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Simultaneous assay of sildenafil and desmethylsildenafil in neonatal plasma by ultra-performance liquid chromatography–tandem mass spectrometry

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ABSTRACT: Sildenafil is used to treat pulmonary hypertension in neonatal and pediatric patients. Pharmacokinetic studies in these patients are complicated by the limited sample volume. We present the validation results of an assay method to quantitate sildenafil and desmethylsildenafil simultaneously in 50 μL of plasma. Deuterated sildenafil was used as an internal standard. After liquid–liquid extraction, analytes were separated on an ultra-performance liquid chromatography (UPLC)-column and quantified via tandem mass spectrometry. The calibration range was linear, with acceptable accuracy and a precision of <15% for both compounds. The lower limits of quantification were 1 ng/mL. Matrix effects were present, but inter-plasma batch variability was under 12%. The method was successfully applied to samples from a pharmacokinetic study into sildenafil pharmacokinetics in neonates, making maximum use of the limited number and amount of plasma samples available.

Keywords: ultra-performance liquid chromatography; mass spectrometry; sildenafil; plasma; neonates; pharmacokinetics

Introduction

Sildenafil is a potent phosphodiesterase (PDE-5) inhibitor of vascular smooth muscle cells and has been licensed under the trade name Revatio® for the treatment of pulmonary hypertension (PH) in adults. In pediatrics, PH treatment options include the off-label use of sildenafil, but so far only one pharmacokinetic study has identified a suitable dosage regimen in a subset of pediatric patients (Mukherjee *et al*., 2009). More studies are needed to define the optimal dose for individual neonatal and pediatric patients (Shah *et al*., 2007; Garg *et al*., 2008). These studies are complicated by the limited amount of biological material (e.g. blood, plasma) available, which poses restrictions on sampling frequency and sample volume. Ideally, analytes of interest should be quantitated simultaneously in as little sample as possible. This limits the burden on individual patients caused by sampling, while maintaining sufficient data points for reliable data analysis.

A sensitive method to quantitate analytes in low volumes of complex matrices is ultra-performance liquid chromatography with tandem mass spectrometry detection (UPLC-MS/MS). With a smaller particle size and higher operating pressures compared with regular HPLC, UPLC provides a shorter runtime and sharper peak shape, which improves sensitivity and reduces potential interference by matrix components (Plumb *et al*., 2004; Churchwell *et al*., 2005; Guillarme *et al*., 2007). UPLC-MS/MS should therefore allow quantitative analysis of multiple analytes with minimal sample preparation and matrix effects.

Several reports on the assay of sildenafil and its active metabolite desmethylsildenafil (Fig. 1) are available, but none describes the combination of UPLC-MS/MS with a low-sample volume and its application in neonatal or pediatric pharmacokinetic studies (Eerkes *et al*., 2002; Al-Ghazawi *et al*., 2007; Wang *et al*., 2005; Sheu *et al*., 2003; Kim *et al*., 2003). We present the validation results of a method for simultaneous assay of sildenafil and desmethylsildenafil in human plasma after liquid–liquid extraction, using ultra-performance liquid chromatography and tandem mass spectrometry. Method applicability is demonstrated with pharmacokinetic curves of sildenafil and desmethylsildenafil in a neonate with PH.

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Abbreviations used: DMS, desmethylsildenafil; ECMO, extracorporeal membrane oxygenation; FA, formic acid; ME, matrix effects; PDE-5, phosphodiesterase; PE, process efficiency; PH, pulmonary hypertension; RE, recovery; SIL, sildenafil citrate.

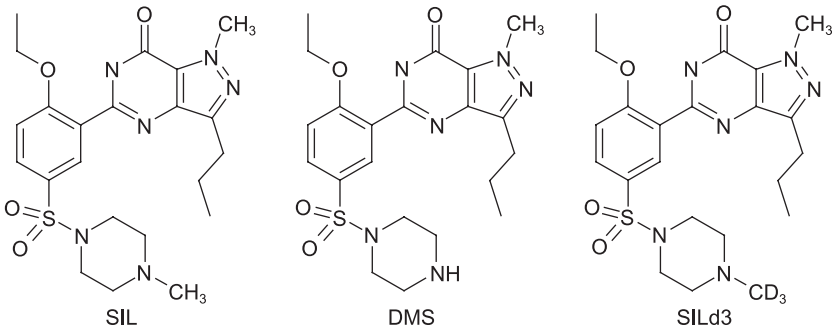

Figure 1. Chemical structures of sildenafil (SIL), desmethylsildenafil (DMS) and internal standard sildenafil-d_3 (SILd3).

