RESEARCH ARTICLE

Developing a phosphodiesterase-5 inhibitor assay to detect counterfeit drugs containing phosphodiesterase-5 inhibitors using spectrophotometry [version 1; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background: Phosphodiesterase-5 inhibitors (PDE5i) are the first line of drugs used for the treatment of erectile dysfunction. PDE5i inhibits the activities of phosphodiesterase-5 (PDE5) found in corpus cavernosum reducing production of guanosine monophosphate (GMP) that results in vasodilation of blood vessels thereby prolonging penile erection. Various counterfeit drugs adulterated with unknown levels of PDE5i pose a danger with reported undesirable adverse effects on patients.

Methods: We developed an assay to detect counterfeit drugs containing PDE5i using spectrophotometry based on a colour change of malachite green from blue to green when exposed to inorganic phosphate (Pi) measured at 630 nm. Initially, a standard graph of a known Pi concentration was established ranging from 0 to 20 μ M followed by a dual biochemical reaction system of converting GMP from cGMP by PDE5, generating Pi from the first reaction's product by calf intestinal alkaline phosphatase (CIAP), and then measuring Pi using an equation derived from the generated standard curve. Dilutions of CX1A, a counterfeit drug sample adulterated with the PDE5i sildenafil, were prepared via ultrasonication and filtration for assay testing and evaluation.

Results: Sil was detected from the two separate samples comprised of low and high dilutions of CX1A quantified as 0.673% and 0.407%, respectively, based on a standard curve of pure sildenafil established from 0.1 nM to 300 μ M.

Conclusions: This PDE5i assay that we developed has the potential to become an alternative analytical method for detecting PDE5i showing comparable results when evaluated using HPLC.

Open Peer Review

Approval Status ? ✓

	1	2
version 1	?	✓
26 Sep 2019	view	view
1. Dirk W. Lachenmeier , Chemical and Veterinary Investigation Agency Karlsruhe, Karlsruhe, Germany		
2. Adi Idris , Griffith University, Southport, Australia		

Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Phosphodiesterase-5 inhibitor, Sildenafil, counterfeit drugs, PDEi assay

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Introduction

Phosphodiesterase-5 inhibitors (PDE5i) are synthetic drugs for clinical treatment of erectile dysfunction prescribed only by a qualified physician¹. As PDE5 regulates penile erection by hydrolyzing cGMP, inhibiting the enzyme elevates cGMP level resulting in vasodilation and maintenance of penile blood flow. PDE5i are widely used in the illegal market as an adulterant in counterfeit drugs, especially in herbal supplements claiming the enhancement of male sexual vitality in a natural way². The use of these counterfeit drugs with unknown content has posed significant health risks to patients. Hence, we developed an assay for easier detection and quantification of counterfeit drugs containing PDE5i using spectrophotometry.

Herein, a known counterfeit herbal drug, CobraX (CX1A), was investigated to develop a potential analytical assay to detect PDE5i (i.e. sildenafil citrate) by utilizing the by-products (i.e. inorganic phosphate (Pi)) that were released from a reaction among the substrates comprised of cGMP, enzyme PDE5 and PDE5i (often adulterated in counterfeit drugs, i.e. CX1A). The colorimetric change in malachite green (MLG) assay was utilized to indirectly quantify the amount of Sil in counterfeit drugs through the detection of Pi released from the PDE5-mediated hydrolysed cGMP. The PDE5i assay that we developed in this study has been evaluated against HPLC and has shown high potential for laboratory and field kit applications to detect and measure the PDE5i content in any drug.

