ORIGINAL ARTICLE

Validation of an HPLC Method for Determining Sildenafil and NDMS Plasma Concentrations in Pulmonary Arterial Hypertension

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Background: Variability in sildenafil pharmacokinetics may impact pulmonary arterial hypertension (PAH) outcomes. According to the limitations of current methods.

Objective: The research developed a simple and accurate method to measure sildenafil and its metabolite (N-desmethyl sildenafil; NDMS).

Materials and Methods: High-performance liquid chromatography (HPLC) with a UV detector at 296 nm and a C18 Kinetex column was used. The mobile phase consisted of 0.05 M potassium dihydrogen phosphate, acetonitrile, and methanol (47: 23: 30 v/v/v), with a 50 µL injection volume and a flow rate of 1 mL/min. Glipizide served as the internal standard.

Results: A linear correlation was observed for sildenafil (10 to 1,500 ng/mL) and NDMS (10 to 500 ng/mL), with R^2 values of 0.9998 and 0.9995, respectively. The lower limit of detection was 5 ng/ml for both compounds. Retention times were 3.25 minutes for sildenafil and 6.48 minutes for NDMS.

Conclusion: This HPLC method with UV detection was validated for its simplicity, reliability, precision, recovery, stability, and specificity for measuring sildenafil and NDMS in plasma from PAH patients.

Keywords: HPLC; Sildenafil; N-desmethyl sildenafil; PAH

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Sildenafil is a phosphodiesterase-5 (PDE-5) inhibitor that acts as a stabilizer of cGMP. PDE-5 inhibitors can slow disease progression by inhibiting vascular remodeling, smooth muscle proliferation, and hypoxia-induced proliferation in vessels, making sildenafil an effective treatment for pulmonary arterial hypertension (PAH). Oral sildenafil is rapidly absorbed, reaching its maximum plasma concentration (Cmax) within 1 hour (range: 0.5 to 2 hours), with a bioavailability of approximately maximum plasma concentration (Cmax) in 1 hour (0.5 to 2 hours), with a bioavailability of 41%. Its major metabolic pathways are via CYP3A4/5 (79%) and CYP2C9 (20%). N-desmethyl sildenafil (NDMS), the active metabolite, has approximately 50% of the potency of sildenafil and about 36% of its pharmacological effects[1].

In the past, methods for detecting sildenafil and its metabolite have been developed using various techniques, including high-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector or mass spectrometry, liquid chromatography-tandem mass spectrometry (LC-MS/MS), and ultra-performance liquid chromatography (UPLC). The specimen, including plasma, semen, or urine, used for detecting sildenafil levels depends on the measurement's specific purpose and the availability of the samples[2-7]. However, studies in PAH patients have focused on sildenafil and its metabolite plasma levels in healthy volunteers. In contrast, investigations of sildenafil and metabolite plasma concentration in PAH patients have been limited. The peak plasma concentration in PAH patients treated with sildenafil 20 mg (orally, three times a day) was 113 ng/mL[8]. The mean NDMS plasma levels in healthy volunteers were 18.64±6.82 ng/mL after a single oral dose

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of 20 mg sildenafil. No data is available on PAH patients[9].

The present study developed and validated a simple method for detecting sildenafil and NDMS plasma levels by using HPLC. Finally, this method could detect sildenafil and NDMS in patients with dementia.

Materials and Methods

Material

Sildenafil citrate was obtained from Sigma-Aldrich (St. Louis, MO, USA). Glipizide was used as an internal standard. Glipizide and NDMS were obtained from TLC Pharmaceutical Standards Ltd. (Michigan, USA). Moreover, other chemicals were of analytical grade.

Equipment

The HPLC system utilized in the present was performed using an auto sampler (717 Plus Autosampler, Millipore, Millford MA, USA), and pump (Waters 600, Millipore, Millford MA, USA) incorporating a wavelength with UV-Visible spectrophotometer (2,487 Dual λ Absorbance Detector and Degasser, Millipore, Millford MA, USA), detector set to operate at a wavelength of 296 nm. Furthermore, the data acquired during the study were subjected to analysis using the Clarity program provided by DataApex, located in Prague, Czech Republic.