Experimental

Reagents

LC-MS-grade water and liquid chromatography-grade methanol were from Biosolve (Valkenswaard, Netherlands). Formic acid (FA, Sigma, Schnelldorf, Germany) was analytical grade. Analytical grade acetic acid, ethyl acetate and sodium hydroxide were from Merck Co. (Darmstadt, Germany). Sildenafil citrate was kindly provided by Pfizer Inc. (Groton, CT, USA). *N*-Desmethylsildenafil citrate was purchased from Bio-Connect (Huissen, Netherlands). Sildenafil-d_3 was from C/D/N Isotopes Inc (Pointe-Claire, Quebec, Canada).

Quality Control Samples And Standard Solutions

Standard stock solutions containing either sildenafil citrate (SIL) or desmethylsildenafil (DMS) citrate (100 and 50 μg/mL respectively, calculated as free base) were prepared in water. These stock solutions were serially diluted and added to blank water or human plasma (ratio 1:9), to produce final concentrations of 1, 3, 10, 20, 70, 100, 300, 600 and 1000 ng/mL for SIL, and 1, 3, 10, 20, 50, 70, 100, 300 and 500 ng/mL for DMS. Calibration standards were prepared along with quality control (QC) samples for intra- and inter-assay comparisons, which were similarly prepared using a separate stock solution. Low (L), medium (M) and high (H) controls were prepared in plasma at concentrations of 5, 400 and 800 ng/mL for SIL and 5, 200 and 400 ng/mL for DMS. All stock and working standard solutions were stored at –80°C. The internal standard (IS) sildenafil-d_3 was dissolved in water to a concentration of 100 μg/mL and diluted with water to prepare a final concentration of 200 ng/mL.

Sample Preparation

To 50 μL of plasma, 20 μL of 0.02 M aqueous sodium hydroxide, 20 μL IS solution and 500 μL of ethyl acetate were added in a glass tube. The sample was vortexed for 20 s. After centrifugation at 16,000***g*** for 10 min, the supernatant organic phase was transferred to a clean glass vial. The solvent was evaporated to dryness at 35°C under nitrogen gas flow, after which the residue was reconstituted in 50 μL of methanol–water–acetic acid solution (40:59:1 v/v/v). When cloudy, samples were centrifuged again at 16,000***g*** for 10 min. The supernatant was transferred to a polypropylene autosampler vial with glass insert and stored at 5°C until assay with UPLC-MS/MS.

UPLC-MS/MS Conditions

The UPLC-MS/MS system consisted of a Waters Acquity Ultra Performance LC coupled to a Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The analytical column was an Acquity UPLC BEH C18 2.1 mm × 100 mm column with 1.7 μm particle size (Waters Ltd, Dublin, Ireland), to which a 0.2 μm pre-column filter unit was added. The mobile phase was a gradient of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol) with an initial composition of 60% B. Mobile phase composition changed linearly from 60% B to 90% B over the course of 1 min. The composition was switched back to 60% B and left to equilibrate. Total runtime was 2 min. The flow rate was 0.3 mL/min with a column temperature of 30°C and an injection volume of 10 μL. Analytes were detected via MS/MS with an electrospray ionization (ESI) interface in positive multiple reaction monitoring (MRM) mode. A solvent delay function was used to divert flow to waste whenever possible. Optimized MRM-settings for the individual drugs, including cone voltage and collision energy, are listed in Table 1. The acquisition settings were: capillary voltage = 3.4 kV; source temperature = 120°C; desolvation temperature = 300°C; desolvation gas flow = 500 L/h; cone gas flow = 50 L/h. Data were acquired using Masslynx V4.1 software and processed using Quanlynx V4.1 (Waters Inc.).

Validation Procedure

The validation procedure included specificity, sensitivity, linearity, accuracy, precision, matrix effects, recovery and autosampler stability.