Methods

Materials and equipment

Potassium phosphate monobasic (KH_2PO_4), guanosine 3',5'-cyclic monophosphate (cGMP), polyvinyl alcohol (PVA), sildenafil citrate salt, calf intestinal alkaline phosphatase (CIAP), human phosphodiesterase 5A1 (PDE5), ammonium molybdate tetrahydrate (MolyB) and malachite green oxalate free base (MLG) were all obtained from Sigma-Aldrich. The adulterated drug, CobraX (CX1A) was kindly donated by Brunei law enforcement agencies. Biotek™ ELx808™ absorbance microplate reader (Thermo Fisher Scientific) was used to read absorbance measured at 630 nm in 96-well microplate-based assays by a spectrophotometric method using MLG as previously described³ was adapted with modifications.

The MLG assay used to detect the counterfeit PDE5i drugs consisted of complex formation between phosphomolybdate (MolyB) with Pi, which was released from the PDE5-mediated hydrolysed cGMP, to produce MolyB-Pi complex, which was subsequently used to bind with MLG oxalate. Under acidic conditions, the bound MLG-MolyB-Pi produced colour changes from blue to shades of green depending on the concentration of Pi³, based on the observation that high concentrations of Pi resulted in high formation of MLG-MolyB-Pi complexes and high absorbance values.

PDE5i detection assay

A functional PDE5i assay consisted of five steps. Figure 1 gives a schematic overview of the protocol.

Step 1: Establishment of standard curve for Pi. A fundamental part of the assay was to establish the limit range of Pi concentrations for the MLG assay. Using n=2 replications, 230 µl of Pi solution (0, 2, 5, 7.5, 10, 12.5, 17.5 and 20 µM) was prepared in 0.5 ml microtubes and 75 µl of stop solution (70% perchloric acid) was added. The solutions were mixed by vortex and 140 µl was aliquoted into each designated well of 96-well microplate for MLG assay.

Step 2: Dual biochemical reaction assays for PDE5-cGMP/GMP-CIAP (PDE5 reaction assay). The optimised PDE5 reaction assay was performed to obtain the total Pi generated per reaction, which was subsequently used to calculate the amount of sildenafil in counterfeit drug samples. For reactions replicated n=3 times, 20 µl of PDE5 solution (2000 unit/µg PDE5) and 0.0001 U/µl CIAP solution were added to a 0.5 ml microfuge tube containing 160 µl reaction buffer (20 mM Tris-HCL at pH 7, 10 mM MgCl_2 , 0.10 mM EDTA). The solutions were vortexed followed by addition of 30 µl of 15 mM cGMP and incubation for 30 min at 37°C. After incubation, 50 µl of stop solution was added to terminate the reaction and vortexed followed by centrifugation at 2000 rpm for 5 min. The solutions were then aliquoted into their designated wells in the 96-well microplate with a total volume of 140 µl per well for the MLG assay.

The percentage yield of Pi was calculated using the following formula:

$$\text{Percentage yield of Pi (\%)} = \frac{\text{No. of moles of generated Pi}}{\text{No. of moles of expected Pi}} \times 100$$

Step 3: Establishment of standard curve for sildenafil. The effect of different concentrations of sildenafil (1, 3, 10, 30, 100 and 300 nM) upon exposure to PDE5 reaction was investigated and subsequently used to generate a standard curve for pure sildenafil with n=4 repeats. First, 40 µl of PDE5-CIAP mixture was added to a 190 µl of sildenafil-cGMP mixture in a 0.5 ml microtube followed by incubation at 37°C for 30 min. After incubation, 50 µl of stop solution was added and mixed well using vortex followed by centrifugation at 2000 rpm for 5 min. The solutions were then aliquoted to their designated wells in a 96-well microplate with a total volume of 140 µl per well followed by MLG assay performance. The percentage of PDE5 activity of each concentration of sildenafil was calculated using the formula below:

$$\text{Percentage of PDE5 Activity (\%)} = \frac{\text{Absorbance of sildenafil}}{\text{Absorbance of optimum PDE5 reaction}} \times 100\%$$

