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Short communication

A simple and sensitive HPLC fluorescence method for determination of tadalafil in mouse plasma

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ABSTRACT

A simple and sensitive high-performance liquid chromatographic (HPLC) method utilizing fluorescence detection was developed for the determination of the phosphodiesterase type 5 inhibitor tadalafil in mouse plasma. This method utilizes a simple sample preparation (protein precipitation) with high recovery of tadalafil (~98%), which eliminates the need for an internal standard. For constituent separation, the method utilized a monolithic C_{18} column and a flow rate of 1.0 mL/min with a mobile phase gradient consisting of aqueous trifluoroacetic acid (0.1% TFA in deionized water pH 2.2, v/v) and acetonitrile. The method calibration was linear for tadalafil in mouse plasma from 100 to 2000 ng/mL ($r > 0.999$) with a detection limit of approximately 40 ng/mL. Component fluorescence detection was achieved using an excitation wavelength of 275 nm with monitoring of the emission wavelength at 335 nm. The intra-day and inter-day precision (relative standard deviation, RSD) values for tadalafil in mouse plasma were less than 14%, and the accuracy (percent error) was within −14% of the nominal concentration. The method was utilized on mouse plasma samples from research evaluating the potential cardioprotective effects of tadalafil on mouse heart tissue exposed to doxorubicin, a chemotherapeutic drug with reported cardiotoxic effects.

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1. Introduction

Tadalafil (6R-trans)-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-2-methyl-pyrazino[1′,2′:1,6]pyrido[3,4-b]indole-1,4-dione (Fig. 1), is a selective reversible phosphodiesterase-5 (PDE5) inhibitor indicated for treatment of erectile dysfunction (impotence) [1–5]. The (PDE5) inhibitors enhance erectile response by increasing levels of penile cyclic guanosine monophosphate (cGMP), which causes smooth muscle relaxation and increases blood flow to the corpus cavernosum [1–6]. Tadalafil (Cialis®), with recent FDA approval (2003), is reported to have a potent PDE5 inhibitory effect and a long duration of action. Tadalafil is hypothesized to possess cardioprotective effects on heart tissue exposed to the cardiotoxic chemotherapeutic drug, doxorubicin [7]. The purpose for development of this test method was to measure levels of tadalafil in small volumes of mouse plasma (e.g. 100–200 μL) obtained during experiments testing its effects on doxorubicin-induced cardiotoxicity.

Contemporary methods for measurement of tadalafil include liquid chromatography–mass spectrometry (LC–MS), liquid chromatography with ultraviolet detection (HPLC–UV), capillary electrophoresis, and chiral chromatography. The LC–MS methods [8,9] were developed for evaluation of dietary supplements and human plasma, respectively. The LC–MS method for dietary supplements was utilized to detect banned additives at the μg/mL levels, and while the LC–MS method for plasma was rapid with high sensitivity (ng/mL), it necessitated a liquid–liquid extraction step as well as an internal standard. The HPLC–UV methods were developed for evaluation of pharmaceutical preparations [10,11] and human and rat plasma [12–14]. These plasma methods also utilized liquid–liquid extraction steps and as well required the use of internal standards. One HPLC–UV plasma method [14] was cited as being simple and sensitive; however, we found it difficult to reproduce the method to the reported sensitivity. Upon further examination of the article [14], it was found that the detector response shown in the figure consisting of chromatogram overlays did not appear very sensitive to tadalafil (i.e. small millivolt full scale response). Other published methods used for pharmaceutical preparations have included capillary electrophoresis [15], chiral chromatography for the separation of tadalafil enantiomers [16], and tadalafil used as an internal standard for determining letrazole levels using fluorescence detection [17].