Table 1. UPLC-MS/MS Acquisition parameters[a] for SIL and DMS

	Q1 (*m/z*)	Q3 (*m/z*)	CV (V)	Dwell time (s)	CE (eV)	R_t (min)
SIL	475.1	99.7	45	0.200	28	1.20
DMS	461.1	283.0	45	0.200	36	1.22
IS (SIL-d_3)	478.2	311.2	50	0.200	30	1.21

[a] Q1, parent-ion mass; Q3, daughter-ion mass; CV, cone voltage; CE, collision energy; R_t, retention time.

Specificity and selectivity. Chromatograms from aqueous calibration standards were compared with those of five batches of blank plasma before and after spiking with SIL, DMS and IS. Ion traces of each analytes mass transition were checked for interferences at the respective retention times.

Limit of quantification. The lower limit of quantification (LLOQ) was defined as the lowest concentration that could be quantified with accuracy and precision within ±20%, as calculated from chromatograms of the five lowest calibration samples.

Standard curves. Curves consisting of nine concentrations were constructed for each analyte, with five independent measurements for each calibration standard. Best fit was selected after exploration of different regression models and weighting factors: the final curves were constructed by weighted (1/*x*) least square linear regression with a fixed origin.

Accuracy and precision. Intra- and inter-day accuracy and precision were calculated for the three quality controls with five measurements each, and in duplicate on six different days, respectively. Accuracy was defined as a percentual deviation from the nominal concentration by quantifying QC samples on a freshly prepared calibration curve. Precision was defined as the relative standard deviation (RSD = standard deviation/mean × 100%).

Matrix effects and recovery. Plasma and solvent components in the ionization chamber cause batch specific ion suppression or enhancement, leading to inter-patient and intra-patient signal variability (Careri and Mangia, 2006; Taylor, 2005). Matrix effects were quantified as previously described (Matuszewski *et al*., 2003). In short, chromatograms were recorded of plasma that was spiked pre-extraction, plasma spiked post-extraction and spiked aqueous eluens. In total, six batches of blank plasma were spiked with low and high concentrations of each analyte in duplicate. Recovery (RE) was defined as the relative signal of samples spiked post-extraction vs pre-extraction. Matrix effects (ME) were similarly defined as the relative signal of post-extraction spiked plasma samples vs. spiked aqueous samples. Process efficiency (PE) was defined as the product of RE and ME, i.e. the overall signal of spiked plasma vs an aqueous standard solution. Average values and coefficients of variation of RE, ME and PE were calculated over the six plasma batches.

Sample stability. Stock stability was tested in QC samples at –20°C (1 month) and compared with reference samples stored at –80°C. Autosampler stability was tested in two QC samples whose extracts were stored at 4°C for up to 3 days. We chose to forego stability testing in plasma and whole blood, since these have been described extensively before. SIL and DMS have been proven to be stable in plasma for at least 3 months at –70 and –20°C, after five freeze–thaw cycles and for 48 h at room temperature (Tesfu, 2004; Wang *et al*., 2005; Eerkes *et al*., 2002; Al-Ghazawi *et al*., 2007). After 7 days at 4°C, >85% of SIL and DMS can be recovered from whole blood (Lewis *et al*., 2000).

Clinical Application

We present data from a term neonate with pulmonary hypertension after extracorporeal membrane oxygenation (ECMO) treatment. This was part of a larger study into population pharmacokinetics during ECMO at the Sophia Children's Hospital; the study protocol was approved by our Institutional Ethics Committee. She received sildenafil treatment starting from a postnatal age of 121 days, with a total body weight of 4.8 kg. Sildenafil therapy (0.5 mg/kg q.i.d. via a nasogastric tube) was started while on ECMO, the dose was incrementally increased based on perceived efficacy up to a total of 28 mg/day. Blood (between 100 and 500 μL) was sampled from an arterial line after ECMO decannulation and consisted of a five-point curve at 0, 1, 2, 4 and 6 h after a dose. In addition, samples were taken at random times, three times daily, for as long as an arterial line was available. After centrifugation, plasma was stored at –80°C until analysis. Plasma levels of SIL and DMS were measured in 50 μL. Individual pharmacokinetic curves were constructed by fitting measured plasma levels to a one-compartment population pharmacokinetic model of 11 patients treated with sildenafil (nine female, median postnatal age 20 days, median weight 4.0 kg) using nonlinear mixed effects modeling (NONMEM, Globomax LLC, Ellicott City, MD, USA).