Step 4: Quantification of sildenafil in CX1A samples via PDE5-CX1A reaction assay. A PDE5-CX1A reaction assay was performed to assess and quantify sildenafil in CX1A samples with n=2 repeats. To a 0.5 ml microtube containing 140 µl of reaction buffer, 20 µl of CX1A solution (0.1 mg/ml and 0.01 mg/ml) and 30 µl of 15 mM cGMP were added and mixed using vortex. For a reaction to start, 40 µl of PDE5-CIAP mixture was added followed by incubation at 37°C for 30 min. After incubation, 50 µl of stop solution was added to the reaction

Figure 1. Schematic summary protocol of the PDE5i assay. This developed protocol can be used to detect other types of PDE5i in any drug by substituting the compound in the reaction assay using the specific PDE5i of interest and counterfeit drug.

and mixed well using vortex followed by centrifugation at 2000 rpm for 5 min. The solutions were then transferred to their designated wells in a 96-well microplate with a total volume of 140 µl per well followed by MLG assay.

$$\text{Percentage of PDE5 activity (\%)} = \frac{\text{Absorbance of CX1A drug solution}}{\text{Absorbance of optimum PDE5 reaction}} \times 100$$

The concentration of sildenafil was calculated using the following formula, which was derived from the standard curve of sildenafil:

$$Y = \text{Bottom} + \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\log \text{IC}_{50} - X) \text{slope}}}$$

Where Y = percentage of PDE5 activity, X = Concentration of sildenafil, top value: 99.99, bottom value: -1.230, $\log \text{IC}_{50}$ (half-maximal inhibitory concentration): -0.505 and slope: -0.722

Step 5: MLG assay. After the desired solutions were aliquoted into their designated well in the 96-well microplate, the MLG assay was performed, where 14 µl of MolyB reagent (50 mM MolyB in 3.4 M sulphuric acid) was added to each well containing the mixture, mixed thoroughly and incubated for

5 min. Then, 26 µl of MLG reagent (1.0 mM MLG, 6.0 mM sulphuric acid, 0.16% PVA) was added, mixed well and incubated for 10 min. After incubation, the absorbance was measured using Biotek™ ELx808™ absorbance microplate reader at 630 nm.

Statistical analysis

Statistical comparisons were made by means of two-way repeated measures Analysis of Variance (ANOVA). The data were analysed with Prism software (Version 6, Graph Pad Inc.). The results were shown as mean ± S.E.M and *n* represents the number of repeats in each experiment. *P* values of 0.05 or lower was considered to be statistically significant.

Results

Establishment of a standard curve for Pi

Pi concentrations ranging from 0 µM to 20 µM were used to generate a standard curve for Pi. Table 1 and Figure 2 show the relative absorbance values for Pi (0 µM to 20 µM) and the linear relationship between absorbance and concentration of Pi, respectively. The equation derived from the standard curve was used to quantify the amount of Pi generated from PDE5 reaction. Raw results of this assay, alongside each of the assays performed in this study, are available as *Underlying data*⁴.

Dual biochemical reaction assays for PDE5-cGMP/GMP-CIAP (PDE5 reaction assay)

Using an optimized PDE5 reaction, 1.842 moles of Pi was generated as shown in Table 2.

Establishment of standard curve of sildenafil

Table 3 shows the percentage of PDE5 activity at each concentration of sildenafil. Using the values obtained, a sigmoidal curve

was established between PDE5 activity and log concentration of sildenafil, with an IC_{50} of 0.3124 μ M (Figure 3).

Quantification of sildenafil in CX1A samples

From the PDE5-CX1A reaction assay, the percentage values of PDE5 activity using low (0.1 mg/ml) and high dilution (0.01 mg/ml) samples of CX1A were 31.33% and 78%, respectively. Using the equation derived from the sigmoidal curve of sildenafil, the concentration of sildenafil was determined as the percentage of sildenafil in low and high dilutions of CX1A samples, i.e. 0.673% and 0.407%, respectively. The mass of sildenafil in CX1A tablet is 3.149 mg. The calculated values were as tabulated in Table 4 and Table 5.

