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Excipient interference on development of a cGMP assay for PDE5

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ABSTRACT

The aims of this study were to develop a simple, cost-effective assay for determination of PDE5 activity *in vitro* for testing of active compounds and excipients used in FDA approved products for treatment of erectile dysfunction. Several excipients used in formulations of these drugs interfered with the PDE5 assay indicating that certain excipients can exhibit pharmacological effects *in vitro* and are therefore not the inert materials they were described as in years past.

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INTRODUCTION

Erectile dysfunction (ED), estimated to affect up to three-quarters of men globally, is a clinical condition where there is inability to achieve and/or maintain an erection sufficient for sexual intercourse in men between 40-71 years. Since the severity of ED increases with age and the presence of underlying comorbidities, such as CVD and diabetes, addressing ED would benefit patients for earlier diagnosis and management of cardiometabolic diseases too (1).

The three known isoforms of phosphodiesterase 5 (PDE5) include PDE5A1, PDE5A2 and PDE5A3 (2). All these isoforms, have similar function and cGMP converting catalytic activity, and are inhibited by the well-studied ED drug sildenafil, but differ at their N-terminal regions (3). Thus, in this present study, PDE5A1 was used as the representative PDE5 isoform that specifically targets cGMP typically generated by nitric oxide (NO)-mediated activation of the soluble guanylyl cyclase. Since cGMP is generated in a variety of physiologically processes, pharmacological inhibition of PDE5 has been demonstrated to have several therapeutic applications in heart failure, immune diseases, cancer therapy, systemic sclerosis, and pulmonary arterial hypertension as well as erectile dysfunction (4-6).

PDE5 is expressed in a variety of tissues including the lung, brain, kidney, cardiac myocytes, gastrointestinal tissue, vascular smooth muscle cells, platelets, skeletal muscles and penile corpus cavernosum (5). Maximum expression is in the vascular smooth muscles of corpus cavernosum and is many fold higher in human male than in female reproductive tracts (7,8). The PDE5 inhibitors relax the penile blood vessel by blocking the hydrolyzation of the second messenger cGMP and hence contribute to increase the blood flow to alleviate erectile dysfunction.

There are many herbal products available on the market that claim to be useful as dietary supplements for providing improved erectile function and improved sexual enhancement in humans (9). Since such products are frequently adulterated with other pharmaceutical ingredients and their analogues, including phosphodiesterase-5 (PDE5) inhibitors, their long-term consumption could lead to adverse events. Fleshner *et al* (10) have shown evidence that of seven oral herbal tablets analyzed, one product contained a mean dose of 30.2 mg sildenafil per tablet, while almost 19.7 mg Tadalafil per tablet was detected in the second tablet, with no evidence of vardenafil being detected in any of the samples tested (11). Also, of ninety-one samples tested, 84% contained PDE5-inhibitor pharmaceutical ingredients, including Tadalafil and/or sildenafil and PDE5 analogues (12). Moser *et al* (13) recently reported that 3 out of 12 investigated herbal medicines contained sildenafil in ranges from 0.5% to 18%. A recent report by Akuamoa *et al* (14), highlights this disturbing issue. Since these products were marketed as safe with no side effects, the current situation indicates a need for stricter regulation of the natural health products industry. One opportunity to impact this problem is development of methods to detect such contaminating analogues of PDE5 inhibitors, and thus a reliable, cost-effective PDE5 assay could be valuable.

Commercially available drugs inhibiting PDE5 (PDE5i) include sildenafil, vardenafil, tadalafil, and the more recently approved avanafil (15). The marketed formulations of these PDE5 inhibitors have the Active Pharmaceutical Ingredient (API) in precise quantities, with the remainder of the weight made up of Pharmaceutical Excipients (PE). Excipients are incorporated in formulations to enhance stability, bioavailability, solubility, physical and chemical properties etc., of the pharmaceutical product and their selection depends on the type of formulation (16). Commonly used excipients for these formulations are binders (e.g., microcrystalline cellulose, povidone, dicalcium phosphate, Hypromellose), fillers (diluent e.g., lactose, calcium phosphate), disintegrants (e.g., sodium starch glycolate, croscarmellose), lubricants (magnesium stearate), glidants (silica colloidal, magnesium stearate), colorants (titanium dioxide, FD&C Blue #2 aluminum lake), surfactants and solubilizers (Tween-80, sodium lauryl sulfate, polyethylene glycol), flavoring and sweetening agents (saccharin sodium), preservatives (sodium benzoate, methyl paraben), antioxidants (butylated hydroxytoluene), coating agents (e.g. triacetin, Hypromellose phthalate, Macrogol), thickening and suspending agents (colloidal silicon dioxide), etc. (17-21). These excipients serve multiple functions in drug products, including but not limited to, providing structural integrity, facilitating drug release, improving drug solubility, and enhancing taste and appearance.

Excipients should ideally be physiologically inert with no effect on either alleviating or exacerbating the disease condition and should not affect the bioactivity of the active drug through drug-excipient interactions. Hence, understanding the role of excipients is crucial for optimizing drug formulations and to ensure that the therapeutic outcomes are consistently achieved. The interactions between excipients and APIs are complex and can be categorized into physical, chemical, and biological mechanisms. Physical interactions may alter drug release profiles due to changes in the material properties of the formulation while chemical interactions could lead to instability or degradation of the API, with implications on drug potency and safety. On the other hand, biological interactions might impact drug absorption, metabolism, and efficacy of a pharmaceutical product.

This study seeks to explore, for the first time, whether excipients present in marketed products for ED directly impact the activity of the target enzyme PDE5. Since the balance between cGMP synthesis by guanylyl cyclase and cGMP hydrolysis by PDE5 regulates the cGMP levels in the penile tissue-corpora cavernosa (7), we used this feature for assay of PDE5 enzyme activity using cGMP as the substrate. HPLC was used as more straightforward method of detection in the assay.

The methodology developed was also applied to examining the ability of different excipients to modulate the performance of drugs *in vitro*. Potential drug-excipient interactions occurring in the PDE5 assay were studied using a range of excipients and several marketed ED compounds.

MATERIALS AND METHODS

Materials

All materials, reagents, APIs, and excipients utilized in this study were sourced from Sigma-Aldrich, USA, unless otherwise mentioned. The human enzyme PDE5A (Cat # E9034) was also obtained from Sigma, USA.

In-vitro PDE5 inhibitory assay

Method of enzyme preparation

The human PDE5 enzyme was diluted 1: 100 with a buffer containing 50 mM Tris, pH 8.0 with 100 mM magnesium chloride, and used fresh every time for the assay.

Method of excipients preparation

All necessary excipients were collected and tested for solubility in either water or DMSO, as these solvents are required for the PDE5 assay. Excipients that dissolved completely in a specific solvent were used to assess their effects in the PDE5 inhibitory assay. Excipients that did not dissolve in either water or DMSO were not evaluated in the PDE5 assay. The excipients as 10 mg/ml stock solutions in either 10 % DMSO or water, were evaluated for their effects on phosphodiesterase enzyme type 5 (PDE5A) by testing using *in vitro* enzyme assays.

