

NATURAL REMEDIES
P R I V A T E L I M I T E D
Bioassay Research Laboratory

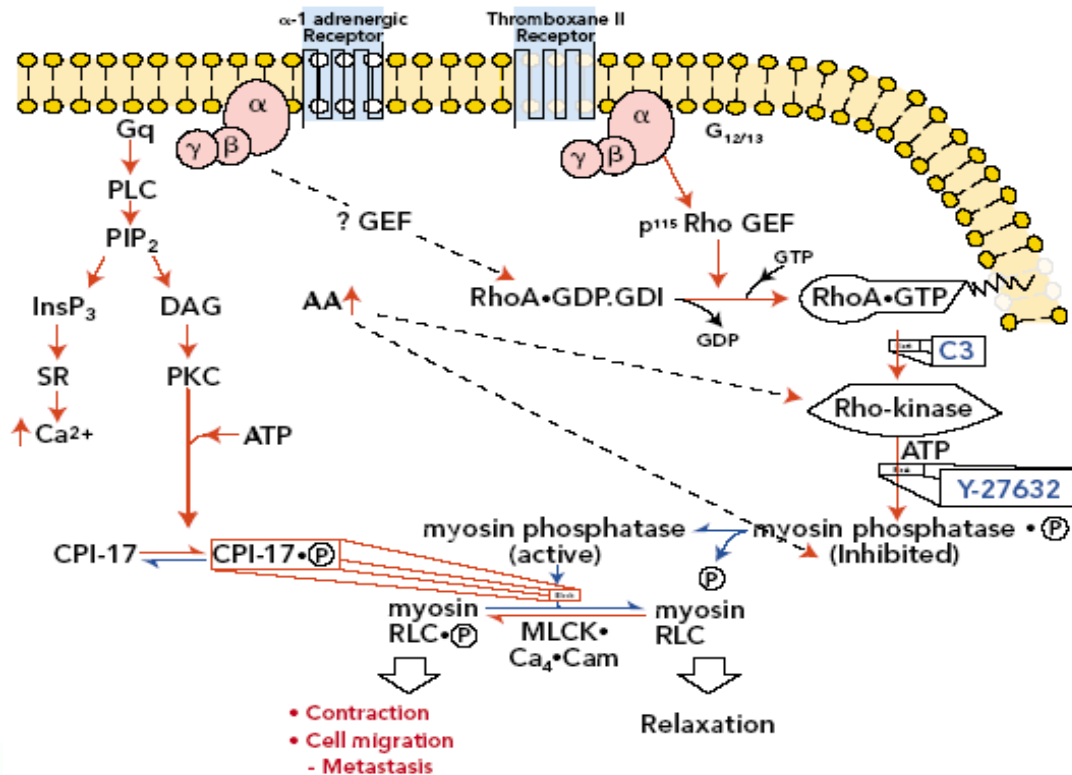
Bioassay Report

Sample	: VigRx Tablet blend	Lot No.	: 120657
		Report No.	: ROK/0701
Assay Performed	: Rho-kinase inhibition assay	Protocol	: NR/BSY/SOP/029/01
Source	: Dr. Alex Schauss, AIBMR Life Sciences	Date	: 16/03/2007

Introduction:

Penile erection occurs in response to cavernous smooth muscle relaxation, increased blood flow to the penis, and restriction of venous outflow. These events are regulated by a spinal reflex relying on visual, imaginative, and olfactory stimuli generated within the central nervous system (CNS) and on tactile stimuli to the penis. Drugs can have a facilitator or inhibitory effect either on the nerves regulating this reflex or on the cavernous smooth muscle. A balance between contractile and relaxant factors governs flaccidity/rigidity within the penis. Drugs that raise cytosolic calcium relax smooth muscle and can initiate penile erection. Increased cavernous smooth muscle tone mediated by norepinephrine and endothelin is the consequence of a rise in cytosolic Ca^{+2} . Following activation of G-protein-coupled receptors such as the α -adrenergic receptor, membrane-associated phospholipase C forms diacylglycerate (DAG) and inositol triphosphate (IP_3). IP_3 triggers release of Ca^{+2} from sarcoplasmic reticulum, thereby raising cytosolic Ca^{+2} . The binding of Ca^{+2} to calmodulin mediates smooth muscle contraction through activation of myosin light chain kinase (MLCK). MLCK phosphorylates myosin heads, which allows interaction of actin and myosin. This interaction can be conceptualized as crossbridge formation that enables smooth muscle contraction and mediates and maintains force. In the penis this translates into detumescence. Conversely, phosphatase and myosin phosphatase (MP) drive this reaction in the opposite direction, resulting in smooth muscle relaxation. Recently, MP activity has been used as a target to induce penile erection. MP is regulated by cytosolic Rho-kinase. Phosphorylation of MP by Rho-kinase maintains myosin light chain phosphatase (MLCP) in its inactive state. However, inhibition of Rho-kinase allows dephosphorylation of MP, prevents intrinsic contractile tone, and allows relaxation of cavernous smooth muscle. The net effect is penile erection *in-vivo*, which occurs even after blockade of L-arginine / NO / cGMP pathways (1) [see figure on page 2]. Rho-kinase inhibitors are thus likely to be helpful in erectile dysfunction; this has been demonstrated *in-vivo* following the administration of the selective Rho-kinase inhibitor, Y-27632 to rats (2)