Chromatographic and HPLC conditions

The separations were performed using a C18 column (Kinetex, 150x4.6 mm, 5 µm particle size) (Phenomenex, Ltd., USA). The mobile phase was 0.05 M potassium dihydrogen phosphate, acetonitrile, and methanol (47: 23: 30 v/v), with the pH adjusted to 6.0. NDMS and sildenafil were eluted from the column with retention times of 3.25 and 6.48 minutes, respectively, at 25°C with a flow rate of 1 mL/min. The total HPLC run time was 15 minutes. The concentrations of sildenafil and NDMS were calculated based on the peak height ratio, with internal standard and standard curves constructed using escalating concentrations of sildenafil (10 to 1,500 ng/mL) and NDMS (10 to 500 ng/mL).

Preparation standards

The preparation of stock standard solutions of sildenafil, NDMS, and glipizide involved dissolving 5 mg of each analyte in 5 mL of methanol, yielding standard solutions with a concentration of 1 mg/mL for the three compounds. Then, stock solutions and internal standard solutions were aliquoted at 500 µL and subsequently stored in NDMS at -80°C. Sildenafil and the internal standard were stored at -20°C until use. To generate the calibration samples, various volumes of the standard stock solutions of sildenafil and NDMS, with concentrations from 10 to 1,500 ng/mL of sildenafil and 10 to 500 ng/mL of NDMS, were prepared. Then, 100 µL of each concentration of sildenafil and NDMS was combined with 50 µL of the internal standard stock solution (100 µg/mL) in 800 µL of drug-free plasma.

For the extraction method, liquid-liquid extraction was performed by mixing 3,000 µL of ethyl acetate, followed by centrifugation at 4,500 rpm and 10°C for 10 minutes. Then, collect the upper layer and transfer it to a new tube, evaporating it under a N_2 stream at 60°C until dry. Reconstitute the sample with 200 µL of mobile phase, dissolve the residual, and subsequently centrifuge at 14,000 rpm for 10 minutes. Collect 120 µL of the supernatant in a vial and place it into an autosampler injector with a 50 µL injection volume. The peak height ratio of sildenafil and NDMS to the internal standard was measured, and a calibration curve was obtained by performing a least-squares linear regression of the peak height ratio against the spiked concentrations.

Sample preparation and HPLC analysis

The protocol was adapted from Kanjanawart et al.(Kanjanawart et al., 2011). The research was conducted at Queen Sirikit Heart Center of the Northeast, Thailand. Blood samples were collected from April 2023 to July 2023. Patients with PAH who received sildenafil therapy and had taken a stable dose of sildenafil for more than 30 days were enrolled. According to the evaluate and validate method, twenty PAH patients had their blood drawn for preliminary results. For the study design, patients with PAH who had given informed consent were asked to collect their blood at the next visit. At the study day, the patients were fasting from midnight before the study day until morning (allowed free water drinking). The patient took sildenafil following their routine dosage of 60 to 240 mg/day, as observed by an investigator. Then, 1 hour later, 3 mL of blood was withdrawn into the heparin-coated tube. The blood tube was centrifuged at 3,500 rpm and 4°C for 15 minutes. The plasma was then transferred to a microcentrifuge tube and stored at -80°C until drug analysis.

Validated method

The validation of the assay was performed, including selectivity, specificity, linearity, and limit of quantification, as well as accuracy, precision, recovery, and stability.

Selectivity and specificity

To demonstrate selectivity and specificity, six different blank human plasma samples were evaluated to assess potential endogenous interference at the retention times of sildenafil, NDMS, and IS. Specificity was confirmed by ensuring that no interfering peaks were present at these retention times at the lower limit of quantification (LLOQ),

demonstrating the method's ability to detect and quantify the analytes reliably.