PDE5 assay

PDE5 assay was carried out using the method described by Li *et al* (22) with minor modifications. Briefly, in a buffer consisting of 50 mM Tris (pH 8.0) and 100 mM $MgCl_2$, 7.5µL of PDE5 enzyme solution (from 1:100 diluted stock solution) and 25µL of the compound solution (inhibitor or diluent) were combined and incubated for 1h at 37°C. After pre-incubation, 3µM cGMP (made in water) was added to the mixture, and the total reaction volume was made up to 500µL with water. The reaction was then allowed to proceed for three hours at 37°C. Taken together, the optimized assay conditions for PDE5A1 inhibitors screening were 0.05µg/mL PDE5A1, 1.14µg/mL cGMP, and 180 min reaction time at 37ºC.

Post-assay, the samples were heated at 100°C for 5 min. to deactivate the PDE5 protein. After cooling, the samples were analyzed using HPLC. A sample without PDE5 was used as the positive control, while 10% DMSO served as the negative control. The percentage inhibition was calculated based on the HPLC peak area obtained. Tadalafil and Vardenafil were used as positive controls for the assay under the conditions described.

Optimization of incubation time, pH and temperature of the assay mixture

A more useful measure of enzyme inhibition with potent lead compounds requires pre-incubation of the enzyme and inhibitor, followed by the initiation of enzyme reaction by substrate addition to measure the residual activity (23). For this reason, the PDE5 inhibitory assay mixture (with PDE5 and test compounds/excipients) was incubated at 37°C for various time points namely 1.5, 3, and 6 hours to optimize the incubation

time required for maximum conversion of cGMP to GMP. The effect of reaction pH on the PDE5 inhibitory assay was also examined by testing the assay at different three pH levels, 7.2, 8.0, and 8.8. The use of elevated column temperature in HPLC is reported to provide better performance since there is lower system backpressure with a temperature increase, along with reduced analysis time (24). To examine if variations in column temperature affect the separation of cGMP and GMP counterparts, the PDE5 assay was also subjected to variations in column temperatures of 30°C, 40°C and 50°C and the cGMP and GMP levels in all samples after the assay period were analyzed by HPLC.

Effect of zinc on PDE5 assay

Bülow *et al* (25) have shown that zinc inhibits the activity of PDE-1, PDE3, PDE4 and PDE5. Zinc was included in the assay mixture at various concentrations with the PDE5 enzyme for a standard time of 1 h at 37°C. After the pre-incubation, the reaction was initiated with the addition of substrate cGMP and the contents incubated for 3 h at 37°C and samples analyzed on HPLC for percentage conversion of cGMP to GMP to quantitate the amount of inhibition of PDE5 compared to a control sample without zinc.

Effect of different concentrations of selected activators and inhibitors on PDE5 activity

Three excipient inhibitors (citric acid, ascorbic acid and sodium lauryl sulphate) and two excipient activators (Tween 80 and Hypromellose) were tested at different concentrations in the reaction mixture and the PDE5 assay was carried out as described previously. This concentration-dependent assay was performed without the inclusion of any standard PDE5 inhibitor drug, unless otherwise specified.

cGMP stability study in the presence of excipients

A standard solution of cGMP at 3 μM was incubated at 37°C with and without excipients such as Hypromellose and citric acid at 2.5 μg and 4 μg/reaction respectively for 3 hours. The resulting samples were analyzed for cGMP degradation by HPLC.

Dose-dependency of activation and inhibition of PDE5 by excipients

To examine if the activation and inhibition of PDE5 is dose-dependent, two randomly chosen excipient activators, and two excipient inhibitors, were tested at different concentrations using the assay conditions described earlier.

Tadalafil's anti-PDE5 activity with and without a selected excipient inhibitor and activator

To examine if an excipients modulation of PDE5 activity remains consistent in the presence or absence of established PDE5 inhibitors like Tadalafil, it was incubated at its IC_{50} value of 62.5 nM with different concentrations of hypromellose/reaction (in μM range). A study was also performed to examine the level of PDE5 inhibition achieved using different amounts of zinc, together with a concentration of Tadalafil, 1.25 μM, that itself fails to show any PDE5 inhibition activity.

HPLC Method

The HPLC method for detecting GMP and c-GMP was developed with modifications to the procedure outlined by Li *et al* (22). The analysis was carried out using a Waters Alliance e2695 HPLC system equipped with a PDA detector and operated with Chromeleon 7 software. The mobile phase used for the HPLC analysis comprised of 0.05 M potassium dihydrogen phosphate (KH_2PO_4) for phase A and absolute methanol for phase B. The column used was an Inertsil ODS, C18, 250 x 4.6 mm, 5.0 μm and the flow rate was maintained at 0.6mL/min. The injection volume was 50 μL and the column temperature was maintained at 40°C throughout the assay. For the gradient the flow rate was maintained at 0.6mL/min with a mobile phase comprising 95% A and 5% B until 3 min after which the mobile phase was changed to 90% A and 10% B and the gradient run for another ~3 minutes. From ~6 min to 10 min, A and B were in the ratio of 85% and 15% respectively and the flow rate was increased to 1.5 mL/min. From ~10.1 min to 12 min the mobile phase composition was adjusted back to 95% A and 5% B, and the flow rate was maintained at 1.5 mL/min. From ~12 to 15 min, the flow rate was reduced to 0.6mL/min with the same mobile phase composition 95% A and 5% B respectively (Table S1).

Table S1: HPLC gradient method

Time	Flow	% A	% B
0.01	0.6	95	5
3.0	0.6	95	5
3.1	0.6	90	10
6.0	1.5	85	15
10.0	1.5	85	15
10.1	1.5	95	5
12.0	1.5	95	5
12.1	0.6	95	5
15.0	0.6	95	5

Docking of ascorbic acid and citric acid with human PDE5A

The objective of this docking study was to explore the potential interaction of ascorbic acid and citric acid with human PDE5A to investigate whether these molecules can bind to the active site of PDE5A, possibly influencing its enzymatic activity. The docking simulations were carried out using AutoDock Vina, a widely used molecular docking tool. The ligand and PDE5A receptor (PDB ID: 1XOZ) were obtained from PubChem and PDB database respectively. Water molecules and co-crystallized ligands were removed, and the protein was prepared for docking using AutoDockTools. The grid box was centered around the known active site of PDE5A to define the docking area and docking was run to explore the best binding for analysis.

RESULTS

Tables S2 to S5 summarizes the details of formulations for Tadalafil, Avanafil, Sildenafil and Vardenafil in the dosage forms marketed commercially for ED and other clinical indications.

Optimization of PDE5 assay parameters

The activity of an enzyme is affected by various factors, including temperature, pH, substrate concentration, and incubation time. In this study, we investigated the impact of incubation time and pH on PDE5A activity and identified the optimal conditions for evaluating the influence of excipients on the target enzyme.

Table S2: List of excipients used in the tablet formulations for the treatment of Erectile dysfunction.