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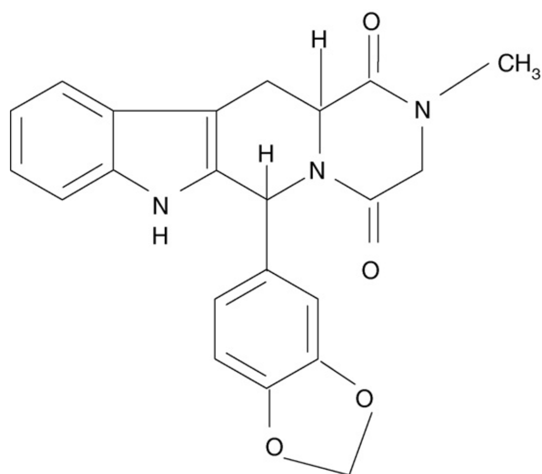

Fig. 1. Chemical structure of tadalafil showing several conjugated moieties (i.e. aromatic rings) which can fluoresce when exposed to UV radiation.

In the present study, we report a simple, sensitive and cost effective HPLC fluorescence method for determination of tadalafil in mouse plasma. The method utilizes a simple sample preparation (e.g. protein precipitation) with high recovery of tadalafil, does not require use of an internal standard, and offers fluorescence sensitivity (ng/mL levels) equivalent to published LC–MS methods. This method employed high efficiency monolithic HPLC column technology, which provided excellent constituent resolution and low LC system backpressures. The method was utilized for mouse plasma samples from research evaluating the potential cardioprotective effect of tadalafil on cardiac tissue exposed to doxorubicin. As tadalafil is currently indicated for treatment of adult male erectile dysfunction, the method was also evaluated on samples of blank human plasma fortified with concentrations (ng/mL) of tadalafil that might be present in treated adult males.

2. Experimental

2.1. Chemicals and blank plasma

Tadalafil standard material (99% purity) was kindly received from Eli Lilly & Company (Indianapolis, IN, USA) [18]. Doxorubicin (≥98% purity) was purchased from Sigma–Aldrich (St. Louis, MO, USA). For mobile phase preparation, concentrated trifluoroacetic acid (TFA) was reagent grade and acetonitrile (ACN) was Optima grade and were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure deionized water (18 MΩ cm) used for all HPLC work was prepared in-house using a US Filter water purification system (Richmond, VA, USA). Blank mouse plasma used for preparation of controls and mouse plasma containing tadalafil was collected from adult male CF-1 outbred mice (Harlan, Indianapolis, IN, USA) treated with saline or Cialis®, respectively. Blank human plasma for evaluation was purchased from VCU Medical Center hospital blood bank (Richmond, VA, USA).

2.2. HPLC equipment and mobile phase

The HPLC equipment consisted of a Varian Solvent Delivery System (ternary) HPLC Model 9010 (Sugarland, TX, USA), Shimadzu SIL-10AD autosampler (Columbia, MD, USA), and Shimadzu RF-535 fluorescence detector. The analytical column used was a Phenomenex® Onyx™ monolithic C_{18} (10 cm × 4.6 mm I.D.) coupled to an Onyx™ C_{18} guard column (5 cm × 4.6 mm I.D.) (Torrance CA, USA). The guard column was replaced after each analytical run of approximately 50 plasma samples. The mobile phase consisted of aqueous trifluoroacetic acid (0.1% TFA in deionized water, pH 2.2, v/v) and acetonitrile (ACN). The mobile phase gradient was programmed with time course as follows: 70:30, 0.1% (v/v) TFA in deionized water: acetonitrile at 0 min and held for 8 min; 10:90, 0.1% (v/v) TFA in deionized water: acetonitrile at 9 min and held for 5 min; 70:30, 0.1% (v/v) TFA in deionized water: acetonitrile at 15 min.