Results and Discussion

The mass transitions of sildenafil (*m/z* 475.1 → 99.7), desmethylsildenafil (*m/z* 461.1 → 283.0) and sildenafil-d_3 (*m/z* 478.2 → 311.2) were selected for electrospray ionization based on their stability and high intensity. Interestingly, SIL's daughter ion of 99.7 provided better stability and intensity than the daughter ion at 311.2, which was selected for its deuterated counterpart. The internal standard and both analytes showed similar chromatographic behavior, which is reflected in the retention times (Fig. 2). There were no discernable interfering compounds in plasma judging from a comparison between spiked plasma, spiked aqueous standards and plasma blanks. The patient samples containing SIL and DMS did not show additional peaks in their total ion currents. From this, we concluded that the combination of liquid–liquid extraction, UPLC and MS/MS detection via multiple reaction monitoring provides sufficient specificity and selectivity. The runtime of 2 min is probably suitable for high sample throughput and could potentially be reduced even further, but this was deemed unnecessary as the total analysis time was mainly dependent on the liquid extraction procedure.

The calibration curves provided reliable linear responses from 1 to 1000 ng/mL for SIL and 1 to 500 ng/mL for DMS, respectively. Mean coefficients of determination (r^2) of the 1/*x*-weighted calibration curves were 0.9990 (range 0.9986–0.9999, $n = 5$) for SIL and 0.9963 (range 0.9936–0.9969, $n = 5$) for DMS, respectively. Plots of weighted residuals vs concentrations did not indicate nonlinearity. Despite the low sample volume, sensitivity is sufficient with an LLOQ of only 1 ng/mL in plasma for both compounds, which is reflected in the precision and accuracy results (Table 2). Intra- and inter-day precision is under 15% for L and H QC samples for both SIL and DMS. Accuracy is between 90 and 110% for all samples, with the exception of inter-day DMS samples. We have no explanation for these aberrant results, but considering that SIL pharmacokinetics is our main focus, we chose to forego extensive efforts into method improvement for DMS and accept the deviations in accuracy. Since we expect DMS plasma concentrations to be in the lower to medium range of the calibration curve, the implications of a reduced accuracy are probably mild. Perhaps a deuterated internal standard for DMS could remedy this problem, but at great financial expense.