Product	Viagra®	Cialis®	Levitra®	Stendra®
API	Sildenafil (100 mg)	Tadalafil (20 mg)	Vardenafil (20 mg)	Avanafil (100 mg)
Tablet core	Microcrystalline cellulose	Microcrystalline cellulose	Microcrystalline cellulose	Mannitol
	Calcium hydrogen phosphate (anhydrous)	Hydroxypropylcellulose	Colloidal anhydrous	Hydroxypropylcellulose
	—	—	Silica	Low substituted hydroxypropylcellulose
	Croscarmellose sodium	Croscarmellose sodium	Crospovidone	Calcium carbonate
	Magnesium stearate	Magnesium stearate	Magnesium stearate	Magnesium stearate
Film coat	Hypromellose	Hypromellose	Hypromellose	—
	Titanium dioxide (E171)	Titanium dioxide (E171)	Titanium dioxide (E171)	—
	Lactose monohydrate	Lactose	—	—
	Triacetin	—	—	—
	Indigo carmine aluminium lake (E132)	Ferric oxide yellow CI77 492	Ferric oxide yellow (E172)	Ferric oxide yellow (E172)
	—	Sodium lauryl sulfate	Ferric oxide red (E172)	—
	—	Talc - purified	Macrogol 400	—
	—	Glycerol triacetate	—	—

Table S3: List of excipients used in formulations for the treatment of Pulmonary hypertension

Product	Revatio® Sildenafil Tablet	Revatio® Sildenafil Powder for oral suspension (10 mg/mL)	Revatio® Sildenafil injection solution (0.8 mg/mL)	Tadliq® Tadalafil USP (0.4 % w/v) oral suspension	Adcirca® Film coated almond shaped Tadalafil tablet (20 mg)	Opsynvi® Macitentan (10 mg), Tadalafil (40 mg) Tablet
Tablet core / Excipients for powder for oral suspension						
	Microcrystalline cellulose	Sorbitol (E420) (250 mg/ml)	Water	Purified water	Microcrystalline cellulose	Microcrystalline cellulose
	Calcium hydrogen phosphate (anhydrous)	Citric acid anhydrous	Dextrose (50.5 mg/ mL)	Citric acid monohydrate	Hydroxypropyl cellulose	Hydroxypropyl cellulose (low substituted)
	Croscarmellose sodium	Sucralose	—	Sucralose powder	Sodium lauryl sulfate	Sodium lauryl sulfate
	Magnesium stearate	Sodium citrate (E331)	—	—	Croscarmellose sodium	Sodium starch glycolate
	—	Xanthan gum	—	Xanthan gum	Magnesium stearate	Magnesium stearate
	—	Titanium dioxide (E171)	—	Trisodium citrate dehydrate	—	Polysorbate 80
	—	Sodium benzoate (E211) (1 mg/ml)	—	Sodium benzoate	—	—
	—	Silica, colloidal anhydrous	—	Simethicone emulsion	Talc	Talc
Film coat / Excipients for powder for oral suspension						
	Hypromellose	Grape flavour: Natural flavouring	—	Glycerin	Hypromellose	Hydroxypropyl methylcellulose
	Titanium dioxide ((E171)	Maltodextrin	—	Polysorbate 80	Titanium dioxide	Titanium dioxide
	Lactose monohydrate	Grape juice concentrate	—	Frozen pepper-mint flavor	Lactose monohydrate	Lactose monohydrate
	Triacetin	Gum acacia	—	—	Triacetin	Triacetin
	—	Pineapple juice concentrate	—	—	Iron oxide	Iron oxide red
	—	Citric acid	—	—	—	Iron oxide yellow

Table S4: List of excipients used in tablet formulations for the treatment of Benign Prostatic Hyperplasia

Name	Tadalafil CIALIS®	Tadalafil CHEWTADZY™
Active	Tadalafil (5 mg) film coated Tablet	Tadalafil (5 mg) uncoated chewable Tablet
Tablet core/Powder for oral suspension		
	Hydroxypropyl cellulose	Hydroxypropyl cellulose
	Microcrystalline cellulose	Microcrystalline cellulose
	Croscarmellose sodium	Croscarmellose sodium
	Magnesium stearate	Magnesium stearate
	Sodium lauryl sulfate	Sodium lauryl sulfate
	Talc	Colloidal silicon dioxide
Film coat		
	—	Bubblegum flavor (acacia, artificial and natural flavors
	Hypromellose	Mannitol
	Titanium dioxide (E171)	FD&C blue No. 1 aluminum lake
	Lactose monohydrate	Lactose monohydrate
	Triacetin	Sucralose
	Iron oxide	—

Table S5: List of excipients used in tablet formulations for the treatment of Raynaud's phenomenon

Name	Sildenafil Viagra®	Tadalafil Cialis®
Active	Sildenafil (100 mg) film coated Tablet	(20 mg) Tadalafil LP film coated Tablet
Tablet core		
	Microcrystalline cellulose	Microcrystalline cellulose
	Calcium hydrogen phosphate (anhydrous)	Hydroxypropylcellulose
	Croscarmellose sodium	Croscarmellose sodium
	Magnesium stearate	Magnesium stearate
Film coat		
	Hypromellose	—
	Titanium dioxide (E171)	Titanium dioxide
	Lactose monohydrate	Lactose
	Triacetin	—
	Indigo carmine aluminium lake (E132)	Iron oxide yellow CI77 492
	—	Sodium lauryl sulfate
	—	Talc
	—	Glycerol triacetate

Optimization of the incubation time on PDE5 assay

The PDE5 assay was performed with varying incubation times of exposure of enzyme with the substrate for 1.5, 3, and 6 h, keeping the other parameters including temperature, cGMP concentration, enzyme concentration, and reaction volume constant. It was observed that PDE5 activity increased as the incubation time of the enzyme with the substrate was extended from 1.5 hours to 6 hours (Fig. 1). In comparison to 22% to 23% conversion of cGMP achieved after 1.5 h (Fig. 1A), the cGMP conversion was 41% and 59% at the end of 3hr and 6hr respectively (Fig. 1B and Fig. 1C). Since the PDE5 activity increase was marginal, i.e.,18%, from 3 hours to 6 hours, a 3-hour incubation time was selected for further studies for reasons of reduced cost and operational efficiency.

Optimization of pH of the PDE5 assay mixture

Enzyme activity is highly sensitive to pH, making the optimization of pH conditions critical for maximizing performance of any enzyme. This optimal pH for

Fig. 1. Effect of assay incubation time on PDE5 activity

Panel A shows the cGMP and GMP peaks using human PDE5A1 enzyme under the conditions described in M & M section. Note the RT of cGMP as 9.94 min while it is 3.77 min for GMP. PDE5 activity of 41 % and 59 % was achieved at 3 h (Panel B) and 6 h of incubation (Panel C) respectively.