The mobile phase was degassed using helium sparging and used at a flow rate of 1.0 mL/min. The HPLC operating pressure was approximately 25 atm and the column temperature was ambient. An injection volume of 30 μL of the prepared plasma sample was delivered using an autosampler. Component fluorescence detection was achieved using an excitation wavelength of 275 nm with monitoring of the emission wavelength at 335 nm. The fluorescence detector operated at high sensitivity with a 1 s response time. A 345 kPa backpressure regulator (SSI, State College, PA, USA) was coupled to the detector outlet to prevent mobile phase out-gassing. Data acquisition and component computations were performed using TotalChrom™ Workstation software (Perkin Elmer™, Norwalk, CT, USA). For UV evaluations, a Varian Model 9050 UV–vis detector (Sugarland, TX, USA) was utilized. To compare the sensitivity of the two HPLC detectors for tadalafil, the UV–vis detector was connected in series with the fluorescence detector and both detectors were set to a 1 V full scale output.

2.3. Standard and control preparation

A stock standard of tadalafil (1.0 mg/mL) can be prepared in dimethyl sulfoxide (DMSO) or methanol and stored at 4 °C. Working standards of tadalafil were prepared at 100, 250, 500, 1000 and 2000 ng/mL in a mixture of deionized water and acetonitrile (50:50, v/v). All working standards were stored frozen at −20 °C. Quality control (QC) samples with fortified amounts of tadalafil were prepared at levels of 200, 750 and 1500 ng/mL using mouse blank plasma. Following preparation, the QC samples were stored frozen at −20 °C and demonstrated stability for at least two months.

2.4. Animal protocol, sample acquisition and processing

All animal experiments were conducted under the guidelines on humane use and care of laboratory animals for biomedical research published by National Institutes of Health (No. 85-23, revised 1996), with animal research protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. For experiments, the animals were either dose with saline (control mouse) or tadalafil (treated mouse) via an oral gavage. Following surgical removal of the mouse heart under pentobarbital anesthesia, pooled blood was collected into BD Diagnostics Vacutainer® tubes containing K_2EDTA (Franklin Lakes NJ, USA). Sample tubes were centrifuged at approximately 600 × *g* for 15 min at 4 °C, with plasma drawn off and stored in polypropylene bullet micro-centrifuge tubes at −20 °C. Prior to HPLC analysis, plasma samples were thawed to ambient temperature, mixed thoroughly by inversion and centrifuged at 1000 × *g* for 10 min to eliminate fibrinous material.

2.5. Sample extraction

Plasma samples were prepared for HPLC analysis by pipetting 150 μL of plasma and 150 μL of acetonitrile into an Eppendorf polypropylene bullet micro-centrifuge tube (Westbury NY, USA). The sample tubes were capped and plasma proteins were precipitated by vortexing for 1 min. The samples were centrifuged at 14,000 × *g* for 10 min at ambient temperature. The clear

filtrates were transferred to deactivated glass autosampler microvials (Waters, Milford MA, USA) and 30 μL was injected for HPLC analysis.

2.6. Stability

The stability of tadalafil in mouse plasma was evaluated at −20 °C for 30 and 60 days. This time frame incorporated the length of time the samples were stored frozen during the study. Control tadalafil samples (e.g. 200, 750, and 1500 ng/mL) were prepared in blank mouse plasma and stored at −20 °C with the study mouse plasma samples. The stored control samples were thawed after 30 and 60 days and evaluated using HPLC. Tadalafil was considered stable if the measured tadalafil values were within the inter-day QC acceptance range.

3. Results and discussion

3.1. Method optimizations

With a method utilizing a sample preparation step involving protein precipitation, the ideal HPLC column should have a sufficiently high efficiency to resolve tadalafil from other endogenous plasma constituents. We utilized a recently introduced HPLC column technology; the Onyx monolithic C_{18} column (10 cm × 4.6 mm I.D.), which provided excellent chromatographic resolution and a low system backpressure of ∼25 atm (gradient time zero condition and flow rate of 1 mL/min). The mobile phase aqueous component, 0.1% TFA in deionized water (pH 2.2), provided good peak shape for tadalafil using the monolithic C_{18} column. The mobile phase organic modifiers (e.g. acetonitrile and methanol) were evaluated to determine which organic solvent would provide the best chromatographic separation of tadalafil from endogenous plasma constituents. Acetonitrile was chosen as the organic modifier as it provided good peak shape and selectivity from other endogenous plasma constituents. Various injection volumes (e.g. 5–30 μL) were evaluated and all can be used without significant effects on chromatographic performance (i.e. constituent resolution, peak shape). This method utilized 30 μL as it provided adequate method sensitivity and autosampler injection precision.

