPLC:	≥ 97%; no individual related substance ≥ 1.0%	QC-010 (System I): 99.3% Largest impurity: 0.32% (RRT 0.84) TMHPLC.THYB4 (System II): 97.7% Largest impurity: 0.53 % (RRT 0.96)
PEPTIDE CONTENT BY CHN (N_{th} 15.80%):	≥ 80%	93.1%

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Certificate of Analysis

LOT NUMBER: FTHYB40602B

TEST	SPECIFICATION	RESULTS
ACETATE CONTENT:	≤ 10%	5.9%
TRIFLUOROACETATE CONTENT:	≤ 0.1%	Not detected (LOD 0.02%)
WATER CONTENT:	≤ 10%	2.55%
MASS BALANCE:	95% to 105%	101.6%
RESIDUAL SOLVENTS:	Acetonitrile ≤0.1% Isopropanol ≤0.1% Methanol ≤0.1% Triethylamine ≤0.1%	< 0.004% < 0.004% < 0.004% < 0.008%
ENDOTOXIN:	< 1 EU/mg	< 0.5 EU/mg
BIOBURDEN:	Total aerobes ≤ 50 cfu/ 100mg *S. aureus* absent / 100mg *P. aeruginosa* absent / 100mg *Salmonella sp.* absent / 100mg *E. coli* absent / 100mg	0 CFU / 100mg Absent / 100mg Absent / 100mg Absent / 100mg Absent / 100mg

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LOT NUMBER: FTHYB40602B

TEST	SPECIFICATION		RESULTS
ADDITIONAL TESTS			
SEQUENCING: (MS-MS)	Amino acid sequence confirmed		Sequence confirmed
HEAVY METALS: (ICP-MS)	Copper:	Report	Not detected (LOD 0.2 ppm)
	Arsenic:	Report	Not detected (LOD 0.09 ppm)
	Molybdenum:	Report	Not detected (LOD 0.3 ppm)
	Silver:	Report	Not detected (LOD 0.09 ppm)
	Cadmium:	Report	Not detected (LOD 0.09 ppm)
	Tin:	Report	Not detected (LOD 0.09 ppm)
	Antimony:	Report	0.20 ppm
	Mercury:	Report	Not detected (LOD 0.09 ppm)
	Lead:	Report	Not detected (LOD 0.09 ppm)
	Bismuth:	Report	Not detected (LOD 0.09 ppm)

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Certificate of Analysis

LOT NUMBER: FTHYB40602B

TEST	SPECIFICATION		RESULTS	
ADDITIONAL TESTS				
CHIRAL AMINO ACID ANALYSIS:	Report	Alanine	0.12%	D-enantiomer
		Threonine	> 99.70%	L-Threonine
			< 0.10%	D-Threonine
			< 0.10%	L-allo Threonine
			< 0.10%	D-allo Threonine
		Isoleucine	> 99.70%	L-Isoleucine
			< 0.10%	D-Isoleucine
			< 0.10%	L-allo Isoleucine
			< 0.10%	D-allo Isoleucine
		Proline	< 0.10%	D-enantiomer
		Leucine	0.44%	D-enantiomer
		Serine	0.97%	D-enantiomer
		Aspartic Acid	0.26%	D-enantiomer
		Methionine	0.23%	D-enantiomer
		Phenylalanine	0.10%	D-enantiomer
		Glutamic Acid	0.36%	D-enantiomer
		Lysine	0.16%	D-enantiomer
UV SPECTRUM:	Report		Maximum absorbance exhibited at 203 nm	

Bassem Atiel
QUALITY CONTROL DEPARTMENT

03/31/07
DATE

Ref. Spec: FTHYB4/v03

BACHEM, Inc.
3132 Kashiwa Street
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16 Appendix C – Analytical Report

ANALYTICAL REPORT

Method Validation and Formulation Analysis of Thymosin beta 4 in HB-PS + 1.0% Sterile Water by HPLC

ChanTest Study Number:	100805.SFI
Study Director:	Cynthia Wladyka, BS
Testing Facility:	ChanTest Corporation 14656 Neo Parkway Cleveland, OH 44128
Sponsor:	RegeneRx Biopharmaceuticals, Inc. 15245 Shady Grove Road Rockville, MD 20850 (Protocol Amendment No. 3)
Dose Analysis Contributing Scientist:	William Giroski, BS
Method Validation Start Date:	21-Oct-2010
Method Validation End Date:	23-Oct-2010
Formulation Analysis Start Date:	29-Oct-2010
Formulation Analysis End Date:	30-Oct-2010
Document Status:	Final
Date:	18-Feb-2011
Number of Pages:	26