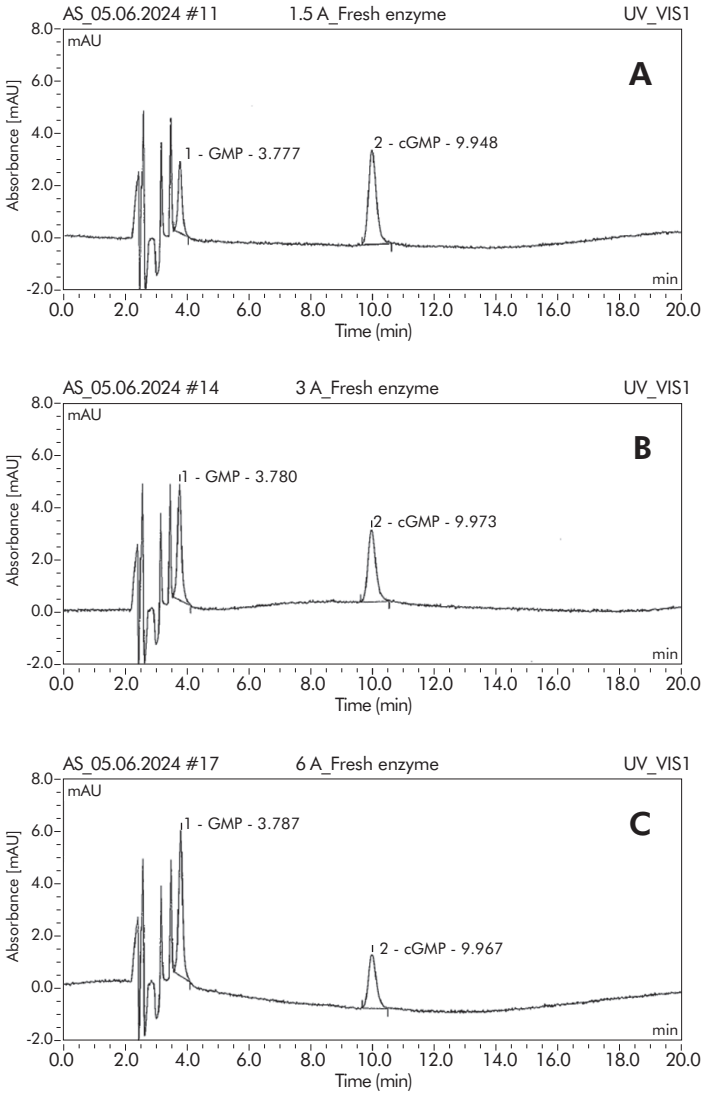
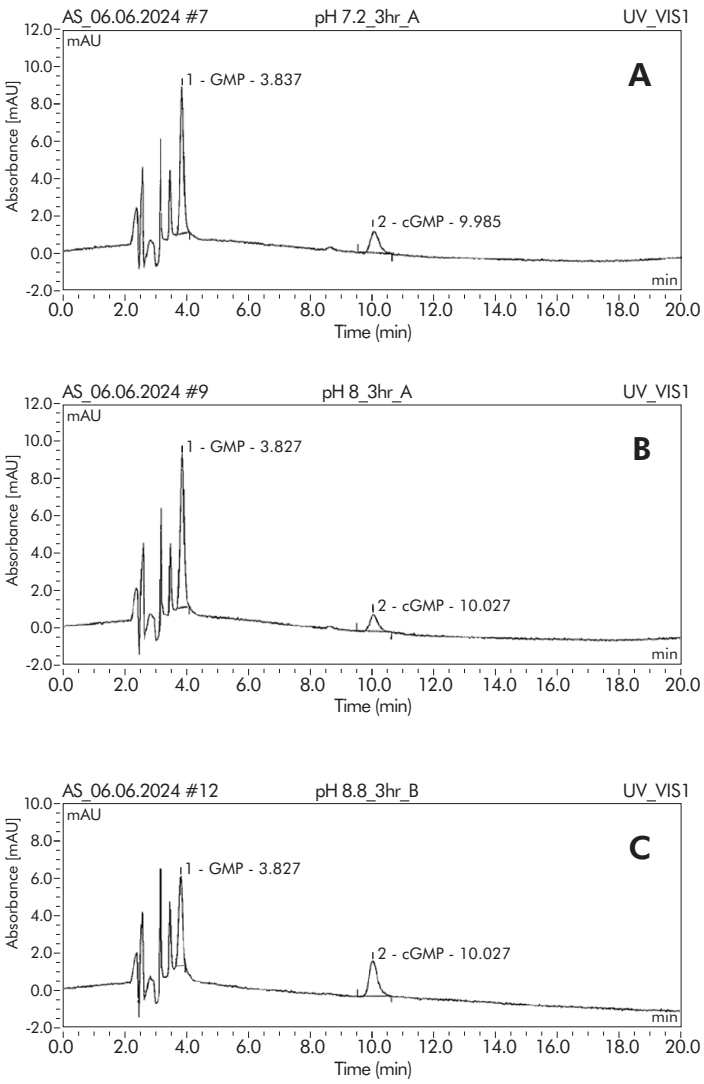


Fig. 2. Effect of pH on PDE5 activity and analysis by HPLC

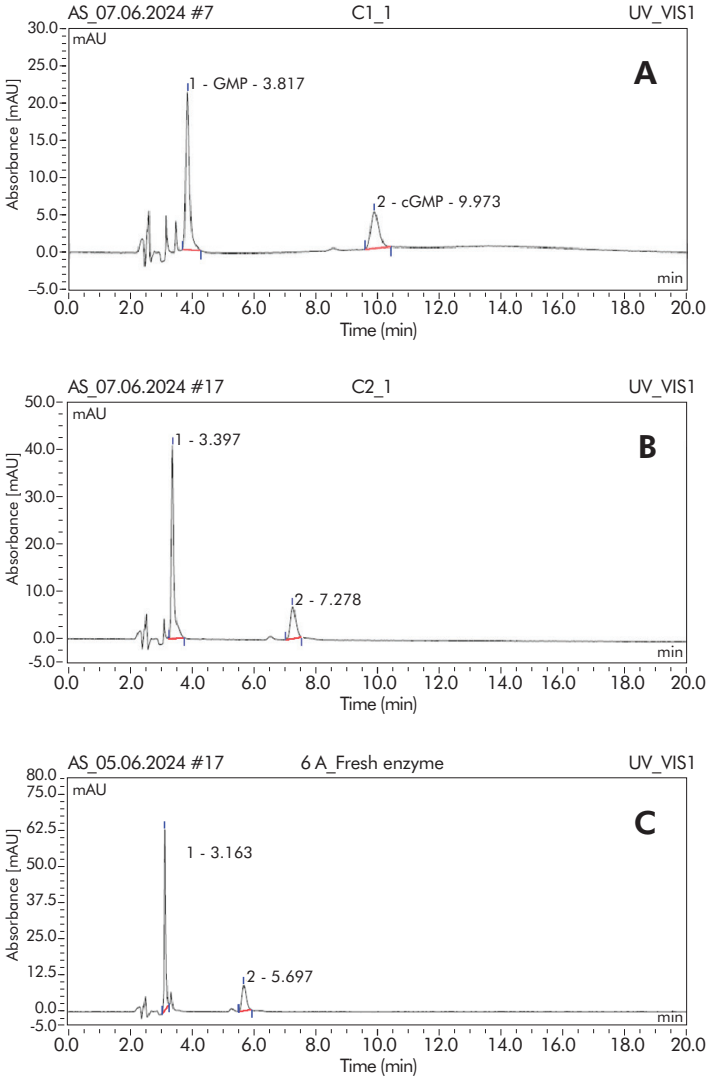
The % PDE5 activity was 75%, 82% and 56% at pH 7.2 (Fig. A), 8.0 and 8.8 (Fig. B) respectively. Note the AUC of the GMP decreasing at pH 8.8 (Fig. C).