Our attempt to reproduce a recently published HPLC–UV method [14] was unsuccessful. The chromatography article reported a sensitive method for measuring tadalafil using HPLC–UV detection; however, we found that UV detection was not sensitive enough to detect tadalafil at low ng/mL concentrations without some form of sample concentration step. Upon further review of the article [14], it was determined that the UV (290 nm) response in the figure of chromatogram overlay for tadalafil was not very sensitive (i.e. small change in mV response per change in tadalafil concentration).

For this reason, we decided to evaluate fluorescence detection and determined that the wavelengths for excitation (275 nm) and emission (335 nm) were optimal for tadalafil in the mobile phase. To compare sensitivity of the detectors, the UV and fluorescence detectors were coupled in series with simultaneous monitoring of both detectors. The observed response (i.e. signal/noise) for tadalafil was significantly higher using fluorescence detection with both detectors outputs set to 1 V full scale. As demonstrated in Fig. 2C, the fluorescence response for tadalafil was approximately 80 fold more than was observed using UV detection at 290 nm (Fig. 2A). When evaluating tadalafil using UV at 220 nm (Fig. 2B), another UV wavelength that tadalafil absorbs, the absorbance is minimal as compared to the response using fluorescence detection; however, this result was not surprising as fluorescence detection is known to be more sensitive than UV detection, primarily due to the higher specificity and lower background of fluorescence measurements (i.e. higher signal/noise ratio).

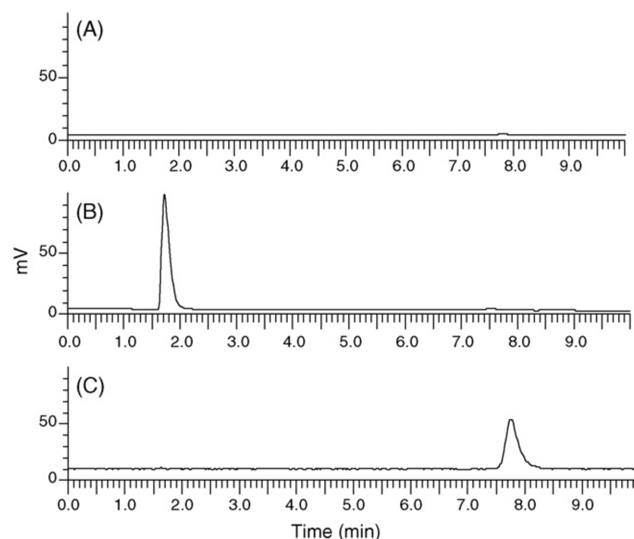

Fig. 2. Overlay chromatograms illustrating (A) 1000 ng/mL tadalafil using ultraviolet detection (UV) with an absorption wavelength of 290 nm and (B) UV at 220 nm, and (C) fluorescence detection with an excitation wavelength of 275 nm and emission wavelength of 335 nm. Tadalafil retention time was ∼7.8 min. The chromatographic detectors were connected in tandem with the HPLC analytical column coupled to the UV detector.

3.2. Linearity, limits of detection and quantitation, computations

The plasma calibration curve was linear for tadalafil throughout the concentration range (100–2000 ng/mL) (Fig. 3), with mean correlation coefficient of 0.9997 (unweighted normal linear regression, $n = 10$ analytical runs). The limit of detection (LOD) for tadalafil was approximately 40 ng/mL and determined by evaluation of mouse blank plasma ($n = 3$). The LOD was calculated using three times the standard deviation of the chromatographic baseline noise and calculation (extrapolation) from the tadalafil standard curve. The lowest standard calibrator (i.e. 100 ng/mL) was used as the limit of quantitation (LOQ) and is administratively defined as a combined accuracy and precision within 30% of the nominal amount. Samples less than the LOQ were reported as an estimated value of tadalafil and used only to aid in determining whether the dosing form of tadalafil was appropriate for the study. For HPLC analysis, external