Personnel

Testing Facility Study Director	Cynthia Wladyka, BS ChanTest Corporation 14656 Neo Parkway Cleveland, OH 44128 Tel. 216-332-1665 Fax 216-332-1706 Email: cwladyka@chantest.com
Dose Analysis Contributing Scientist	William Giroski, BS ChanTest Corporation 14656 Neo Parkway Cleveland, OH 44128 Tel. 216-332-1665 Fax 216-332-1706 Email: bgiroski@chantest.com
Testing Facility Management	Jessica Brimecombe, PhD ChanTest Corporation 14656 Neo Parkway Cleveland, OH 44128 Tel. 216-332-1665 Fax 216-332-1706 Email: jbrimecombe@chantest.com
Sponsor Study Monitor	Christian B. Allan, PhD RegeneRx Biopharmaceuticals, Inc. 15245 Shady Grove Road Rockville, MD 20850 Tel. 301-208-9191 Email: callan@regenerx.com

1 Signature

This report was reviewed, approved and submitted by:

William Giroski, BS
Dose Analysis Contributing Scientist

ChanTest Corporation

William Giroski 18-Feb-2011
Signature Date

2 Quality Assurance Statement

Method Validation and Dose Formulation Analysis Inspections

Inspection Date	Inspection Type	Date Reported to:		
		Dose Analysis Contributing Scientist	Study Director	Testing Facility Management
29-Oct-2010	Test Article Preparation	29-Oct-2010	29-Oct-2010	29-Oct-2010
29-Oct-2010	Data Acquisition	29-Oct-2010	29-Oct-2010	29-Oct-2010
09-Nov-2010	Data	09-Nov-2010	10-Nov-2010	10-Nov-2010
09-Nov-2010	Draft Report	09-Nov-2010	10-Nov-2010	10-Nov-2010
15-Feb-2011	Final Report	15-Feb-2011	17-Feb-2011	17-Feb-2011

The Quality Assurance Unit has reviewed the report and has determined that the results incorporated into this report accurately reflect the study raw data.

Alicia DePlatchett, BA, RQAP-GLP
Quality Assurance Supervisor
ChanTest Corporation

ADePlatchett 18-Feb-2011
Signature Date

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3 Introduction

This report describes the method validation, concentration verification and homogeneity analyses of the Thymosin beta 4 dosing formulations samples for ChanTest study 100805.SFI. All dose formulation samples were analyzed by high performance liquid chromatography (HPLC) using ChanTest Corporation Analytical Test Method 100805.SFI.00 (Appendix A).

4 Test and Control Articles

4.1 Test Article

Test Article ID:	Thymosin beta 4
Alternate Test Article ID:	TB4
Lot Number:	FTHYB40602B (Protocol Amendment No. 1)
Retest Date:	July-2011
Form:	Acetate salt
Molecular Weight:	4963.55 g/mol (free peptide)
Peptide Purity:	98.4% [1]
Peptide Content:	93.1%
Storage Conditions (bulk):	Frozen (set to maintain -10 to -30 °C); under argon
Carrier:	Sterile water for injection, USP (sterile water)
Solubility:	Approximately 660.0 µM in HB-PS + 1.0% water as per ChanTest non-GLP study 100805.SFI.PRE

[1] Formulations were not corrected for peptide purity. Only a correction for peptide content was used in this study.

4.2 Test Article Carrier

Name:	Sterile water for injection, USP
Source:	Moore Medical / Hospira
Lot Number:	86-502-DK
Expiration Date:	01-Feb-2013
Molecular Weight:	N/A
Storage Conditions (bulk):	Room temperature
Rationale for Selection:	The test article is water-soluble.

4.3 Vehicle

Test article formulations were prepared in a vehicle consisting of a HEPES-buffered physiological saline (HB-PS) solution (composition in mM): NaCl, 137; KCl, 4.0; $CaCl_2$, 1.8; $MgCl_2$, 1; HEPES, 10; Glucose, 10; pH adjusted to 7.4 with NaOH (prepared weekly and refrigerated until used), supplemented with 1.0% sterile water. Chemicals used in solution preparation were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted, and were of ACS reagent grade purity or higher.

Name:	HB-PS + 1.0% sterile water
Source:	ChanTest Corporation
HB-PS Batch Numbers:	101014NEA410 (Expiration Date: 11-Nov-2010) 101021NEA418 (Expiration Date: 18-Nov-2010)
Storage Conditions (bulk):	Refrigerated
Rationale for Selection:	HB-PS provides the appropriate ionic composition for *in vitro* recording.

5 Experimental Conditions

5.1 100805.SFI.00 Analytical Test Method Summary

The test method procedure was validated to establish stability, linearity, accuracy and intra-assay precision (repeatability), quantifiable range, and lack of interference in the chromatographic region of interest (Figures 1 through 3).

Analysis of the electrophysiology dose formulations was performed by high-performance liquid chromatography (HPLC) using method 100805.SFI.00: "Determining the Concentration of Thymosin Beta 4 (TB4) in HB-PS + 1.0% sterile water by HPLC." Elution of the Thymosin beta 4 peak occurred under linear gradient conditions (Mobile Phase A: Water with 0.1% Trifluoroacetic acid (TFA); Mobile Phase B: Acetonitrile with 0.1% TFA). The method utilized a 6-point standard curve. System suitability criteria were met prior to acceptance of data.