PDE5A1 enzyme activity was identified by systematically varying pH levels and evaluating their effects on enzymatic performance. The pH of the PDE5 assay mixture was optimized by studying various pH values 7.2, 8.0 and 8.8, while keeping the other parameters constant. It is depicted in Fig. 2, that PDE5 activity was decreased as pH of the assay mixture increased. The % cGMP conversion at pH 7.2, 8.0 and 8.8 was 75%, 82% and 56% respectively as seen in Figures 2A, 2B and 2C, respectively. This is consistent with reports of similar pH values being used for PDE5 assays using radiolabeled and protein substrates (26-29).

Fig. 3. Effect of HPLC column temperature on efficiency of separation of GMP and cGMP on HPLC

PDE5 assay was subjected to variations in column temperatures of 30°C, 40°C and 50°C and the cGMP and GMP levels in all samples after the assay period were analyzed by HPLC. Note the closer elutions of both the substrate and the product as the HPLC column temperature increases. The retention time of cGMP and GMP was 9.8 min and 3.8 min when the column temperature was 30°C (panel A) while it was 7.28 min and 3.39 min (panel B) at 40°C column temperature and it was 5.69 min and 3.16 min, at the column temperature was 50°C.



Optimization of column temperature on PDE5 assay

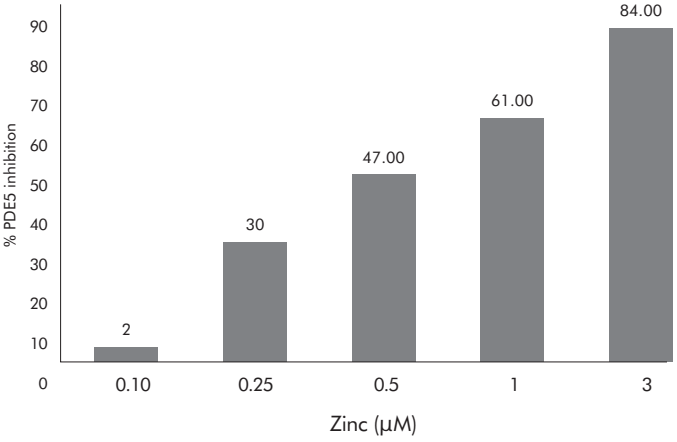
Nearly 34% less conversion of cGMP to GMP was observed at 30°C (Fig 3A) in comparison to the assay with the column temperature of 40°C (Fig. 3B) and 23% of activity observed at column temperature of 50°C (Fig. 3C). A 40°C column temperature was selected for subsequent experiments because retention times between the substrate and the product were shorter as the temperature of the column increased.

Effect of zinc on PDE5 activity

Conducting the PDE5 assay in the presence of zinc chloride showed that Zn increases the inhibition of PDE5 in a dose-dependent manner (Fig. 4). A concentration of 0.5 µM Zn inhibited ~ 47 % of PDE5 activity.

Fig. 4. Effect of zinc concentrations on PDE5 activity

PDE5 assay was carried out at different zinc concentrations in the assay mixture. It is evident from the data that almost 84% PDE5 inhibition was observed when the zinc concentration was 3 µM, and up to 30% inhibition was observed with 0.25 µM zinc concentration. At 0.1 µM, there was negligible effect of zinc on PDE5 activity.



Excipients used in various formulations of drugs affecting PDE5 activity

To investigate the influence of excipients on PDE5A activity, excipients listed in Tables S2 to S5, that are included in formulations of PDE5 inhibitors, were assessed for their solubility. Water and DMSO were chosen as solvents due to their compatibility with the PDE5 assay. Excipients were dissolved at a concentration of 10 mg/ml in these solvents, with stirring conducted at 70°C for an appropriate duration to ensure complete solubilization. The resulting clear solutions of excipients were then used for the PDE5 assay after dilution to a final concentration of 0.05% (w/v).

Effect of excipients on the PDE5 assay

Excipients dissolved in either water or DMSO were assessed for their impact on the PDE5 assay. The excipients listed in Table 1 demonstrated PDE5 inhibitory activity by effectively slowing or reducing the conversion of cGMP to GMP. Of mention would be excipients such as citric acid, benzalkonium chloride and sodium lauryl sulphate that caused 100% inhibition of the PDE5 activity under the conditions tested. It is also interesting to note that excipients such as Tween 20, Tween 60 and Tween 80, produced more than 200% activation of the PDE5 activity , with similar effects being activity observed with PEG 6000 and Hypromellose Table 2. The excipients that were found to be inert for PDE5 activity are listed in Table 3.

Table 1: List of excipients found as PDE5 inhibitors

Sr. No.	Excipients*	% Inhibition
1	Hydroxypropyl β-cyclodextrin	18.54 (0.324 mM)
2	Citric acid (2.6 mM)	100
3	Benzalkonium Cl (1.6 mM)	100
4	Sodium lauryl sulfate (1.7 mM)	100
5	Sodium hyaluronate (1.19 mM)	68.89
6	Thymol (3.32 mM)	100
7	Ascorbic acid (2.83 mM)	92.89
8	Sodium propyl paraben (2.47 mM)	100
9	Sodium starch glycolate 3668 (0.0004 mM)	39.11
10	Starch (1.46 mM)	66.91
11	Stearic acid 352754 (1.75 mM)	50.9
12	Zinc (0.5 µM)	47

*Figure in brackets refers to excipients concentration per assay

Table 2: List of excipients found as PDE5 activators

Sr. No.	Excipients*	%Inhibition
1	α-Cyclodextrin (0.514 mM)	24.39
2	Methyl β-cyclodextrin (0.34 mM)	58.31
3	Polysorbate/tween-20 (0.41 mM)	319.25
4	Polysorbate/tween-60 (0.38 mM)	259.42
5	Polysorbate/tween-80 (0.38 mM)	298.96
6	Sodium benzoate (E211) (3.47 mM)	16.22
7	Polyethylene glycol-6000 (0.08 mM)	219.78
8	Carrageenan-I (0.002 mM)	20.67
9	Carrageenan-λ (0.863 mM)	120.69
10	Mannitol (2.74 mM)	27.2
11	Hypromellose (0.39 mM)	224
12	Carbomer	27.2
13	tri-Sodium Citrate (1.93 mM)	15.06
14	Ethyl cellulose(1.1 mM)	27.2
15	Cetosteryl alcohol BP (0.97 mM)	19.15