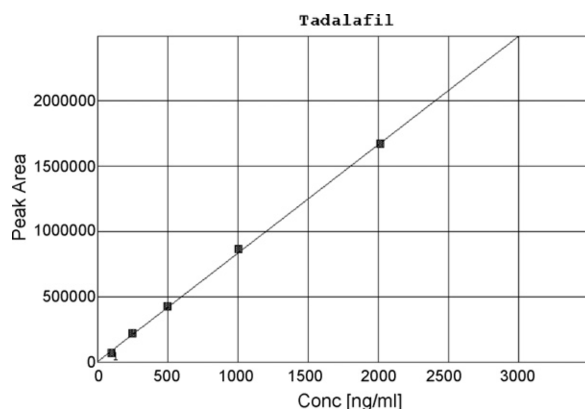

Fig. 3. A typical calibration curve for tadalafil using peak area, external standardization and normal linear regression. The standards used for calibration were prepared at concentrations of 100, 250, 500, 1000 and 2000 ng/mL.

Table 1
Combined intra-day and inter-day accuracy and precision for tadalafil controls prepared in mouse plasma.

Component	Fortified concentration (ng/mL) (n = 9)	Calculated mean concentration (ng/mL) (n = 9)	% Error	% RSD
Tadalafil	200	171.4	−14.3	13.3
Tadalafil	750	720.2	−4.0	3.6
Tadalafil	1500	1479.4	−1.4	1.6

Table 2
Intra-day accuracy and precision for tadalafil controls prepared in human plasma.

Component	Fortified concentration (ng/mL) (n = 3)	Calculated mean concentration (ng/mL) (n = 3)	% Error	% RSD
Tadalafil	200	202.6	1.3	4.4
Tadalafil	750	688.2	−8.2	1.2
Tadalafil	1500	1438.8	−4.1	3.4

standardization and peak area response were used for all sample computations.

3.3. Accuracy, precision and recovery

The accuracy and precision for the method was determined by evaluation of replicate plasma control samples at fortified tadalafil concentrations of 200, 750 and 1500 ng/mL (n = 9 at each level). The combined intra-day (within day) and inter-day (between day) accuracy of the method was reported as percent error of the measured concentration relative to the nominal concentration. The combined intra-day and inter-day precision of the method was reported as the percent relative standard deviation (% RSD). Using the same plasma extraction procedure to evaluate mouse or human samples, the method demonstrated sufficient accuracy and precision for tadalafil with results listed in Tables 1 and 2, respectively. The absolute recovery of tadalafil from mouse or human samples was evaluated by comparing extracted controls prepared in blank plasma versus unextracted standards prepared in acetonitrile and deionized water (200, 750 and 1,500 ng/mL, n = 3 at each level). The mean absolute recovery of tadalafil for both mouse and human plasma control samples throughout the concentration range was determined to be >98%. For additional QC, the control samples used for analysis were prepared and handled identical to the test samples, thus controlling for potential errors in sample processing (e.g. micropipetting, centrifugation step, and sample storage temperature). The results of the stability study indicated that tadalafil in mouse plasma at 200, 750, and 1500 ng/mL was stable for at least 60 days. All control sample values were determined to be within the inter-day QC acceptance range, indicating stability when stored frozen at −20 °C.

3.4. Chromatography

The method demonstrated excellent chromatographic selectivity without endogenous plasma interferences at the retention time of tadalafil (∼7.9 min) (Fig. 4). The analytical run time of ∼21 min allowed for mobile phase gradient re-equilibration between sample injections. With a method that does not employ a formal sample cleanup step and to extend the column lifetime, the guard column was changed and the analytical column was flushed after each analytical run (∼50 injections) with acetonitrile: deionized water (90:10, v/v).