Linearity and LLOQ

Linearity was assessed by constructing calibration curves using seven concentration levels, analyzed over three separate days. The calibration range was 10 to 1,500 ng/mL for sildenafil and 10 to 500 ng/mL for NDMS. Calibration curves were plotted using the peak height ratios of the analyte to IS versus the nominal concentrations. The regression analysis was performed using a quadratic model with a $1/x^2$ weighting factor. The slope, intercept, and correlation coefficient (R2) were determined, with r required to be greater than 0.99. The LLOQ was defined as the lowest concentration on the calibration curve that met the acceptance criteria for accuracy and precision. To comply with validation guidelines, accuracy and coefficient of variation (CV) should be within ±15% of the nominal concentration, except at the LLOQ, where deviations up to ±20% are acceptable.

Recovery

The recovery of sildenafil and NDMS was measured when both substrates were added at low, medium, and high concentrations, with calculations based on the calibration curve. The mean measured total and mean %recovery were evaluated. The percentage of recovery should not be over 15%.

Precision and Accuracy

To investigate the accuracy and precision of the HPLC method, data from low-, medium-, and high-quality controls (QCL, QCM, and QCH, respectively) of sildenafil and NDMS were observed over three days, both intra-batch and inter-batch. Each concentration was performed in 4 replicates and was prepared from the same stock solution. Both intra- and inter-batch were reported as the mean concentration (ng/mL), percentage of coefficients of variation (% CV), and percentage of accuracy (% accuracy). The accepted %CV must be lower than 15% and the % accuracy must be between 85.0% and 115.0%.

Stability

The stability of sildenafil and NDMS was assessed under four conditions: autosampler (25°C), freeze-thaw (three cycles), short-term (6 hours at room temperature), and long-term (30 days at -80°C). In all cases, analyte concentrations were compared to those of freshly prepared QCL and QCH samples. Stability was considered acceptable when the accuracy was within 85.0 to 115.0%, the coefficient of variation (% CV) was ≤15%, and the percentage variation did not exceed ±15%.

Ethics

The study protocol was approved by the Khon Kaen University Ethics Committee for Human Research, Khon Kaen University, Thailand (HE651115). Informed consent was obtained from 20 patients who enrolled in the present study.

Statistic

The demographic data were described by Descriptive statistical analysis. The plasma sildenafil and NDMS concentrations were shown as mean ± SD.

Results

The validation of the assay was performed, including selectivity, specificity, linearity, and limit of quantification, as well as accuracy, precision, recovery, and stability. The chromatogram of the blank plasma spiked with Sildenafil and NDMS is shown in Figure 1. No additional peaks of endogenous substances were observed interfering with the assay.

The calibration curve was linear, with the mean correlation coefficients of 0.9998 for sildenafil and 0.9995 for NDMS. The LLOQ concentration was 10 ng/mL for sildenafil and NDMS. Both the limit of detection (LOD) of sildenafil and NDMS were 5 ng/mL.

Accuracy and precision

The concentration of QCL, QCM, and QCH was 30, 700, and 1,200 ng/mL for sildenafil (Table 1) and 30, 250, and 400 ng/mL for NDMS (Table 2). Both intra- and inter-batch were shown the accepted %CV and %accuracy.

Recovery

The recovery of sildenafil and NDMS was measured when both substrates were added to the concentrations QCL, QCM, and QCH, with calculations from the calibration curve. The mean measured total, mean percentage recovery, and coefficient of variation are shown in Table 3.

Stability

The stability tests (including autosampler, freeze-thaw, short-term, and long-term stability) of sildenafil and NDMS

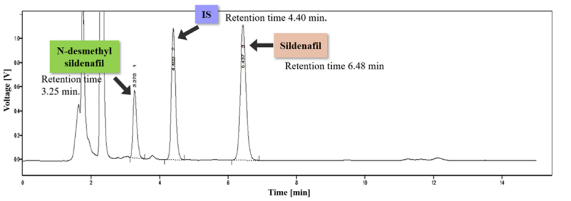

Figure 1. Chromatogram of blank plasma spiked with Sildenafil, NDMS, and Internal standard (IS).