HPLC conditions for analysis of Thymosin beta 4 in electrophysiology formulations were as follows:

Column:	Waters Delta Pak C18 5µm 2.1 X 150 mm 300 Å
Flow Rate:	0.50 mL/min
Injection Volume:	50.0 µL
Detector Wavelength:	205 nm
Column Temperature:	30.0 °C
Autosampler Temperature:	4.0 °C
Needle Wash Solution:	70% Methanol in water

Gradient Table:

Time (min)	Flow (mL/min)	%A	%B	Curve
-	0.50	85.0	15.0	-
9.00	0.50	70.0	30.0	6
9.50	0.50	15.0	85.0	6
10.50	0.50	15.0	85.0	6
11.00	0.50	85.0	15.0	6

Run Time: 11.00 minutes; Injection Delay Time: 7.00 minutes

5.2 Calibration Standard Curve and Quality Control Samples

5.2.1 Calibration Standards

Six non-zero calibration standards covering the concentration range from 0.84 µM to 12.0 µM were prepared by diluting the 20 µM calibration standard working stock solution in HB-PS + 1.0% sterile water (Appendix A).

5.2.2 QC Samples

Thymosin beta 4 QC samples at concentrations of 1.0, 5.0 and 10.0 µM were prepared by diluting the 20 µM QC sample working stock solution in HB-PS + 1.0% sterile water. In addition, ULOQ QC samples were prepared at 660.0 µM by diluting the QC primary stock solution to a final solution matrix of HB-PS + 1.0% sterile water (Appendix A).

5.3 Data Collection and Calculation

Peak areas of Thymosin beta 4 were measured using Empower software, Version 2, from Waters Corporation. The linear equation and equation of determination (R^2) for the calibration curve were calculated by the software.

System suitability, i.e. USP plate count and USP tailing factor of the middle calibration standard and subsequent standard checks (equivalent to the concentration of the middle calibration standard) were generated by Waters Corp. Empower software. Descriptive statistics including arithmetic mean (average) and standard deviation, % RE, % CV and % Change were conducted using Microsoft Excel, version 2003.

% Relative Error (%RE) = 100 * (measured mean concentration – nominal concentration)/nominal concentration

% Coefficient of Variance (% CV) = 100 * (std. dev. of concentration of replicates/mean concentration of replicates)

% Change = 100 * (measure concentration at T*n* – mean concentration at T_0)/mean concentration at T_0

Where T*n* = time of the non-zero time point

6 Results and Discussion

6.1 Method Validation

Test and Acceptance Criteria	Result
System Suitability USP Plate Count > 10460 (of the standard checks)	14710 to 15266
System Suitability USP Tailing Factor < 2.00 (of the standard checks)	1.26 to 1.28
System Suitability Check standard reproducibility % RE of the standard checks injected throughout the run must be ± 15.0% of back-calculated value of 6.0 µM calibration standard.	0.0 to 1.2%
Chromatographic Non-Interference There are no interfering peaks in the matrix blank within the retention time window of the analyte.	Passed
Linearity Coefficient of Determination, $R^2 \geq 0.990$	0.999976 to 0.999990
Accuracy (Calibration standards) % RE ± 15.0%	-0.9 to 2.2%
Accuracy (QC samples) % RE ± 15.0%	-7.4 to -4.2% for accuracy and precision samples; -5.8 to -1.4% for freshly prepared QC samples analyzed during method validation.
Repeatability (QC samples) % CV ≤ 15.0%	0.1 to 7.5% for accuracy and precision samples; 0.0 to 1.7% for freshly prepared QC samples analyzed during method validation.
Range The range was determined based on the results from the linearity, accuracy, and repeatability sections.	0.84 to 660.0 µM
Accuracy/Precision at Time 0 (QC samples) % RE ± 15.0%; % CV ≤ 15.0%	-7.4 to 1.8% (% RE) 0.1 to 3.7% (% CV)
Stability (Stock solution(s))	See Section 9, Table 1

% RE ± 15.0%; % CV ≤ 15.0%	
Stability (QC samples) % Change ± 15.0% of Time 0 Stability QC samples; % CV ≤15.0%	See Section 9, Table 2

6.1.1 Determination of Stability of Thymosin beta 4 in Sterile Water

The stability of Thymosin beta 4 in sterile water stored at room temperature (15 – 30 °C) for approximately 7 hours followed by additional room temperature storage of approximately 23 hours was evaluated during method validation. A 65.9 mM primary stock solution was prepared by adding the appropriate amount of sterile water to Thymosin beta 4. The 1.0 mM secondary stock solution was prepared by diluting the 65.9 mM primary stock with sterile water. After each storage interval, triplicate working stock solutions were freshly prepared from the 65.9 mM primary stock. These working stocks were then diluted further to a nominal concentration of 6.0 µM in HB-PS + 1.0% sterile water. After each storage interval, the 1.0 mM secondary stock was diluted to fit within the range of the calibration curve. The final matrix was HB-PS + 1.0% sterile water.