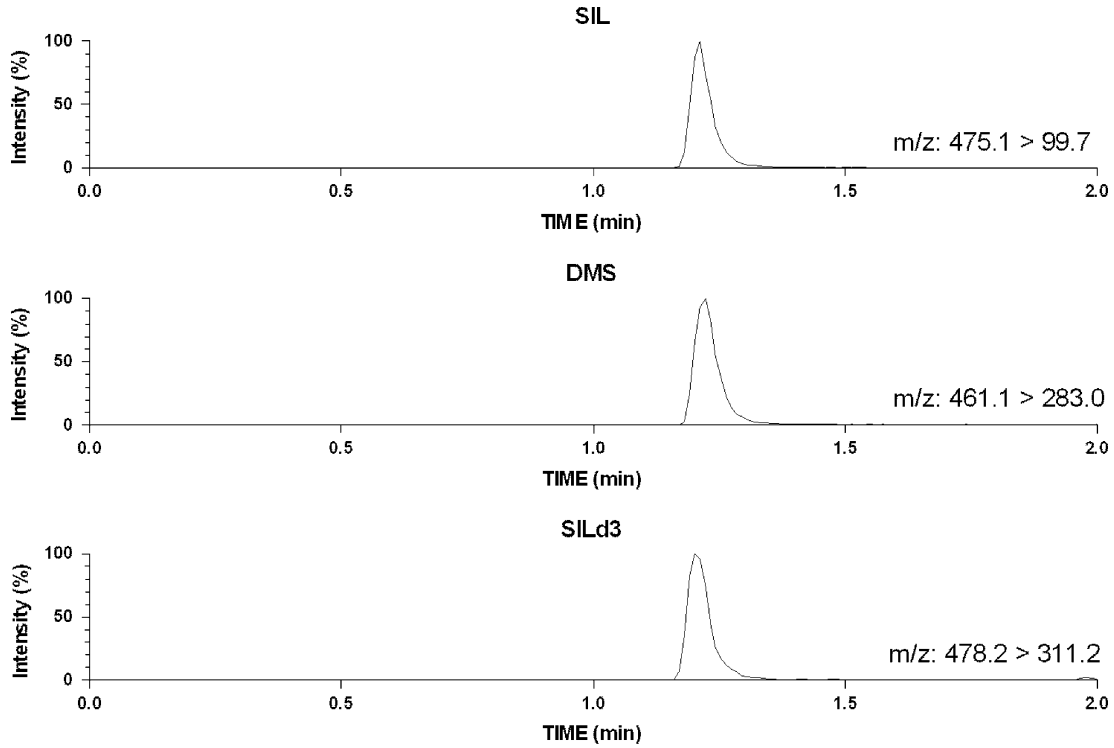

Figure 2. Chromatogram of sildenafil (SIL), desmethylsildenafil (DMS) and internal standard sildenafil-d_3 (SILd3). Each curve is the ion trace of a mass transition specific for each analyte.

Table 2. Intra- and inter-run precision and accuracy data for SIL and DMS

	Added (ng/mL)	Precision		Accuracy (%)
		Measured (ng/mL)	RSD (%)	
Intra-day (n = 5)				
SIL	4.99	4.84	3.46	97.0
	400	409	3.11	102
	799	769	3.47	96.3
DMS	5.01	5.20	8.60	104
	200	206	10.8	103
	400	418	3.47	104
Inter-day (n = 12)				
SIL	4.99	4.85	13.4	97.2
	400	383	9.95	96.0
	799	813	7.07	102
DMS	5.01	5.34	10.7	107
	200	232	13.9	116
	400	501	13.6	125

Matrix effects were extensively evaluated with low and high QC-dilutions in six batches of blank plasma (Table 3). Although plasma components appear to cause significant signal loss (on average 52% for SIL and 28% for DMS) and recovery is below 65%, the liquid extraction and UPLC-separation appear to lead to good reproducibility: inter-batch variability for ME, RE and PE is under 15% for both analytes. This might be a result of the use of a deuterated internal standard vs a structural analog.

We evaluated autosampler stability to estimate the potential autosampler storage period between sample processing and UPLC-MS/MS injection. After three days at 4°C, signal intensity of processed L, M and H QC samples was within 90–110% of the reference value, indicating that samples can be processed beforehand as long as they are stored at 4°C. Stock stability was satisfactory as well: after one month of storage at either –80 or –20°C, we could identify no degradation of SIL or DMS.

This method was applied to a pharmacokinetic study of sildenafil in neonatal patients with pulmonary hypertension. We successfully modeled SIL and DMS plasma concentrations on a one-compartment model for each compound, with oral absorption and first-order elimination. Using the population model, individual Bayesian pharmacokinetic parameters were calculated for a neonatal PH patient. Concentration–time curves were constructed using the dose history, see Fig. 3. Pharmacokinetic parameter estimates for the population and the selected individual are given in Table 4. Inter-patient variability in clearance and distribution volume estimates were large (from 62% for $CL_{desmethylsildenafil}$ up to 94% for $V_{sildenafil}$), which explains the difference between the population parameters and the selected individual. These data show that the LC-MS method can be used in combination with PK modeling software to deduce PK parameter estimates using sparse sampling designs with few samples per individual.

Conclusion

We present a sensitive, simple UPLC-MS/MS method for simultaneous quantification of sildenafil and its main metabolite in 50 μL of plasma. With an LLOQ of 1 ng/mL for both analytes and an instrument runtime of only 2 min, this method allows high

Table 3. Matrix effects (ME), recovery (RE) and process efficiency (PE) for the assay of SIL and DMS in plasma, $n = 6$ batches of human plasma

	SIL		DMS	
	L	H	L	H
ME (inter-batch RSD)	0.50 (3.3%)	0.47 (1.3%)	0.81 (6.9%)	0.64 (2.5%)
RE (inter-batch RSD)	0.44 (10.5%)	0.64 (10.4%)	0.23 (7.8%)	0.32 (5.2%)
PE (inter-batch RSD)	0.22 (11.0%)	0.30 (10.5%)	0.18 (10.4%)	0.21 (5.7%)