Table 1. Relative absorbance values for Pi from 0 μ M up to 20 μ M.

[Pi], μ M	Relative absorbance, AU
0	0.000 $\pm$ 0.014
2	0.052 $\pm$ 0.014
5	0.115 $\pm$ 0.003
7.5	0.177 $\pm$ 0.013
10	0.239 $\pm$ 0.009
12.5	0.280 $\pm$ 0.006
15	0.333 $\pm$ 0.011
17.5	0.379 $\pm$ 0.002
20	0.416 $\pm$ 0.011

AU, arbitrary units.

Figure 2. The linear relationship between absorbance and Pi concentration from 0 to 20 μ M. The correlation allows the establishment of standard curve of Pi.

Table 2. Amount of Pi generated from PDE5 reaction.

Variable	Value
Mean absorbance	0.224 $\pm$ 0.005 AU
Concentration of Pi*	10.236 μ M
Amount of generated Pi	1.842 mole

*Calculated using an equation derived from standard graph of Pi.

Table 3. Percentage of phosphodiesterase-5 (PDE5) activity of sildenafil (Sil) (1 nM–300 μ M).

Sil, μ M	PDE5 activity, %
1×10^{-3}	98.93
3×10^{-3}	98.59
1×10^{-2}	89.40
3×10^{-2}	85.19
1×10^{-1}	64.44
3×10^{-1}	57.59
1×10^0	25.43
3×10^0	15.06
1×10^1	5.60
3×10^1	2.93
1×10^2	0.55
3×10^2	0.13

Figure 3. The standard curve of sildenafil (Sil) from 0.1 nM to 300 μ M with IC_{50} : 0.3124 μ M. Sigmoidal relationship was established for standard curve of pure Sil.

Table 4. Determination of concentration of sildenafil (Sil) in CX1A sample.

Tablet	Dilution	PDE5 activity, %	Final [Sil], μM	Actual [Sil], μM
CX1A	1:10	31.33	0.878	10.1
	1:100	78	0.053	0.61

PDE5, phosphodiesterase-5.

Table 5. Determination of mass of sildenafil (Sil) per tablet of CX1A.

Tablet	Sil in sample, nmol/ml	Sil in sample, ng/ml	Drug amount in sample, ng/ml	Percentage of Sil in CX1A sample, %	Mean percentage of Sil, %	Mass of CX1A tablet, mg	Mass of Sil per tablet, mg
CX1A	10.1	6.7337	1000	0.6734	0.540	583.2	3.149
	0.61	0.4067	100	0.4067			

Discussions and conclusion

Colour changes from blue to shades of green were observed with increasing Pi concentrations. This indicates that a complex of Pi with MolyB, and subsequently MLG, was formed. Low concentrations of Pi resulted in less formation of the Pi-MolyB-MLG complex, hence the observation of a lighter coloration with lower absorbance values. Increasing Pi concentrations led to greater binding and higher formation of complexes, indicated by high absorbance values. However, the linearity between concentration and absorbance was only observed up to 20 μM , indicating that beyond this maximum concentration range, the coloration of MLG with Pi-MolyB complexes was completely saturated. The Pi concentrations from 0 μM to 20 μM were used to develop the standard curve for Pi, which was needed in the later stages to quantify the amount of Pi released from PDE5 reaction. An important step to validate the reliability of this method is the linear correlation between sample concentration and the amount of activity measured. Reproducible satisfying linear correlations were observed using 0 μM to 20 μM Pi, with line of regression of 0.993 ± 0.001 ($n=2$) demonstrating that the established standard curve for Pi was robust and reliable to measure Pi.