The % CV and % RE results for the stock solutions met the specified acceptance criteria. The data demonstrated that the 65.9 mM primary stock solution and the 1.0 mM secondary stock solution in sterile water were stable for approximately 30 hours from time of preparation when stored at room temperature (15 - 30 °C).

The stability data for the test article primary and secondary stock solutions prepared in sterile water are presented in Table 1.

6.1.2 Determination of Stability of Thymosin beta 4 in HB-PS + 1.0% Sterile Water

The stability of Thymosin beta 4 in HB-PS + 1.0% sterile water at concentrations of 1.0, 10.0 and 660.0 µM stored at room temperature (15 – 30 °C) for approximately 6 and 28 hours and refrigerated (2 - 8 °C) for approximately 28 hours was evaluated during method validation.

The % RE and % CV at time 0 of the stability QC samples were determined. The results indicated that the samples were accurate at the start of analysis.

The % Change and % CV results for the Thymosin beta 4 in HB-PS + 1.0% sterile water met the specified acceptance criteria. The data demonstrated that the Thymosin beta 4 in HB-PS + 1.0% sterile water at concentrations of 1.0, 10.0 and 660.0 µM was stable for approximately 28 hours when stored at room temperature and refrigerated.

The stability data for the test article in the formulation matrix determined for 6 and 28 hours during method validation are presented in Table 2.

6.2 Electrophysiology Dose Formulation Analysis

Test and Acceptance Criteria	Result
System Suitability USP Plate Count > 10460 (of the standard checks)	14907 to 15320
System Suitability USP Tailing Factor < 2.00 (of the standard checks)	1.25
System Suitability Check standard reproducibility % RE of the standard checks injected throughout the run must be ± 15.0% of back-calculated value of 6.0 µM calibration standard.	0.5%
Chromatographic Non-Interference There are no interfering peaks in the matrix blank within the retention time window of the analyte.	Passed
Linearity Coefficient of Determination, $R^2 \geq 0.990$	0.999988
Accuracy (Calibration Standards) % RE ± 15.0%	-0.9 to 0.4%
Accuracy (QC Samples) % RE ± 15.0%	-5.5 to 4.6%
Repeatability (QC Samples) % CV ≤ 15.0%	0.1 to 0.8%

6.2.1 Concentration Verification

Samples of the electrophysiology dose formulation solutions collected from the outflow of the perfusion apparatus were analyzed for concentration verification. Sample concentrations higher than 10.0 µM were diluted with HB-PS + 1.0% sterile water in duplicate, to fit within the range of the calibration curve.

The mean (n = 2) measured concentrations for the 100 and 600 µM samples were 115.0 and 691.6 µM, respectively. The 100 µM samples were within ± 15.0% RE; however the 600 µM samples resulted in % RE of 15.3%. A Laboratory Failure Investigation Form OOS-110110-01 is filed with the raw data. The cause of the laboratory failure was unknown. No retest was mandated because nominal concentrations would be reported in the electrophysiology report. For the vehicle (0 µM) samples tested, there was no detectable peak in the retention time of the analyte.

The electrophysiology dose formulation concentration verification data are presented in Table 3.

6.2.2 Homogeneity

Samples for homogeneity determination collected from the top, middle, and bottom electrophysiology dose formulation reservoir were analyzed. Sample concentrations higher than 10.0 µM were diluted with HB-PS + 1.0% sterile water in duplicate, to fit within the range of the calibration curve.

The samples met the % CV acceptance criteria of ≤ 15.0%, indicating that all dose formulations were homogeneous. The % CV (n = 6) results for the 100 and 600 µM samples were 0.3 and 0.7%, respectively.

The electrophysiology dose formulation homogeneity data are presented in Table 4.

7 Conclusions

The results presented in this report show that the test method is validated and is suitable for the analysis of Thymosin beta 4 in HB-PS + 1.0% sterile water at concentrations ranging from 0.84 to 660.0 µM.

Stability was demonstrated for Thymosin beta 4 in sterile water at concentrations of 65.9 and 1.0 mM for approximately 30 hours stored at room temperature.

Stability was demonstrated for Thymosin beta 4 in HB-PS + 1.0% sterile water at concentrations of 1.0, 10.0 and 660.0 µM for approximately 28 hours when stored at either room temperature or refrigerated.

The results of the analysis of the electrophysiology dose formulation samples demonstrated that the samples met the acceptance criteria for homogeneity. The concentration verification of 100 µM sample met the %RE ± 15.0% acceptance criteria (% RE was 15.0%). However, the acceptance criteria of %RE ± 15.0% was not met for the 600 µM sample (%RE was 15.3%).

8 Amendments and Deviations

Protocol amendments and/or deviation(s) are attached to the final electrophysiology report as an appendix.