Biochemistry of myosin, the Rho-kinase pathway, and maintenance of tone: Myosin light-chain kinase (MLCK), acting through Ca^{+2} and calmodulin (Ca_4CaM), is responsible for phosphorylation of myosin. Phosphorylated myosin interacts with actin to increase smooth muscle tone. This maintains the penis in a detumescent state. Conversely, myosin phosphatase (MP) dephosphorylates myosin to reduce tone. Rho-kinase, in combination with ATP, prevents phosphorylation of MP, thereby maintaining the latter molecule in the inactive state. Thus, constant tone is maintained even in the absence of NE. This may explain how the penis is kept in the detumescent state. Rho-kinase inhibitors *in-vivo* and *in-vitro* relax cavernous smooth muscle, raise intracavernous pressure, and trigger penile erection. A variety of signal transduction pathways (shown) are responsible for maintaining a constitutive increase in Rho-kinase. Gq, G-protein; PLC, phospholipase C; PiP_2 , inositol biphosphate; DAG, diacyl glycerate; SR, sarcoplasmic reticulum; PKC, protein kinase C; GEF, guanine nucleotide exchange factor; Aa, arachadonic acid; RLC, regulatory light chain; GDP, guanidine diphosphate; Y-27632, selective inhibitor of Rho-kinase, NE- Norepinephrine. Reproduced from Steers WD [(2002) 'Pharmacological treatment of Erectile dysfunction' *Reviews in Urology* 4 (3) S17-S25].

Principle of the assay:

Plates are pre-coated with a substrate corresponding to recombinant the C terminus of MBS (myosin-binding subunit of Myosin phosphatase), which contains a threonine residue that may be phosphorylated by DMPK family members, including Rho-kinase, MRCK (Mytonic Dystrophy kinase-related Cdc42-binding kinase) and DMPK (Mytonic Dystrophy Protein kinase). The detector antibody is AF20, an antibody that specifically

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detects only the phosphorylated form of threonine-697 on MBS. The amount of phosphorylated substrate is measured by binding it with a horseradish peroxidase conjugate of AF20, a anti-phospho-MBS threonine 696 specific antibody, which then catalyses the conversion of the chromogenic substrate tetra methyl-benzidine (TMB) from a colorless solution to a blue solution (or yellow after addition of stopping solution). The color is quantified by spectrophotometry and reflects the relative amount of Rho-kinase activity.

Reagents / chemicals used:

1. Reagents are prepared as per Rho-kinase assay kit Cat. no. CY-1160, lot no. 08F06A, Cyclex Co. Ltd., Japan, stored at 4°C.
 - a. Microplate-wells coated with recombinant MBS C terminus(758-1032 a.a),
 - b. 10X wash buffer
 - c. Kinase buffer
 - d. 20X ATP
 - e. HRP conjugated Detection antibody
 - f. Substrate solution
 - g. Stop solution
2. Rho -kinase II (cat. no. CY-E 1160-1, lot no. 11D05, Cyclex Co.Ltd, Japan, store at -70°C).
3. Rho-kinase specific inhibitor Y-27632 (cat. no. 688001, lot no.B40256 Calbiochem, Germany, store at -20°C)
4. Ultra pure water (cat. no. 400000, lot no. 166329-30 Cayman Chemicals Company, USA)

Sample preparation: 2000mg of the VigRx Tablet blend was sonicated for 8minutes in 20ml of methanol and made up the volume to 100ml with ultra-pure water, filtered & the filtrate was used for the assay. 22.5% was soluble (450 mg was soluble out of 2000mg).

Procedure:

The assay is carried out as per the protocol of Rho-kinase Assay Kit (catalog no. CY-1160) from Cyclex Co. Ltd., Japan.

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Briefly, A total reaction mixture of 100 µl in kinase reaction buffer contained varying concentrations of sample / positive control, 10munits of Rho-kinase II enzyme and 100 µM of ATP were incubated at 30°C for 30 minutes in substrate coated plate supplied along with the kit. Following incubation, the degree of substrate phosphorylation was determined using the standard ELISA protocol provided with the kit.