Results of several preliminary studies using different types of buffer, working pH and types of substrate showed that a reaction buffer with pH 8 and cGMP were the most suitable conditions for the PDE5 reactions. The reaction buffer consisting of Tris-HCl, EDTA affected the activity of CIAP and MgCl_2 , provided cGMP-dependent Mg^{2+} that further supported the catalytic activity of PDE5. Although pH 8 favored CIAP's activity more than PDE5 limiting GMP production, it allowed both dual reactions to occur and produce better results. Based on specificity of cGMP to PDE5, cGMP was used as the substrate.

Approximately 100 units (a conversion of 1 picomole of cGMP to GMP per minute is defined as one unit) of PDE5 with a 30 min incubation was used for each PDE5 reaction. To

completely allow all generated GMP to release Pi, about 10-fold of 0.001 unit CIAP concentration (where conversion of 1 μM of GMP to Pi per minute is defined as one unit) was used with 30 min incubation time. Therefore, 3 nmol of both cGMP and Pi production was expected per PDE5 reaction. However, the observed efficiency of the reaction was only 60% of the expected amount (1.842 moles), suggesting that the reaction conditions, although operational, did not yet achieved the optimal level for the dual biochemical reaction assay. This is despite the fact that results were within the acceptable range with more than half of the expected cGMP-GMP-Pi conversion taking place.

Sildenafil was used as the sole reference for PDE5i in this study. Having similar structures, sildenafil competes with cGMP for the access to the catalytic site and is therefore known as a 'competitive inhibitor'. Sildenafil does not interact with the allosteric cGMP-binding site in PDE5⁵. Among the members of PDE enzyme families, sildenafil is highly selective towards PDE5, with a high inhibitory affinity for the catalytic site, as compared with the ~600 to 25,000-fold lower affinity of the natural substrate, cGMP⁶. In general, PDE5 activity decreases as sildenafil concentration increases. At lower concentrations of sildenafil, the amount of cGMP surpasses sildenafil, increasing the chance of cGMP binding to PDE5, thus allowing greater PDE5 activity. However, as the concentration of sildenafil increases, it displaces cGMP and binds to PDE5, causing a decrease in PDE5 activity. The total loss of PDE5 activity would occur when sildenafil completely inhibits all PDE5 molecules.

A sigmoidal relationship was established between PDE5 activity and different concentration of sildenafil, demonstrating positive cooperative binding. This means that the binding of the enzyme with its substrate at one binding site affects the affinity of other sites for their substrates, allowing a rapid and increased coordinated enzymatic activity. This is consistent with

a PDE5 profile that has both catalytic and allosteric binding sites for its substrates. However, since sildenafil is considered a competitive inhibitor, which binds at the catalytic site of PDE5, the sildenafil inhibitory profile shows negative cooperative binding (represented by negative hill slope value) presumably reflecting the changes in kinetic characteristics caused by cGMP-induced PDE5 activation⁷. The IC_{50} of sildenafil towards PDE5 in this study of 312.4 nM was about ~40-70-fold higher than previous studies that showed IC_{50} of around 3.5–8 nM⁸. This variation could be attributed to the dependency of IC_{50} values on several factors such as cGMP concentration, the source and extraction method of enzymes, the reaction condition, the number of samples and other factors affecting the experimental design⁹.

The amount of sildenafil in CX1A samples determined in this study was validated using HPLC producing comparable results (data not shown). The low and high dilutions of CX1A resulted in 0.673% and 0.407% of the calculated percentage of sildenafil in CX1A, respectively. Other types of PDE5i and counterfeit drugs could also be tested and investigated using the PDE5i assay and employing optimal conditions and appropriate controls. In conclusion, the PDE5i assay developed in this

study has the potential to become an alternative method for the detection of counterfeit drugs containing PDE5i.

Data availability

Underlying data

Open Science Framework: PDE5i Assay. <https://doi.org/10.17605/OSF.IO/PV59F4>.