9 Tables

Table 1 – Stability Analysis of Thymosin beta 4 Sterile Water Stock Solution

Primary Stock Date Prepared: 21-Oct-2010

Concentration (mM)	Time and Storage Condition	Mean[a] (n=3)	Std. Dev.[a] (n=3)	% CV[a] (n=3)	% RE[a] (n=3)
65.9 (primary stock)	~7 hours Room Temperature (15 – 30 °C)	65.0	2.41	3.7	-1.4
	~23 hours[b] Room Temperature (15 – 30 °C)	66.3	0.65	1.0	0.5
1.0 (secondary stock)	~7 hours Room Temperature (15 – 30 °C)	1.0	0.01	0.9	0.0
	~23 hours[b] Room Temperature (15 – 30 °C)	1.0	0.01	1.1	1.0

a: Calculations based on unrounded values obtained from raw data.
b: Stability analysis was performed for a total of approximately 30 hours from time of preparation.

Table 2 – Stability Analysis of Thymosin beta 4 in Formulation Matrix

Dates Acquired: 21-Oct-2010 through 23-Oct-2010

Concentration (µM)	Storage Condition	Mean[a] (n=3) at T_0	6 hrs		28 hrs	
			% Change[a] from T_0	% CV[a]	% Change[a] from T_0	% CV[a]
Low QC 1.0 µM	Room Temperature (15 – 30 °C)	1.0	1.6	0.6	1.8	0.9
	Refrigerated (2 - 8 °C)		N/A[b]	N/A[b]	2.9	0.9
High QC 10.0 µM	Room Temperature (15 – 30 °C)	9.8	0.5	1.2	3.1	0.1
	Refrigerated (2 - 8 °C)		N/A[b]	N/A[b]	3.2	0.1
ULOQ QC 660.0 µM	Room Temperature (15 – 30 °C)	611.4	-0.3	1.0	0.8	1.2
	Refrigerated (2 - 8 °C)		N/A[b]	N/A[b]	0.3	2.6

a: Calculations based on unrounded values obtained from raw data.
b: Not applicable; time point not analyzed.

Table 3 – Concentration Verification of Thymosin beta 4 Dose Formulations

Samples collected from the outflow of the perfusion apparatus

Formulations in Delivery Solutions Collection Date: 29-Oct-2010

Nominal Concentration (µM)	Test Results		Mean[a] (n=2)	% RE[a] (n=2)
	Rep 1	Rep 2		
Vehicle (0 µM)	DNS[b]	DNS[b]	N/A[c]	N/A[c]
100 µM	115.5	114.4	115.0	15.0
600 µM	698.7	684.4	691.6	15.3[d]

a: Calculations based on unrounded values obtained from raw data.
b: Detection not significant.
c: Not applicable.
d: Did not meet acceptance criteria %RE ±15.0%. Refer to section 6.2.1.

Table 4 – Homogeneity of Thymosin beta 4 Dose Formulations

Collection Date: 29-Oct-2010

Nominal Concentration (µM)		Test Results		Mean[a] (n=6)	Std. Dev.[a] (n=6)	% CV[a] (n=6)
		Rep 1	Rep 2			
100 µM	Top	115.6	116.1	116.2	0.38	0.3
	Middle	116.4	116.7			
	Bottom	115.9	116.4			
600 µM	Top	684.5	682.9	685.5	4.67	0.7
	Middle	688.2	689.7			
	Bottom	689.6	677.8			

a: Calculations based on unrounded values obtained from raw data.

10 Figures

Figure 1 – Representative Plot of Thymosin beta 4 Calibration Curve

Acquired: 29-Oct-2010
$Y = 5.27e^{5}X - 5.18e^{4}$; Weighting: none
$R^2 = 0.999988$