A control reaction was carried out without the test sample. The % inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Absorbance (control)} - \text{Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

IC₅₀ was calculated using log-probit analysis.

Results:

→ Refer table 01 for IC₅₀ data.

Table 01: IC₅₀ data of the tested sample in Rho-kinase II inhibition assay

Tested material	Concentration tested µg/ml	% inhibition (n=2)	IC ₅₀ µg/ml (95% confidence intervals)
Rho-kinase specific inhibitor (Y-27632)	0.0032	0.0	0.199 (0.13 – 0.31)
	0.032	40.54	
	0.32	56.13	
	3.2	78.23	
VigRx Tablet blend*	125	11.54	965.12 (796.12 – 1217.39)
	250	16.26	
	500	28.61	
	1000	54.41	
	2000	68.88	

* **Percent yield-** 2000mg of sample was sonicated for 8minutes in 20ml of methanol, the volume was made up to 100ml with ultra-pure water and filtered. The residue was evaporated to dryness to determine the undissolved solids (1550mg was the undissolved solids, *i.e* 77.5%). For calculation purposes the stock solution (2000mg/100ml) was assumed to be 100% soluble.

Fig. 01 Effect of VigRx Tablet blend on Rho-kinase II activity

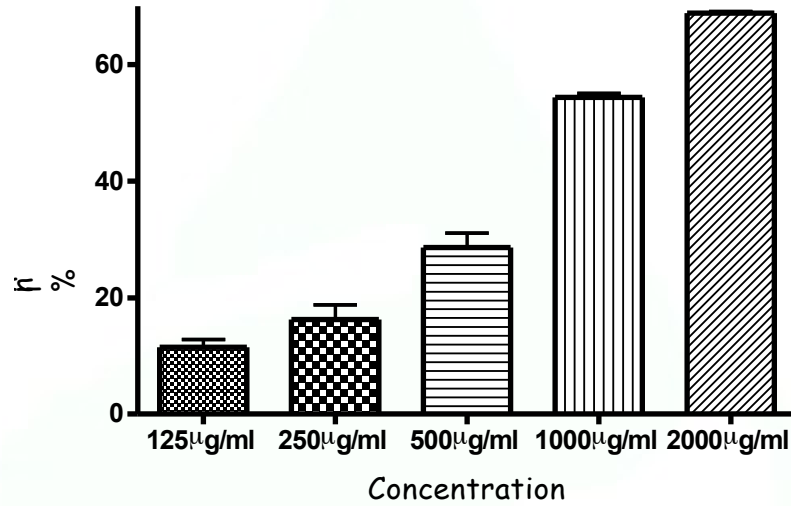
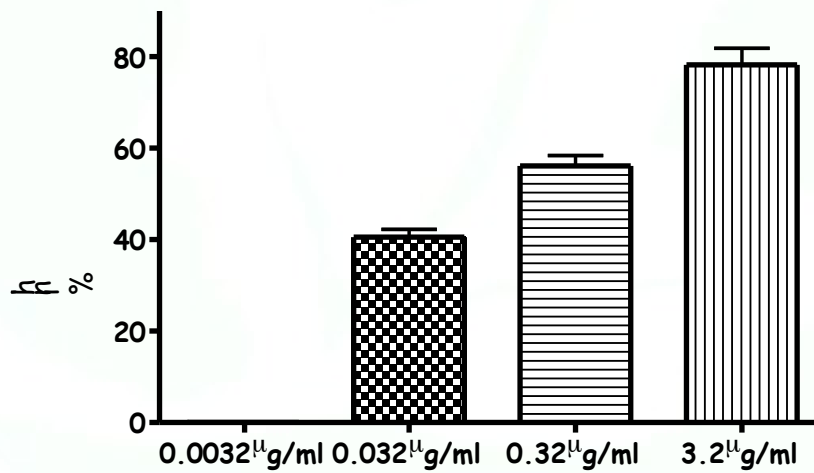


Fig. 02 Effect of Y-27632 on Rho-kinase II activity



References:

- 1] Steers WD, (2002) Pharmacologic treatment of ED. *Reviews in urology* 4(3): S17-S25.
- 2] Rajasekaran M, White S, Baquir A and Wilkes N (2005) Rho-kinase inhibition improves erectile function in aging male Brown-Norway rats. *J.Androl* 26(2):182-8.

Disclaimer:

1. The results listed above pertain only to the tested samples and applicable parameters
2. Samples will be disposed after one month from the date of issue of test report unless otherwise specified.
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