*Figure in brackets refers to excipients concentration per assay

Table 3: List of excipients found inert to PDE5 activity

Sr. No.	Excipients*	% Inhibition
1	Hydroxyethyl β-cyclodextrin (0.36 mM)	ND
2	Betadex sulfobutyl ether sodium (0.37 mM)	ND
3	Sodium citrate (1.93 mM)	ND
4	Xanthan gum (2.1 mM)	ND
5	Polyethylene glycol-400	ND
6	Polyethylene glycol-4000	ND
7	Carrageenan-K (0.63 mM)	ND
8	Glycerin (5.42 mM)	ND
9	Sorbitol (2.74 mM)	ND
10	L-arginine (2.87 mM)	ND
11	Mixed fruit flavour	ND
12	Cetyl Alcohol (2.06 mM)	ND
13	Agar Agar (1.48 mM)	ND
14	Orange flavor	ND
15	Lactose	ND

*Figure in brackets refers to excipients concentration per assay

Table S6: Comparison of concentrations of selected excipients in ED formulations with concentrations influencing in vitro results for the PDE5 assay

Sr. No.	Literature reference	Excipient	In-Vitro PDE5 assay; Conc. of excipients/well	Conc. of excipients in Tablet/ suspension	API used in formulation
1	WO2017168174A1	Sodium lauryl sulphate	1.734 mM	112.45 mM	Sildenafil
2	ZIMAGRA	Starch	1.46 mM	1.05 M	Sildenafil
3	ZIMAGRA	Sodium starch glycolate	0.4 µM	8.56 µM	Sildenafil
4	US7182958	Polysorbate-80	0.382 mM	2.13 mM	Tadalafil
5	WO2017168174A1	Citric acid	2.6 mM	1.41 M	Sildenafil
6	AU2017368232A1	Benzalkonium chloride	1.603 mM	0.48 M	Tadalafil
7	US6943166	Hypromellose	0.396 mM	15.36 mM	Tadalafil
8	ZIMAGRA	PEG-6000	0.08 mM	0.143 mM	Sildenafil

To ensure that the concentration of the excipients used for the PDE5 assays is well within the acceptable limits of these excipients found in the various dosage forms, we examined various label claims of several ED products for the exact concentration of excipients. No useful information was obtained from these sources so information from published patents was used to estimate the molar concentrations of these agents in ED patented formulations. The data presented in Table S6 clearly show that the concentration of excipients used for testing in the PDE5 assay *in-vitro* is lower than the molar concentrations in the ED formulation patents.

cGMP stability study in the presence of excipients

No significant changes were observed in the HPLC area of cGMP afer incubation with excipients, suggesting that cGMP remains stable under the conditions of this test. After incubation with the four excipients (two inhibitors and two activators) the cGMP peak area was at least 97.3% of the cGMP control (Table S7).

Table S7: Stability of cGMP in the presence of different excipients under conditions used for the complete assay process for HPLC detection of PDE5 activity.

Sr. No.	Combination	HPLC Area (%)
1	CGMP	84742 (100.0)
2	Citric acid + cGMP	84163 (99.1)
3	Ascorbic acid + cGMP	82477 (97.3)
4	Hypromellose + cGMP	82805 (98.0)
5	Tween-80 + cGMP	84539 (99.8)

Figure 5: PDE5 inhibition study in presence of (A) Citric acid, (B) Ascorbic acid while panels C and D shows PDE5 activation of PDE5 enzyme in the presence of (A) Tween-80 and (B) Hypromellose under the experimental conditions described in M & M section activation. The concentrations of 0.5 µg, 2 µg and 4 µg of citric acid/reaction equates to 5.2 µM, 20.8 µM and 41.66 µM respectively while 4 µg, 7.5 µg and 10 µg ascorbic acid/reaction equates to 45.45 µM, 85.22 µM and 113.63 µM respectively taking the molecular weight of citric acid and ascorbic acid as 192 and 176 daltons respectively. Similarly molecular weight of hypromellose was taken as 1261 daltons and its concentrations of 0.25µg, 1 µg and 2.5 µg 2.5 / reaction equates to 396 nM, 1.5 µM and 3.9 µM respectively while tween 80 (MW 1310), at 0.25 µg and 2.5 µg/ reaction refers to 381 nM and 3.8 µM respectively.

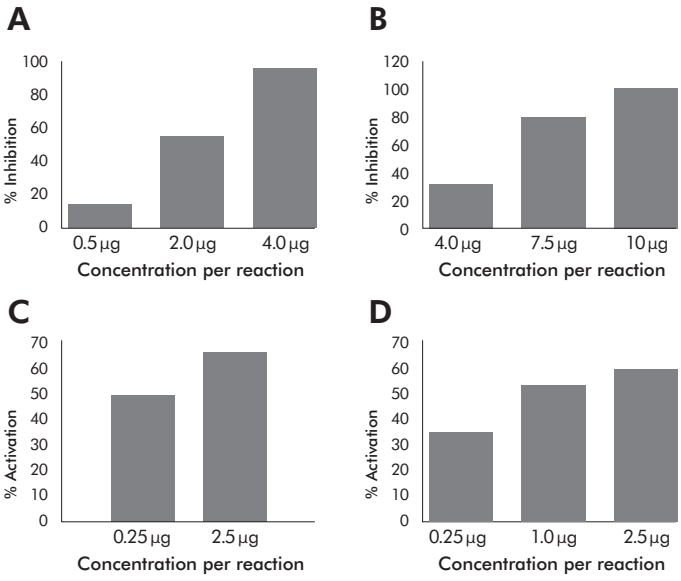
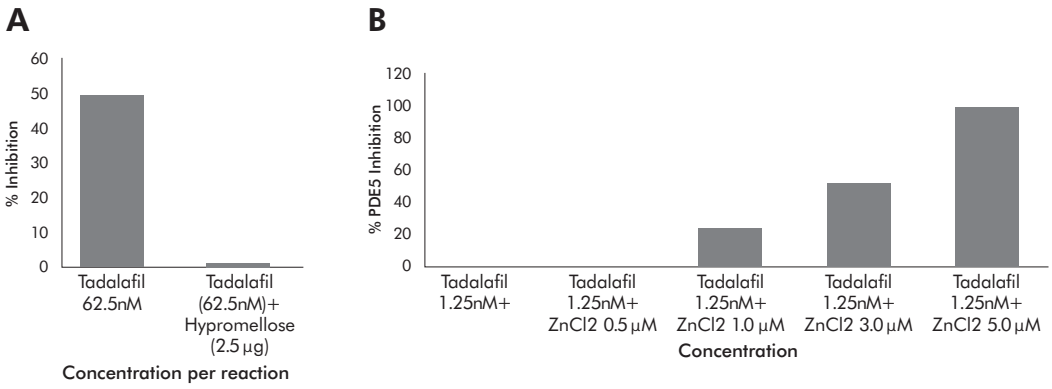


Figure 6: PDE5 inhibition activity of Tadalafil in absence and presence of excipient activator and excipient inhibitor. Panel A shows effect of hypromellose on % activation of PDE5 causing loss of Tadalafil's anti-PDE5 activity while panel B shows the dose dependence PDE5 inhibition activity of zinc on Tadalafil's anti-PDE5 activity.