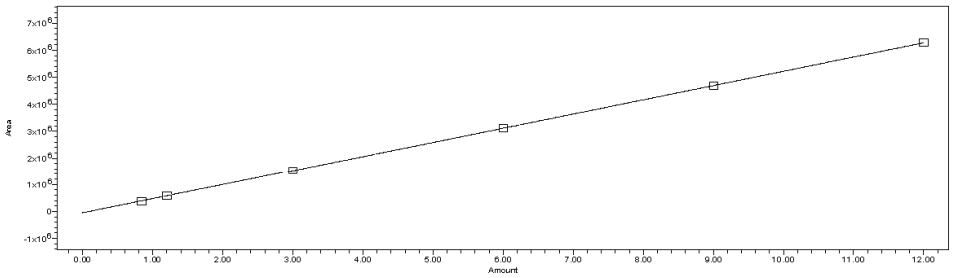


Figure 2 – Chromatogram of a Matrix Blank

Acquired: 29-Oct-2010

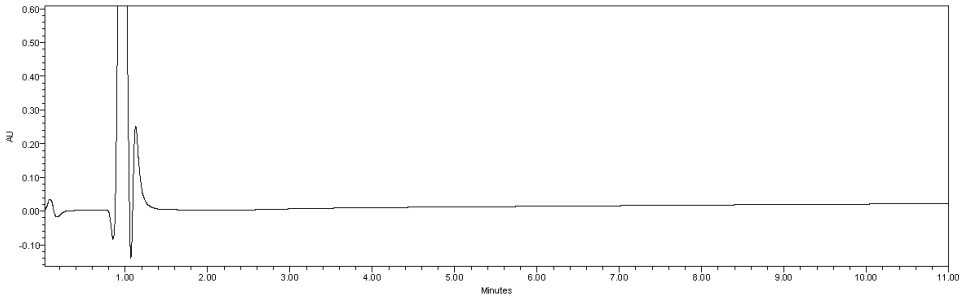
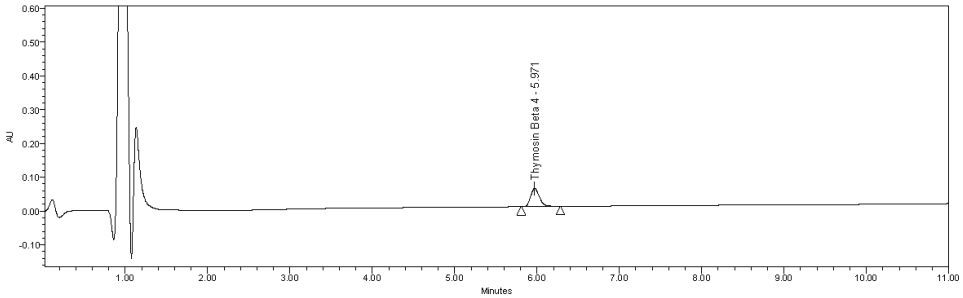


Figure 3 – Chromatogram of 0.84 µM Thymosin beta 4 Calibration Standard

Acquired: 29-Oct-2010



11 Appendix A. Test Method

ChanTest
THE ION CHANNEL EXPERT

ANALYTICAL TEST METHOD

Test Method: 100805.SFI.00

Title: Determining the Concentration of Thymosin Beta 4 (TB4) in HB-PS + 1.0% sterile water by HPLC

Supersedes: None

Prepared By: Bill Giroski, BS
Analytical Chemist/Dose Solution Analyst
Signature 27-Oct-2010 Date

Approved By: Jessica Brimecombe, PhD
Head, Laboratory Operations
Signature Jessica Brimecombe 27-Oct-2010 Date

Purpose:
To determine the amount of Thymosin Beta 4 (TB4) in HB-PS + 1.0% sterile water by HPLC analysis.

Scope:
This Test Method applies to the analysis of Thymosin Beta 4 (TB4) at ChanTest Corporation in HB-PS + 1.0% sterile water over a calibration range of 0.84 µM to 12.0 µM.

Training Requirements:
Personnel conducting this test method should be familiar with the referenced standard operating procedures and the operation/use of the equipment and materials listed below.

References:
ChanTest Corporation SOP 2301 "Development and Preparation of Analytical Test Methods"
ChanTest Corporation SOP 2303 "Operation and Maintenance of the Waters HPLC System"
ChanTest Corporation SOP 2501 "Test & Control Article Preparation"
ChanTest Corporation Study 100805.SFI
United States Pharmacopeia <621> Chromatography
United States Pharmacopeia <1225> Validation of Compendial Methods

Equipment and Materials:
Waters HPLC system (Alliance 2695 System with Waters 2996 or 2998 Photodiode Array Detector using Empower Software) or equivalent HPLC system
HPLC Column: Waters Delta Pak 2.1 mm X 150 mm C18, 5 µm, 300 Å (Catalog No. WAT023650 or equivalent)
Analytical balances (As appropriate)
Vortex mixer
Volumetric Glassware, various volumes
Intra-Laboratory Vacuum

Volumetric Glassware, various volumes
Intra-Laboratory Vacuum
Mobile Phase Filter 0.45 μm, PTFE or equivalent
Amber glass screw-top vials with caps, various volumes
Clear glass screw-top vials with caps. various volumes
Polypropylene centrifuge tubes, various volumes
Single channel digital microliter pipettes
Pipette Tips
Appropriate HPLC autosampler vials and caps

Reagents and Standards:
Sodium Chloride, ACS reagent or better
Potassium Chloride, ACS reagent or better
Magnesium Chloride Hexahydrate, ACS reagent or better
Calcium Chloride Dihydrate, ACS reagent or better
Glucose, Minimum 99.5%
HEPES, Minimum 99.5%
Sodium Hydroxide, ACS reagent or better

Milli-Q Water or LC/MS grade water (called water)
Acetonitrile, HPLC grade or better
Trifluoroacetic acid, HPLC grade or Minimum 99%
Test article: Thymosin Beta 4 (TB4)
Sterile Water for Injestion, USP

Preparation of Mobile Phase:
Mobile Phase A: Water w/ 0.1% TFA

1. Prepare 0.1% TFA v/v in water. (For example, in a 1000 mL volumetric flask, add ~900 mL of water. Add 1 mL of TFA. Fill flask to volume with additional water. Mix well.)
2. Filter and degas.