This project contains 'Aziemah *et al.* 2019 Raw_data for developed PDE5i assay.xlsx', which includes raw data for all experiments performed in this study.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Current Peer Review Status: ? ✓

Version 1

Reviewer Report 01 October 2019

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Adi Idris

Menzies Health Institute Queensland, School of Medical Science, Griffith University, Southport, QLD, Australia

Work presented by Azizi A *et al* described a spectrophotometric assay to detect the presence of phosphodiesterase-5 inhibitors. This could be used as a basis in the early stages in a pipeline to develop a method to detect PDE5i in suspect counterfeit drugs. Though preliminary and brief in nature, these findings shed light on the potential use of this assay from an application standpoint. However, the authors are asked to address some comments below before this manuscript is fully acceptable:

- Grammatical and language mistakes found throughout. Recommend English proofreading
- Authors are asked to define CX1A in the abstract
- The term "Sil" was used in the abstract and introduction. Please define at first use or change to Sildenafil.
- Table 1 is unnecessary as Fig 2 clearly depicts this more clearly. This also applies to Table 3. If need be put the raw data as supplementary data
- The results section was poorly written. It is too short and reads like a technical document. It lacks thought and short conclusive statements. Either re-write this section or consider merging the results section with the discussion section (if allowed by journal guidelines)
- Table 4 and 5 should be merged into one
- The beginning of the discussion section reads poorly, making it hard for readers to grasp the essence of the paper. Authors should firstly highlight the main findings from their study then critically analyse their data.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Innate immunity

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 30 September 2019

<https://doi.org/10.5256/f1000research.21379.r54386>

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Dirk W. Lachenmeier

Chemical and Veterinary Investigation Agency Karlsruhe, Karlsruhe, Germany

The authors describe an application for spectrophotometric analysis of PDE5 inhibitors in counterfeit drugs or food supplements. The application could be interesting in settings where more advanced analysis such as HPLC or LC/MS is unavailable.

Major deficits:

1. No validation data at all is presented besides linearity (e.g. detection limits, accuracy, precision, selectivity)
2. The discussion section does not provide a comprehensive discussion at all. The results should be placed into the state of the art, i.e. compared to other methods such as HPLC or NMR. Furthermore, the advantages and limitations of the current research must be discussed. A major limitation is, for example, the lack of a real applicability study apart from

one single sample. It is not demonstrated that the method works “in the real world” with all problems regarding matrix interferences or interferences from other similar compounds or isomers. For example, the RASFF system of the EU reports that currently sildenafil thiono analogues have a high incidence in illegal food supplement (i.e. hydroxythiohomosildenafil, hydroxyhomosildenafil and thiosildenafil)[[reference](#)],[[reference](#)]. Would such compounds be detected by your assay?

The following revisions are required:

Throughout: There is a constant mismatch in units, which makes the paper difficult to follow. % should be avoided at all cost and replaced by meaningful units such as g/100 g, while μM could be recalculated to mg/L.

Throughout: I dislike the non-chemical abbreviation Pi for inorganic phosphate. If the ionic state is known, e.g. PO_4^{2-} , this should rather be stated.

Throughout: please only present significant decimals in numbers considering validation results

Title: The title is rather long. Can the two times repetition of PDE5I be avoided perhaps?

Abstract, conclusion: The data about “comparable results with HPLC” is not in the paper and should be deleted (or shown in the paper).

Introduction, 1st paragraph, 2nd last line: “easier detection”. Why is it easier? Compared to what it is easier? The sample preparation appears to be tiresome and could be more complicated than the sample preparation (dilution) required for HPLC or NMR analysis (e.g. see Monakhova Y *et al.*, 2012 ¹).

Page 3, right column, 2nd paragraph: HCL should read HCl

Table 2: mole should read “mol” (or M) but see comment on units above?

Table 4: what is the difference between final and actual concentration? Add footnotes

Table 5: can the final result be reported as mg/kg?

Page 7: “data no shown” for HPLC: why?

Figure 1: explain abbreviation rxn

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

No

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: analytical chemistry; food chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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