Dose-dependency of activation and inhibition of PDE5 by excipients

Fig. 5 shows the dose dependency of several excipients (inhibitors and activators) on PDE5 activity. Panel A and panel B show %PDE5 inhibition by citric acid and ascorbic acid respectively at three different concentrations while panels C and D show PDE5 activation by Tween 80 and Hypromellose at different concentrations. The effect on PDE5 activity was observed in the micromolar range for these excipients.

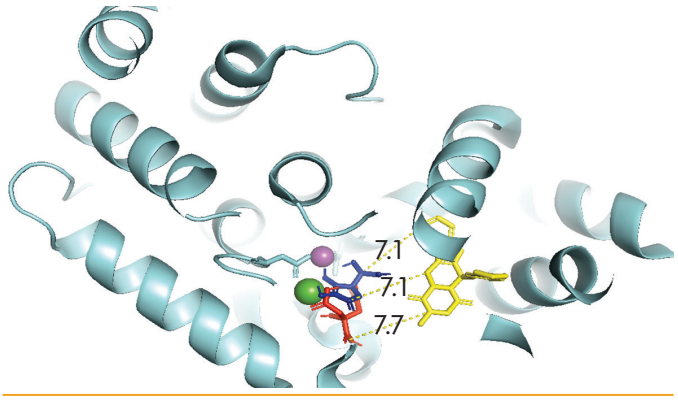
Tadalafil's anti-PDE5 activity with and without a selected excipient inhibitor and activator

It is evident from Fig. 6, panel A, a reduction of around 50% in PDE5 inhibition was observed when 62.5 nM Tadalafil was tested without Hypromellose. However, this effect was reduced when the same concentration of Tadalafil was tested in the presence of Hypromellose (7.9µM). In contrast, using a concentration of Tadalafil,1.25nM, producing negligible PDE5 inhibition activity on its own, however the coadministration of zinc gave dose-dependent increases in PDE5 inhibition, Fig. 6, panel B.

Docking of ascorbic acid and citric acid with human PDE5A:

The binding energies for the docking of PDE5A with ascorbic acid and citric acid were found to be -5.6 kcal/mol and -5.2 kcal/mol, respectively. These favorable docking scores suggest that ascorbic acid and citric acid could potentially inhibit the PDE5A enzyme. Citric acid forms multiple hydrogen bonds with key amino acids in the active site of PDE5A, including Asp654, His657, Glu682, Thr723, Leu725, and Asp764. In contrast, ascorbic acid forms hydrogen bonds with His613, His617, Asp654, and Asp764 of PDE5A. The docked structures of citric acid and ascorbic acid with human PDE5A were compared to the structure of human PDE5 bound with Tadalafil (PDB ID: 1XOZ), using structural superimposition in PyMOL (a protein structure visualization tool). Both citric acid and ascorbic acid docked at the catalytic site of human PDE5A, near the tadalafil binding site, in the same catalytic pocket with a distance of 7.0–7.7 Å towards the magnesium binding site (Fig. 7) indicating that the observed inhibition of PDE5 activity is through specific affinity of these excipients to the catalytic site of PDE5. It may also be possible that some other excipient molecules may bind to different sites, potentially leading to inhibition or activation through alternative mechanisms, but such speculation requires further investigation.

Figure 7: The catalytic site of human PDE5A (represented as a cyan cartoon) is shown with docked ascorbic acid (blue stick) and citric acid (red stick). Structural superimposition with PDB structure 1XOZ shows Tadalafil (yellow stick) positioned approximately 7.0 Å from the docked site at the catalytic binding site. Magnesium and Zinc are shown with green and magenta sphere.



Synergistic effect in PDE5 inhibition by citric acid and ascorbic acid.

The data presented in Table S8 demonstrates a synergistic effect in PDE5 inhibition by citric acid and ascorbic acid. When used separately, citric acid at 5.2µM and ascorbic acid at 45µM both inhibit PDE5, but their combined effect at the same concentrations leads to enhanced inhibition, suggesting a synergism between the two.

Table S8: Synergistic effect in PDE5 inhibition by citric acid and ascorbic acid

Excipients	% PDE5A inhibition
Citric acid (5.2 µM)	14
Citric acid (20.8 µM)	54
Ascorbic acid (45 µM)	31
Ascorbic acid (85 µM)	79
Citric acid (5.2 µM)+ Ascorbic acid (45 µM)	82
Citric acid (5.2 µM)+ Ascorbic acid (85 µM)	100
Citric acid (20.8 µM+ Ascorbic acid (45 µM)	100
Citric acid (20.8 µM)+ Ascorbic acid (85 µM)	100

DISCUSSION

An alternative PDE5 assay has been developed that is both facile and cost effective. Overall this method utilizes three times less enzyme per reaction and a seven fold reduction in the amount of cGMP as the substrate compared to other published assays (22). In the optimized conditions described in the current work the amount of PDE5A1 enzyme used per assay is well within the linear range of 0.025 ~ 0.30µg/mL as described by Li and co-workers (22). Our HPLC method for the PDE5 assay is less costly to implement and run compared to assays using LC-MS methods (30) and can be easily adopted in labs engaged in similar research.

The PDE5 assay developed was used in the evaluation of several PDE5 inhibitors for relative potency using *in vitro* methods. Given that all commercially available products of PDE5 inhibitors are formulated with excipients, we also wanted to investigate whether these important materials would have any influence on the *in vitro* performance of ED inhibitors.

Results demonstrate that some of the excipients used in formulations of ED drugs enhance PDE5 activity *in vitro*, while some inhibit the PDE5 activity *in vitro*. It is not possible to determine any clinical significance based on these findings, but they highlight an opportunity for a simple test to determine whether certain excipients could potentially interfere with the performance of a PDE5 drug/product in terms of efficacy or in terms of affecting side effects commonly associated with existing PDE5 inhibitors, such as headaches, vision impairment etc. (31). Our new method might also be valuable for the evaluation of herbal products that may be adulterated with approved ED drugs (11-14).

Results from testing several different classes of excipients also showed that certain excipients that had no effects on *in vitro* performance of several ED drugs. These alternative "inactive" excipients provide opportunities to design drug products that would be less likely to influence *in vitro* performance of PDE5 drugs, which theoretically could provide benefits *in vivo* but this is highly dependent on a lot of other factors (see later). For example, PEG-400 and PEG-4000 could serve as superior alternatives to Hypromellose for film coating. Similarly, Hydroxyethyl-cyclodextrin, Betadex sulfobutyl ether sodium, PEG-400, PEG-4000, and xanthan gum could replace Tween, β-cyclodextrin, methyl β-cyclodextrin, PEG-6000, carbomer and cetosteryl alcohol as solubilizers, wetting agents, stabilizers, emulsifier etc. Benzalkonium chloride could be a replacement for sodium benzoate as a preservative and xanthan gum could be a gelling agent in place of carrageenan.