Mobile Phase B: Acetonitrile w/ 0.1% TFA

1. Prepare 0.1% TFA v/v in acetonitrile. (For example, in a 1000 mL volumetric flask, add ~900 mL of acetonitrile. Add 1 mL of TFA. Fill flask to volume with additional acetonitrile. Mix well.)
2. Filter and degas.

Preparation of Needle Wash Solution:

Prepare a needle wash solution of 70% Methanol in water. (For example, measure 700 mL of methanol in a 1000 mL volumetric flask and bring to volume with water).

Preparation of Matrix Solution (Matrix Blank):

Prepare a solution of HB-PS + 1.0% sterile water. (For example, pipette 1000 µL of sterile water in a 100 mL volumetric flask and bring to volume with HB-PS.)

Preparation of Primary Stock Solutions:

Solution worksheets are used where appropriate.

Thymosin Beta 4 (TB4) Stock Solutions (for Calibration Standards):

~ 66 mM Calibration Primary Stock: Prepare a solution of Thymosin Beta 4 (TB4) in sterile water at a concentration of ~ 66 mM by weighing a known amount of Thymosin Beta 4 (TB4) to a known amount of sterile water.

Thymosin Beta 4 (TB4) Stock Solutions (for Quality Control (QC) Samples):

~ 66 mM QC Primary Stock: Prepare a solution of Thymosin Beta 4 (TB4) in sterile water at a concentration of ~ 66 mM by weighing a known amount of Thymosin Beta 4 (TB4) to a known amount of sterile water. The ULOQ QC may be prepared from a separate higher concentration primary stock solution.

If test article supply is not sufficient for preparation of two primary stocks then only one primary stock will be used for preparation of calibration standards and QC samples.

Preparation of Working Stock Solutions:

Thymosin Beta 4 (TB4) Working Stock (WS) solutions (Calibration Standards):

Prepare working stock solutions of Thymosin Beta 4 (TB4) by diluting aliquots of the Calibration Primary Stock Solution with HB-PS and sterile water to achieve final concentrations of 20 µM of Thymosin Beta 4 (TB4) in HB-PS + 1.0% sterile water.

Thymosin Beta 4 (TB4) Working Stock (WS) solutions (Quality Control Samples):

Prepare working stock solutions of Thymosin Beta 4 (TB4) by diluting aliquots of the QC Primary Stock Solution with HB-PS and sterile water to achieve final concentrations of 20 µM of Thymosin Beta 4 (TB4) in HB-PS + 1.0% sterile water.

Preparation of Calibration Standards:

Thymosin Beta 4 (TB4) Calibration Standards:

Calibration range: 0.84 µM to 12.0 µM.

Prepare the calibration standards using the table below:

Concentration of Calibration Standard (µM)	Volume of HB-PS + 1.0% sterile water needed (µL)	Volume of Thymosin Beta 4 (TB4) Working Stock Solution needed (µL)
0.84	958	42
1.2	940	60
3.0	850	150
6.0	700	300
9.0	550	450
12.0	400	600

Preparation of Quality Control Samples (QCs):

Prepare the QC samples at concentrations sufficient to cover the anticipated range of the test article formulation samples.

Low QC1 (1.0 µM): add 50 µL of the 20 µM working stock and 950 µL of HB-PS + 1.0% sterile water to a HPLC vial, vortex. (Prepare 3 to 5 replicate samples).

Mid QC2 (5.0 µM): add 250 µL of the 20 µM working stock and 750 µL of HB-PS + 1.0% sterile water to a HPLC vial, vortex. (Prepare 3 to 5 replicate samples)

High QC3 (10.0 µM): add 500 µL of the 20 µM working stock and 500 µL of HB-PS + 1.0% sterile water to a HPLC vial, vortex. (Prepare 3 to 5 replicate samples).

If the concentration of actual test article formulations is expected to be greater than the high QC sample, prepare 3 to 5 replicate ULOQ QC samples at a concentration sufficient to cover the range of the anticipated test article formulations (for example at 110 to 120% of the nominal concentration of the highest test article formulation.). Dilute each ULOQ QC sample with HB-PS + 1.0% sterile water to fit within the range of the standard curve.

Preparation of Samples:

All dose solution test article formulations should be analyzed in duplicate (unless dose solution sample volume is not sufficient). If nominal concentrations of the test article formulation samples are above the high QC sample, dilute the samples with HB-PS + 1.0% sterile water to fit within the range of the standard curve.

Analytical Procedure:

Operating Parameters

Flow Rate:	0.50 mL/min.
Injection Volume:	50.0 µL
Detector Wavelength:	205 nm
Column Temperature:	30.0 °C
Autosampler Temperature:	4.0 °C

Gradient Table:

Time (min.)	Flow (mL/min)	%A	%B	Curve
-	0.50	85.0	15.0	-
9.00	0.50	70.0	30.0	6
9.50	0.50	15.0	85.0	6
10.50	0.50	15.0	85.0	6
11.00	0.50	85.0	15.0	6

Run Time: 11.00 minutes.
Injection Delay Time: 7.00 minutes.