Table 1. Intra-batch and inter-batch of sildenafil of QCL, QCM, and QCH

Sildenafil						
Sample type	**QCL**					
Sample batch	1	%Accuracy	2	%Accuracy	3	%Accuracy
Measured concentration (ng/mL)	27.33	91.11	29.33	97.77	28.78	95.94
	30.02	100.07	28.77	95.90	29.98	99.93
	27.26	90.86	29.99	99.98	29.05	96.82
	29.03	96.76	28.79	95.98	28.53	95.09
Intra-batch mean (ng/mL)	28.41		29.22		29.08	
Intra-batch precision (%CV)	4.75		1.97		2.18	
Intra-batch accuracy (%)	94.70		97.41		96.94	
Inter-batch mean (ng/mL)	28.91					
Inter-batch precision (%CV)	1.50					
Inter-batch accuracy (%)	96.35					
Sample type	**QCM**					
Sample batch	1	%Accuracy	2	%Accuracy	3	%Accuracy
Measured concentration (ng/mL)	746.22	106.60	727.03	103.86	733.54	104.79
	669.07	95.58	694.04	99.15	716.17	102.31
	664.46	94.92	716.46	102.35	734.77	104.97
	679.01	97.00	736.68	105.24	641.26	91.61
Intra-batch mean (ng/mL)	689.69		718.55		706.43	
Intra-batch precision (%CV)	5.53		2.55		6.27	
Intra-batch accuracy (%)	98.53		102.65		100.92	
Inter-batch mean (ng/mL)	704.89					
Inter-batch precision (%CV)	2.06					
Inter-batch accuracy (%)	100.70					
Sample type	**QCH**					
Sample batch	1	%Accuracy	2	%Accuracy	3	%Accuracy
Measured concentration (ng/mL)	1,194.62	99.55	1,260.29	105.02	1,134.33	94.53
	1,185.08	98.76	1,228.02	102.34	1,184.61	98.72
	1,216.92	101.41	1,273.34	106.11	1,096.37	91.36
	1,198.94	99.91	1,231.15	102.60	1,125.15	93.76
Intra-batch mean (ng/mL)	1,198.89		1,248.20		1,135.12	
Intra-batch precision (%CV)	1.11		1.78		3.24	
Intra-batch accuracy (%)	99.91		104.02		94.59	
Inter-batch mean (ng/mL)	1,194.07					
Inter-batch precision (%CV)	4.75					
Inter-batch accuracy (%)	99.51					

*%CV= percentage of coefficient variants; **QCL, QCM, and QCH of sildenafil were 30,700,1200 ng/ml, respectively

were performed and summarized in Table 4. All accuracy and variation of sildenafil and NDMS were accepted.

The stability of sildenafil and NDMS in the autosampler injector at 25°C remained stable for up to 6 hours in the autosampler (Table 4). Freeze-thaw in 3 cycles for sildenafil, and NDMS were indicated to have reliable stability (Table 4). Short-term stability confirmed that both sildenafil and NDMS remained stable for 6 hours (Table 4). Storage of both sildenafil and NDMS in -80°C demonstrated long-term stability for over 30 days (Table 4).

The present study validated a method for measuring plasma concentrations of sildenafil and NDMS in 20 patients with pulmonary arterial hypertension (PAH). The demographic data are presented in Table 5. Among the 20 patients, 80% were women, with a mean age of 42.60±14.06 years. No significant differences were observed in the demographic data across the groups. Plasma concentrations of sildenafil and NDMS are reported as mean ± SD in Table 5.