There is an increasing number of published reports highlighting putative pharmacological activity of excipients. Our study provides further evidence of excipients having the potential to engage with a pharmacological target *in vitro*. This knowledge could be useful to raise awareness for design of formulations. For instance, zinc might be regarded as a appropriate supplement in formulations for erectile dysfunction because a deficiency of Zn is one of the causes of ED and zinc promotes initiation and retention of penile erection (32). Although Zn is reported to inhibit ~50 % of PDE5 activity at 3 µM concentration (25), our paper discloses that Zn as low as 0.1 µM shows almost 25% inhibition of PDE5 activity. Since zinc promotes initiation and helps to retain penile erection, this metal ion might

appear to offer advantages as an additive/excipient in formulations for erectile dysfunction drugs. However, while there appear to be potential merits to this proposal, the other reported properties of zinc need to be understood. It plays an essential role in male sexual function, such as in the testicular, prostate glandular epithelium, generation of testosterone, erectile function, and sexual behavior (33). High zinc levels have shown a negative effect of increasing doses of zinc on sperm count, quality, and motility; hence the use of zinc needs careful consideration (34).

A report by Pottel *et al.* (35) highlights an important discovery regarding excipients wherein their large-scale computational screening identified nearly 38 excipients with potential activity against 44 biological targets. Notably, some of these excipients exhibited tissue-level toxicity, and two are indicated to reach *in vivo* concentrations that may overlap with their observed *in vitro* activities. Our study highlights the impact of excipients on PDE5 activity *in vitro* and describes a method for conducting specific activity studies on formulation components of marketed products for ED.

Evidence that excipients possess some level of pharmacological activity may also imply a possible influence on the side effect profile of a medicinal product. This could be particularly relevant for chronic use medications that patients need to take for a long time with consequent ingestion of excipients for years and years (36). A review of the interaction of selected excipients on pre-systemic metabolism by CYP450 enzymes also demonstrates the need for careful selection of excipients to maximize drug bioavailability and minimize side effects (37). Our disclosure of unreported effects of certain excipients on PDE5 activity *in vitro* is another example of the importance of understanding the effects of all formulation components in a drug product, not just the API.

Other published work highlighting the impact of excipients on pharmacological activity of an active substance includes a recent study by Zakowiecki *et al* (38) describing excipients in formulations that allow tableting over a wide range of compaction pressures without adversely affecting pancreatin content and its enzymatic activity. Furthermore, Löhr *et al* (39) found significant variation in the enzyme activity of marketed formulations compared to label claims such as for lipase that changed from 93% to 115%, for amylase that ranged from 97% to 233%, and for protease that varied from 120% to 281%. These differences could be attributed to role of excipients affecting the pharmacological action of the active. Biological products currently represent a huge percentage of pharmaceutical portfolios and recently marketed products. In a study by Ashutosh *et al* (40), currently used excipients in various protein therapeutic products were assessed leading to recommendations to use common excipients across biotherapeutic products to minimize adverse reactions, interactions, or problematic quality profiles associated with various biotechnology-derived drug products.

Development of a straightforward and cost-effective assay is essential for facile investigation of possible drug-excipient interactions. Several procedures have been developed for analyzing PDE5 inhibitor activity and each assay offers advantages and limitations. Published methods include using liquid scintillation analyzer for radioactive substrates (41), HPLC with diode array detection and liquid chromatography-electrospray ionization tandem mass spectrometry (42), liquid chromatography-high-resolution mass spectrometry (LC-HRMS) (43), HPLC with diode array detection (44), High-Performance Liquid Chromatography with Ultraviolet Detection using fluorescent substrates (45), U-HPLC–HRMS/MS technique (46). Selection of an effective PDE5 assay method relies on achieving specific experimental requirements e.g., for specificity and sensitivity, and the assay method should be free from interferences, user-friendly and cost-effective. Compared with the assays involving isotope labeling or fluorescent tagged cGMP, assays involving substrate conversion of cGMP using HPLC is simpler and easy to operate. Therefore, our current study highlighting the use of simple HPLC to measure PDE-5 inhibitor activity represents a straightforward approach for researchers in regulatory agencies and laboratories concerned with the safety and quality of these specific therapeutics.

Translation of drug-excipient interactions *in vitro* to performance of drugs, or products, *in vivo* is difficult. Many factors influence the behavior of a drug at its target site including its activity, specificity, ADME of the molecule, and other factors such as the performance of the dosage form/delivery system in which it is administered. These factors also apply to an excipient in terms

of its distribution around the body and the concentration it achieves at various sites. The current study provides a good example of why caution is needed when extrapolating from *in vitro* data. That is, Hypromellose was shown to completely abolish the PDE5 inhibition activity of Tadalafil using an *in vitro* assay. However the likelihood that this effect of Hypromellose would have any significance on performance of oral doses of Tadalafil in humans is very low because Hypromellose is not absorbed from the human GI tract. There are other excipients in our work that showed apparent activity *in vitro* as well as being orally available in humans (such as ascorbic acid and citric acid). While the results indicate there is chance that these materials could impact performance of a co-administered PDE5 drug, the ramifications of these findings should be put into context with the multitude of factors that would need to be overcome for this interaction to occur at precisely the right order of magnitude of effect, at the right time and at the right location in the body. While it is challenging to determine any likely clinical implications of results from the *in vitro* work, the findings are nevertheless valuable to increase awareness of how certain excipients may have pharmacologic effects and are not simply inert carriers/fillers.

CONCLUSION

An alternative method has been developed for detecting the reduction of cGMP caused by PDE5 hydrolysis. This simple and straightforward approach is based on using HPLC as the detection method for determination of enzyme reaction activity. Subsequent work was focused on exploring the effects of various excipients on PDE5 activity, and the impact of these materials on the in vitro activity of a widely used PDE5 inhibitor, Tadafil. Overall, the article provides an in-depth analysis of the excipients used in FDA-approved PDE5 inhibitor formulations and the effect these excipients can have on modulating PDE5 activity. It identifies several excipients (eg. Hypromellose, ascorbic acid etc.) that can either inhibit or activate PDE5, with corresponding effects on the activity of ED drugs *in vitro*. It is not possible to determine any clinical ramifications of the results *in vitro* but the data are consistent with increased published evidence of drug-excipient interactions *in vitro*. At the very least, the data presented in our work highlights that many excipients can interfere with the PDE5 assay, indicating pharmacological effects *in vitro,* and therefore are not the inert carriers and fillers that they were described as in the past.

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AUTHORS' CONTRIBUTIONS

Aditya Dev planned and optimized all experiments related to PDE5 assays and analyzed the experimental data. While Somesh Shintre and Sanman Kolhe carried out the HPLC assays, Damini Jadhav carried out solubility of the excipients and carried out experiments on their effects on PDE5 activity by HPLC. Komal Bhagwat supervised experimentations of Ms. Damini Jadhav, formatted the tables, and prepared the first draft of the manuscript. The project conceptualization, experimental design, data interpretation and manuscript preparation was carried out by Sriram Padmanabhan.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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