EVALUATION OF RHO KINASE INHIBITORY POTENTIAL OF PROSOLUTION PILLS USING *IN VITRO* ENZYME ASSAY

STUDY NO: 110503/DM/PC

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STATEMENT OF COMPLIANCE

The Study Director hereby declares that the work was performed under her supervision and in accordance with the mutually agreed study plan and the standard operating procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study. The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

Jayesh Chaudhary
MD, Vedic Lifesciences Pvt. Ltd.

Vijay Gokarn
Assistant project manager- Technical

CERTIFICATE

We certify that the work reported here is a true and authentic report of the study entitled, ‘‘EVALUATION OF RHO KINASE INHIBITORY POTENTIAL OF PROSOLUTION PILLS USING IN VITRO ENZYME ASSAY”, based on the experiment conducted in one of the partnered Toxicology Laboratory Services of VEDIC LIFESCIENCES PVT LTD (B-203 Morya Landmark I, Off New Link Road, Andheri (W), Mumbai - 400 053,) India. The results presented here are faithful reflection of data collected during the study.

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QUALITY ASSURANCE STATEMENT

The study entitled "EVALUATION OF RHO KINASE INHIBITORY POTENTIAL OF PROSOLUTION PILLS USING IN VITRO ENZYME ASSAY was inspected and findings were reported to Management and to the Study Director.

Inspections were performed according to the Standard Operating Procedures of the Quality Assurance Unit. The report was audited against the approved study plan and pertinent raw data and accurately reflects the raw data.

STATEMENT OF CONFIDENTIALITY

This report which contains **CONFIDENTIAL** and **PROPRIETARY** information of **LEADING EDGE MARKETING** will not be disclosed to anyone except the employees of this company wherever necessary or to persons authorized by law or judicial judgment without the expressed or written approval of Sponsor.

DECLARATION

The Study Director hereby declares that the work was performed under his supervision and in accordance with the described procedures. It is assured that the reported results faithfully represent the raw data obtained during the experimental work. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study.

The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

LIST OF COMMONLY USED SYMBOLS AND ABBREVIATIONS

ABBREVIATION	DISCRIPTION
˚C	Degree centigrade
ATP	Adenosine triphosphate
HRP	Horseradish concentration
IC_{50}	50% inhibitory cncentration
µg	Microgram
µl	Microlitre
MBS	Myosin binding unit
mg	Milligram
min	Minute
ml	Millilitre
MLC	Myosin light chain
ROCK	Rho kinase

1. STUDY DETAILS

Study Title	:	Evaluation of Rho kinase inhibitory potential of Prosolution pills using *in vitro* enzyme assay
Test Item	:	Prosolution pills
Study Number	:	110503/DM/PC
Sponsor	:	DM CONTACT MANAGMENT 100-645 TYEE ROAD, VICTORIA BC V9A6X5, CANADA
Test Facility	:	Vedic Lifesciences 203, Morya Landmark-I, Off Link Road, Andheri (W) Mumbai - 400053 India

2. MONITORING PERSONNEL

Sr. No.	Designation	Personnel	Signature with date
1.	Assistant project manager-Technical	VIJAY GOKARN VEDIC LIFESCIENCES PVT.LTD MUMBAI	
2.	Managing Director	JAYESH CHAUDHARY VEDIC LIFESCIENCES PVT.LTD MUMBAI	

3. SUMMARY

The present study was conducted to evaluate the Rho kinase inhibitory potential of Prosolution pills using Rho kinase II (ROCK- II) recombinant protein with Rho-kinase assay Kit. Proslotion Pills were tested at concentrations ranging from 1 mg/ml to 5 mg/ml (w/v) and the concentration leading to 50 % enzyme inhibition was calculated.Prosolution Pills was found to inhibit ROCK- II activity at all the concentrations tested in a dose dependent manner. IC_{50} value of prosolution pills was determined to be 3.024 mg/ml. Hence, it can be concluded that prosolution pills possesses considerable ROCK- II inhibition potential

4. OBJECTIVE

To evaluate the Rho kinase inhibitory potential of Prosolution Pills using *in vitro* enzyme assay.

5. MATERIAL AND METHODS

5.1 MATERIALS

5.1.1 Test Item Information

Name of test item (TI)	:	Prosolution Pills
Batch No.	:	T/122
Physical Description of TI	:	White, coated, oval shaped tablet
TI Manufacturing Date	:	December 2010
TI Expiry Date	:	November 2012
Storage Condition	:	RoomTemperature (27℃)

5.1.2 Test Item Analysis

Analysis for the identity and purity of the test item was not conducted as part of this study, and is the responsibility of the sponsor.

5.1.3 Reference Item Information

H1152

Name of Reference Item	:	H-1152 dihydrochloride [H-1152P.2HCl, (S)-(+)-2-Methyl-1-[(4-methyl-5-

		isoquinolinyl)sulfonyl]homopiperazine.2HCl]
Product Code	:	ALX-270-423
Physical description	:	White to off-White solid
Solubility	:	Soluble in water
Storage Condition	:	-20°C
Manufactured By	:	Enzo Lifesciences

5.1.4 Test System

Rho kinase II Protein

Name of test system protein	:	Rho kinase II Recombinant
Code	:	CY-E1160-1
Lot. No.	:	30E11A
Storage Condition	:	-70°C
Manufactured By	:	Cyclex Co. Ltd.
Expiry Date	:	May 2013

5.1.5 Chemicals/Reagents/Kit

Rho kinase Assay Kit

Name of the Kit	:	Cyclex Rho kinase Assay kit
Cat. No.	:	CY-1160
Lot No.	:	26D11A
Manufactured By	:	Cyclex Co. Ltd.
Storage Condition	:	4°C
Expiry Date	:	April 2012

5.2 METHODS

5.2.1 Outline of the method

The Rho kinase inhibitory potential of Prosolution Pills was evaluated using ROCK II recombinant protein *in vitro.* The potential of Prosolution Pills as a ROCK II inhibitor was evaluated by comparison of ROCK II activity in the absence (control) and presence of

test item.