Discussion

The present study aimed to develop and validate an

Table 2. Intra-batch and inter-batch of NDMS of low, medium, and high-QCs (QCL, QCM, QCH, respectively)

NDMS						
Sample type	**QCL**					
Sample batch	1	%Accuracy	2	%Accuracy	3	%Accuracy
Measured concentration (ng/mL)	28.41	94.71	32.36	107.88	31.14	103.81
	27.95	93.16	33.67	112.22	33.05	110.17
	28.14	93.81	31.70	105.67	30.13	100.43
	28.54	95.15	32.98	109.95	32.97	109.91
Intra-batch mean (ng/mL)	28.26		32.68		31.82	
Intra-batch precision (%CV)	0.95		2.58		4.50	
Intra-batch accuracy (%)	94.21		108.93		106.08	
Inter-batch mean (ng/mL)	30.92					
Inter-batch precision (%CV)	7.57					
Inter-batch accuracy (%)	103.07					
Sample type	**QCM**					
Sample batch	1	%Accuracy	2	%Accuracy	3	%Accuracy
Measured concentration (ng/mL)	243.91	97.57	245.11	98.04	256.99	102.79
	246.50	98.60	269.95	107.98	268.79	107.52
	232.41	92.96	264.32	105.73	269.49	107.80
	251.80	100.72	227.28	90.91	267.86	107.14
Intra-batch mean (ng/mL)	243.66		251.66		265.78	
Intra-batch precision (%CV)	3.36		7.72		2.22	
Intra-batch accuracy (%)	97.46		100.67		106.31	
Inter-batch mean (ng/mL)	253.70					
Inter-batch precision (%CV)	4.42					
Inter-batch accuracy (%)	101.48					
Sample type	**QCH**					
Sample batch	1	%Accuracy	2	%Accuracy	3	%Accuracy
Measured concentration (ng/mL)	415.70	103.93	408.51	102.13	361.35	90.34
	400.69	100.17	416.82	104.21	354.01	88.50
	417.29	104.32	423.32	105.83	435.35	108.84
	372.61	93.15	413.92	103.48	360.00	90.00
Intra-batch mean (ng/mL)	401.57		415.64		377.68	
Intra-batch precision (%CV)	5.16		1.48		10.22	
Intra-batch accuracy (%)	100.39		103.91		94.42	
Inter-batch mean (ng/mL)	398.30					
Inter-batch precision (%CV)	4.82					
Inter-batch accuracy (%)	99.57					

*%CV = percentage of coefficient variants; **QCL, QCM, and QCH of NDMS were 30,250,400 ng/ml, respectively

HPLC method with UV detection for the quantification of sildenafil and its active metabolite, NDMS, in plasma samples. Notably, this is the first validated HPLC method explicitly developed for measuring sildenafil and its metabolite in plasma from patients with PAH.

Sildenafil is widely known as a medication for erectile dysfunction. However, it is also commonly used in PAH therapy, with lower doses than those used for erectile dysfunction. Previous studies typically investigated sildenafil in 50 or 100 mg tablet forms, while PAH therapy uses 20 mg tablets. Data on sildenafil 20 mg are scarce. Furthermore, most prior studies used healthy male subjects. The present study is the first to utilize samples from patients with PAH, and the authors included both male and female participants.

The demographic characteristics of our study population were predominantly middle-aged women, which is consistent with the epidemiology of PAH reported in a previous study[10]. Plasma samples from all 20 participants were successfully analyzed for both sildenafil and NDMS peak concentrations, with all values falling within the established calibration range. This demonstrates that the

Table 3. Recovery of sildenafil and NDMS

	QC concentration (ng/mL)	Mean measured total (ng/mL)	%CV	Mean recovery (%)
Sildenafil	30	29.22	1.97	97.41
	700	718.55	2.55	102.65
	1,200	1,248.20	1.78	104.02
NDMS	30	32.68	2.58	108.93
	250	251.66	7.72	100.67
	400	415.64	1.48	103.91

calibration range used in our validated HPLC method was sufficiently broad to encompass clinically relevant drug levels observed in patients with PAH.