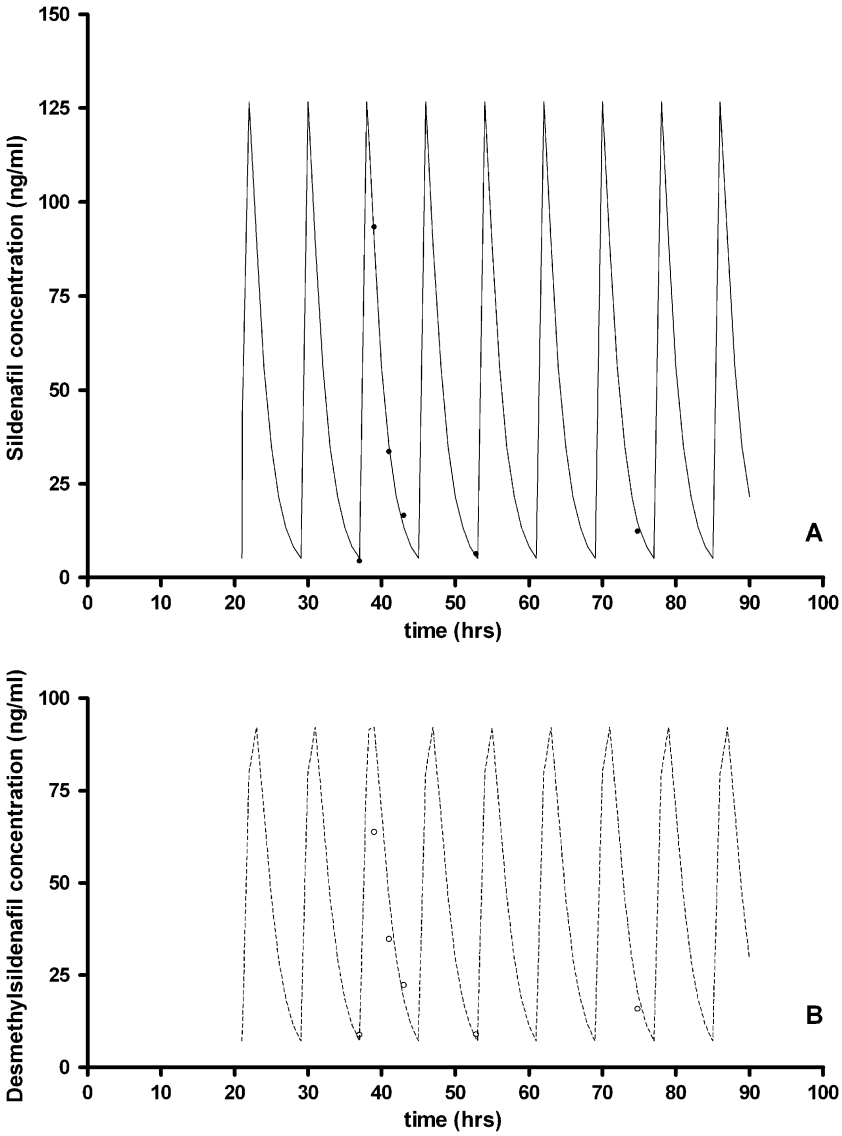

Figure 3. Concentration-time curves of sildenafil (A) and desmethylsildenafil (B) in a neonate with pulmonary hypertension. Circles are measured concentrations; the curves were constructed using the recorded dose regimen and the individual Bayesian predicted PK parameters derived from a population model of 11 individuals.

sample throughput. In combination with pharmacokinetic modeling software, the low sample requirements make it particularly suitable for pharmacokinetic studies in neonates, making maximum use of the limited number and amount of plasma samples available.

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Table 4. Pharmacokinetic parameter estimates[a]

	Population estimates	Individual estimates
Sildenafil		
k_a (/h)	2.4 ± 4.2%	2.4
V (L)	34 ± 5.0%	37.6
CL (L/h)	7.3 ± 17%	18
Desmethylsildenafil		
V (L)	14 ± 35%	13.9
CL (L/h)	9.7 ± 19%	19.6

[a] k_a, Absorption rate constant; *V*, distribution volume; *CL*, clearance. Population estimates are given as medians ± their corresponding standard deviations. *V* and *CL* estimates are displayed assuming a biological availability of 100% and a SIL to DMS conversion fraction of 100%.

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