Fig. 4 depicts overlay chromatograms illustrating (A) prepared 100 ng/mL tadalafil (RT ∼ 8.0 min) standard in deionized water and acetonitrile, (B) prepared 2000 ng/mL tadalafil standard in deionized water and acetonitrile, (C) blank mouse plasma, (D) mouse plasma sample with ∼553 ng/mL tadalafil, and (E) blank human plasma. As the mice were treated with the co-medication, doxorubicin, we evaluated doxorubicin to ensure that there were no chromatographic interferences. A 10 μg/mL doxorubicin standard was injected and did not fluoresce at the optimal tadalafil wavelengths for excitation (275 nm) and emission (335 nm); therefore, no chromatographic interference was evident with doxorubicin.

3.5. Sample preparation optimization and solubility

As tadalafil is an organic substance with hydrophobic properties (Fig. 1) and reported to be practically insoluble in water [17], a study was performed to evaluate the solubility of tadalafil in the preparation of plasma control samples (three levels). Our laboratory standard operating procedure (SOP) for preparing plasma control samples is normally to evaporate (e.g. via nitrogen) the organic solvent from the pipetted aliquot of a standard solution prepared in methanol, followed by reconstitution with the biological matrix (e.g. blank plasma). However, when preparing plasma controls with tadalafil using the SOP, we observed that approximately 20–30% of the tadalafil was not recovered from the extraction, as determined by comparison to standards prepared in acetonitrile:deionized water (1:1, v/v). When using deionized water instead of plasma as the matrix, we found about the same reduction in tadalafil recovery, thus demonstrating the insolubility of tadalafil in an aqueous milieu. To overcome this solubility issue in the preparation of control samples, fortified amounts of stock tadalafil standard prepared in methanol were directly added to the plasma without the

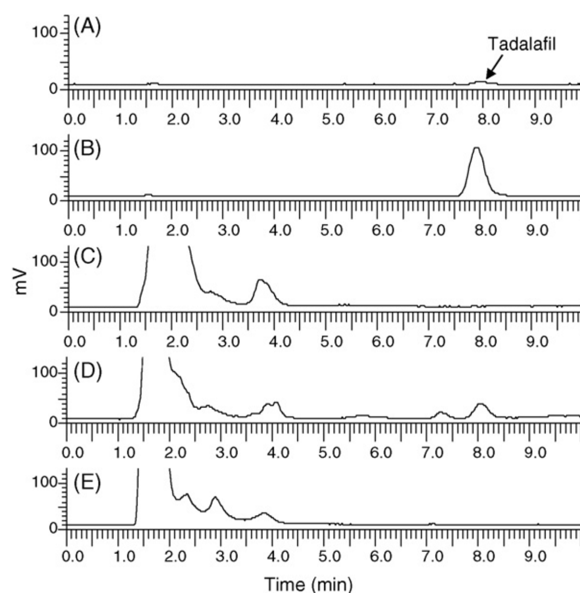

Fig. 4. Chromatograms illustrating (A) prepared 100 ng/mL tadalafil (RT ∼7.9 min) standard in deionized water and acetonitrile, (B) prepared 2000 ng/mL tadalafil standard in deionized water and acetonitrile, (C) blank mouse plasma, (D) mouse plasma sample with ∼553 ng/mL tadalafil, and (E) blank human plasma.

methanol evaporation step. This resulted in plasma control samples having between 2% and 15% (v/v) methanol:plasma ratio and >98% recovery of tadalafil from the extracted control samples.

4. Conclusions

A simple and sensitive HPLC method using fluorescence detection was developed for determination of tadalafil in mouse plasma samples. The method employs a simple sample preparation using protein precipitation with high recovery of tadalafil, thus eliminating the need for an internal standard. In addition, the method utilizes recently introduced HPLC monolithic column technology, which provided sufficient selectivity to separate tadalafil from other endogenous plasma constituents. The method was employed without methodological problems in the evaluation of limited volume mouse plasma samples containing tadalafil, obtained in the setting of doxorubicin-related cardiac damage.

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