The HPLC system used in this present study is a simple technique for measuring drug levels in plasma, though it has lower sensitivity than LC-MS/MS. Many healthcare centers with access to LC-MS/MS use it for drug-level measurements due to its higher sensitivity. However, LC-MS/MS is costly and not available in all centers or hospitals. This study aimed to develop a method using HPLC, which is more affordable and accessible in many settings, as an alternative to LC-MS/MS. Our validated method is both simple and accurate. The results were consistent with previous studies regarding stability and intra- and inter-batch quality controls.

Although this method has a higher LLOQ than LC-MS/MS, its LLOQ is lower than previously reported for HPLC methods[11]. Additionally, the authors established a wide linear correlation range for sildenafil and NDMS (10 to 1,500 ng/mL and 10 to 500 ng/mL, respectively), covering the reported plasma concentrations. Our method also showed the lower LOD for sildenafil and NDMS compared to previously validated HPLC methods[12]. Stability at room temperature was consistent with that reported in the previous study[13].

The main limitation of our study is the inherent sensitivity of the HPLC system, which is generally lower than that of LC-MS/MS, resulting in a higher LLOQ or LOD. However, the validated HPLC method used in this study reflects the optimal performance achievable within HPLC-based techniques.

Conclusion

This HPLC method with UV detection was validated for its simplicity, reliability, precision, recovery, stability, and specificity for measuring sildenafil and NDMS in plasma from PAH patients.

What is already known on this topic?

Previous methods are complex, costly, and limited to detecting NDMS, with restricted accessibility. Moreover,

Table 4. Summary of sildenafil and NDMS stability tests

Stability test	Sildenafil				NDMS			
	QCL		QCH		QCL		QCH	
	% Accuracy	% Variation	% Accuracy	% Variation	% Accuracy	% Variation	% Accuracy	% Variation
Autosampler stability at 6 hours	104.78	5.39	102.25	-2.14	100.63	-2.65	106.67	0.99
Freeze-Thaw stability at 3 cycles	101.27	0.50	100.45	0.65	100.05	5.29	98.52	-4.70
Short-term stability at 6 hours	103.61	8.94	103.39	10.30	100.94	0.31	108.91	2.27
Long-term stability at 30 days	90.62	5.61	96.88	2.75	98.55	0.91	113.39	14.48

Table 5. Demographic data

Demographic data	Male, n=4 (Mean±SD)	Female, n=16 (Mean±SD)	Total, n=20 (Mean±SD)	p-value
Aged (years)	40.25±5.74	43.19±15.55	42.6±14.06	0.553
BMI (kg/m^2)	23.21±7.57	21.46±3.40	21.81±4.32	0.679
RHC parameter; mPAP (mmHg)	53.00±13.34	55.38±18.65	56.67±17.34	0.780
Sildenafil dosage per day (mg)	160.00±89.35	123.44±49.90	130.75±58.74	0.481
Peak sildenafil conc. (ng/mL)	473.18±540.70	418.39±522.49	429.93±511.45	0.864
Peak sildenafil C/D ratio (ng/mL/mg)	2.66±1.92	3.24±2.71	3.12±2.52	0.639
Peak NDS conc. (ng/mL)	375.59±450.55	122.65±149.41	173.24±245.87	0.345
Peak NDS C/D ratio (ng/mL/mg)	1.79±1.70	0.91±0.76	1.08±1.02	0.383

BMI=body mass index; C/D ratio=concentration per sildenafil dosage per day; conc.=concentrations; mPAP=mean pulmonary artery pressure; RHC=right heart catheterization

previous studies used plasma from healthy volunteers rather than PAH patients, highlighting a significant research gap.

What this study adds?

The authors developed a precise, reliable, and accessible HPLC method to measure sildenafil and NDMS in PAH patient plasma, enabling the effective detection of drug levels across all guideline-recommended doses.

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Conflicts of interest

The authors declare no conflict of interest.

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