Assay:

Inject Matrix Blank(s) (for equilibration). After equilibration of the system, inject/re-inject matrix blank(s), calibration standards, matrix blank(s), QC samples and formulation samples. Throughout the run, inject/re-inject the middle (6.0 µM) calibration standard (standard check) after approximately every 10 formulation samples and at the end of the run. Additional standard checks and/or matrix blanks can be added to the run at the discretion of the analyst.

System Suitability: Use Empower Software Package or equivalent.

USP Tailing Factor

Tailing factor for the Thymosin Beta 4 (TB4) peak in the middle (6.0 µM) concentration calibration standard must be less than 2.00.

USP Tailing Factor = $W_{0.05}$ / 2f

Where:

$W_{0.05}$ = Peak Width at 5% of Peak Height

f = distance from the peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from the baseline

USP Plate Count

USP plates (N) for the Thymosin Beta 4 (TB4) peak in the middle (6.0 μM) concentration calibration standard must be greater than 10460.

USP plates (N) = $16(Rt/W)^2$

Where:

Rt = retention time

W = width of peak measured by extrapolating the relatively straight sides to the baseline. Empower Software calls this "Width @ Tangent (USP Plate Count)"

Coefficient of Determination

The coefficient of determination (R^2) for the calibration standards must be greater than or equal to 0.990.

Data Analysis, Acceptance Criteria, and Calculations:

Peak areas of Thymosin Beta 4 (TB4) are measured using Empower Software manufactured by Waters Corp or equivalent. The equation of the line and the coefficient of determination (R^2) is calculated by the software.

The concentrations of the formulation samples are calculated by the Empower Software using the equation of the line calculated from the calibration standards. The peak area found for each formulation sample is used to determine the concentration using the equation of the line. If a dilution factor is necessary, the concentration found is multiplied by that dilution factor to result in a final concentration.

Matrix Blank must contain no interfering peak(s) in the window of interest (retention time window) for the analyte.

The % Relative Error (% RE) is used to demonstrate the accuracy of the method. The % RE of all QC samples, standard checks and back-calculated calibration standards should be within ±15.0% of target concentration. The % Relative Error (% RE) is calculated using the following equation:

% RE = 100 * (measured concentration – nominal concentration)/nominal concentration

The % Coefficient of Variance (% CV) is used to demonstrate the precision of the method. The % CV of each concentration level of QC samples should be ≤ 15.0%. The precision of the QC samples is calculated at each QC level as follows:

% CV = 100 * (std. dev. of concentration of replicates/mean concentration of replicates)

Data Recording and Reporting Results:

Worksheets will be used to capture the execution of the procedures outlined in this test method. The results of the execution of this test method will be captured on worksheets as well. All worksheets and critical raw data will be included in the study folder.

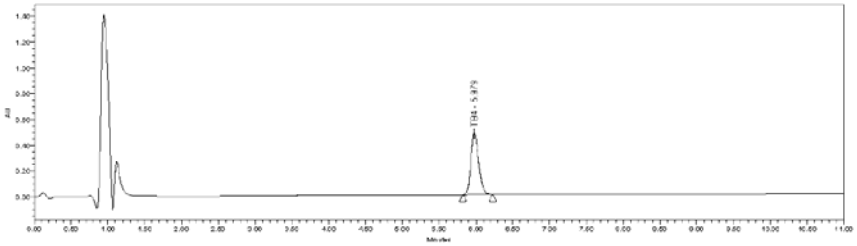
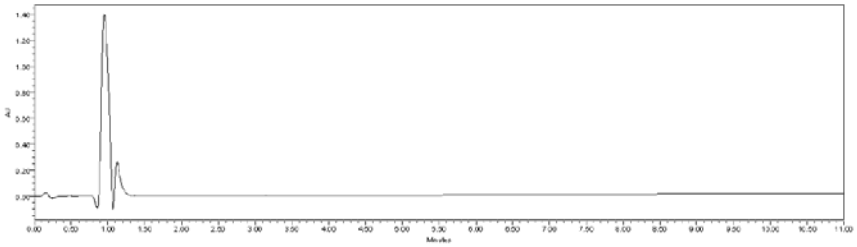
Example Chromatograms:

Typical chromatograms of a calibration standard and a matrix blank are shown in Attachment I and II, respectively.

Document Revision History:

Version	Date	Primary Author	Section	Changes
Draft 1A	10-11-10	BG	All	New Method
00	10-27-10	BG	Throughout	Formatting and grammatical errors

Attachment I: A typical chromatogram of Thymosin Beta 4 (TB4) calibration standard 6.0 μM



Attachment II: A typical chromatogram of the matrix blank.