H-1152 dihydrochloride was used as a reference ROCK

II inhibitor.

5.2.2 Description of the test procedure

5.2.2.1 Test item preparation

5.2.2.1.1 One tablet (1300 mg) of Prosolution Pills was dissolved in water to obtain stock solution corresponding to concentration of 50 mg/ml.

5.2.2.1.2 The stock solution was diluted and used for the preparation of subsequent dilutions in water to achieve final concentrations ranging from 1 to 5 mg/ml (w/v).
Figure 1: Dilution scheme for Prosolution Pills.

5.2.2.2 Reference item preparation

5.2.2.2.1 H-1152 was weighed and dissolved in water to obtain stock solution of 1 mg/ml.

5.2.2.2.2 This stock solution was diluted in water to achieve solution of final concentration of

0.319µg/ml.

5.2.2.3 Rho kinase assay procedure

The inhibition of recombinant ROCK II enzyme activity by Prosolution Pills was determined using Rho kinase assay kit as per manufacturer's instructions.

5.2.2.3.1 10 µl of different concentrations of prosolution pills ranging from 1 to 5 mg/ml or 0.319µg/ml concentration of H-1152 dihydrochloride were added to 80 µl of kinase reaction buffer.

5.2.2.3.2 The above solution was incubated with 10 µl (10 mU) of ROCK II recombinant protein

for 30 min at room temperature in 96 well plate which was pre-coated with the recombinant C terminus of the myosin binding subunit (MBS) of myosin phosphatase

5.2.2.3.3 After the incubation, the wells were washed five times with wash buffer containing 2 %
Tween®-20.

5.2.2.3.4100 µl of HRP conjugated anti-phospho-specific detection antibody AF 20 was added to each well and incubated for 60 min at room temperature.

5.2.2.3.5 After the incubation, wells were washed five times with wash buffer containing 2 % Tween®-20.

5.2.2.3.6 Subsequently, 100 µl of substrate reagent was added to each well and the plate was incubated at room temperature for 5 min.

5.2.2.3.7 After the incubation, 100 µl of stop solution was added to each well.

5.2.2.3.8 Absorbance was measured at 450 nm using multiplate reader.

5.2.2.4 Evaluation of ROCK II recombinant protein activity *in vitro* using Rho kinase assay kit

The potential of Prosolution Pills as a ROCK II recombinant protein inhibitor was determined by calculating its IC_{50} value (concentration at which 50 % of ROCK II activity is inhibited). The % inhibition was calculated at each concentration tested as per formula given below:

$$\% \text{ Inhibition} = \frac{[\text{Absorbance of control} - \text{Absorbance of Test item}] \times 100}{\text{Absorbance of control}}$$

IC_{50} value was determined using the Graph pad prism TM software v 4.01

6. RESULTS

The potential of Prosolution Pills as a Rho kinase inhibitor was evaluated using ROCK II

recombinant protein. Reference ROCK II inhibitor (H-1152 dihydrochloride) at a concentration of 0.319µg/ml was found to inhibit 95.19 % ROCK II activity. The percentage inhibition of ROCK II activity mediated by prosolution pills at different concentrations ranging from 1 to 5 mg/ml is shown in table 1. Prosolution Pills was found to exhibit concentration dependent inhibitory effects on ROCK II activity. IC_{50} value was determined to be 3.024 mg/ml.

Table 1: Percent ROCK-11 activity inhibition by Prosolution Pills

Concentration (mg/ml)	Percentage inhibition (%)
1.0	21.77
2.0	35.83
3.0	46.42
3.5	53.79
4.0	58.13
4.5	60.85
5.0	69.69

7. CONCLUSION

Data obtained from the present study revealed that Prosolution Pills inhibited Rho kinase activity in a dose dependent manner with IC_{50} value of 3.024 mg/ml. This value is comparable to the activity as reported for other herbal mixtures known to cause inhibition of Rho Kinase [1]. Thus this data suggests that Prosolution Pills possesses substantial ROCK II inhibition potential for further development.

8. STUDY DEVIATION

1) Preparation of main stock of test item Prosolution Pills at 20 mg/ml as proposed in the study plan in section 7.2.2.1.1 was refined in the report to 50 mg/ml in order to meet the test condition.

2) The incubation of the test item Prosolution pills or reference item H-1152 dihydrochloride with the ROCK II recombinant protein at 30°C as proposed in the study plan in section 7.2.2.3.2 was refined in the report to room temperature.

9. REFERENCES

1. Smitasiri Y, *et al*., An Initial Evaluation of the Safety, Efficacy and Purity of VigRX, an Herbal Combination Formula, for the enhancement of Male Sexual Health. *The Open Natural Product Journal,* 2010; 3, 10-19.
2. Ikenoya M, *et al*., Inhibition of Rho-kinase-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation in human neuronal cells by H-1152, a novel and specific Rho kinase inhibitor. *Journal of Neurochemistry*, 2002; 81, 9-16.

10. ARCHIVING

The following items will be archived at the test facility for 6 years after completion of the study: study plan; raw data and final report.

11. REPORT DISTRIBUTION

The final report (original copies) will be distributed as follows:
Sponsor: One signed final report in original (Copy No. 1/2).
Test Facility: One signed final report in original (Copy No. 2/2).