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Short communication

Quantitative analysis of sildenafil and desmethylsildenafil in human serum by liquid chromatography–mass spectrometry with minimal sample pretreatment

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ABSTRACT

A simple, sensitive and specific liquid chromatography tandem mass spectrometry method with minimal sample pretreatment was developed for the simultaneous analysis of sildenafil and its metabolite desmethylsildenafil in human serum. Sample pretreatment consisted of adding a methanolic solution of the internal standard vardenafil to the samples. After vortexing and centrifugation the samples were directly injected onto the C18 column using gradient elution. The aqueous and organic mobile phases were ammonium acetate 2 mM supplemented with 0.1% formic acid in water and methanol, respectively. The detection by a triple quadrupole mass spectrometer in positive ESI ionization mode was completed within 5 min. The lower limits of quantification for sildenafil and desmethylsildenafil are 1.0 ng/ml. The intra- and inter-day precisions measured as relative standard deviation were within 10% for both compounds over the linear range. Intra- and inter-day accuracy of sildenafil and desmethylsildenafil ranged from 92 to 103%. This method has been used in a clinical pharmacokinetic study of sildenafil in intensive care patients.

1. Introduction

Sildenafil (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo-[4,3-d]pyrimidin-5yl)-phenylsulphonyl]-4-methylpoperazine) is a type 5 phosphosiesterase (PDE_5) inhibitor. It is registered for the treatment of erectile dysfunction and recently for the treatment of pulmonary hypertension. The effectiveness of sildenafil in the treatment of pulmonary hypertension, is based on vasodilatation in well ventilated areas in the diseased lung. It decreases pulmonary arterial pressure with only a modest systemic effect [1–3].

After oral administration sildenafil is rapidly absorbed with a bioavailability of approximately 45%. It is metabolised in the liver by CYP3A4 and is converted into the active metabolite N-desmethylsildenafil (Fig. 1). Sildenafil is excreted predominantly as metabolites in faeces (80%) and to a lesser extent in urine (13%).

Several methods have been described to quantify sildenafil and its metabolite in plasma samples, using liquid chromatography and mass spectrometry [4–9]. These methods include a pretreatment of the samples with liquid–liquid extraction (automated) solid phase extraction or automated sequential trace enrichment. This paper describes an analytical method for the measurement of sildenafil and desmethylsildenafil in human serum which is fast, has minimal sample pretreatment and does not need specific pretreatment equipment. The assay has successfully been applied to a clinical pharmacokinetic study of sildenafil and desmethylsildenafil in intensive care patients with Acute Respiratory Distress Syndrome (ARDS).

2. Experimental

2.1. Materials

Reference compounds sildenafil and desmethylsildenafil (purity 99.3 and 100%) were a gift from Pfizer Global Research & Development (Sandwich, UK). Vardenafil (purity 99.1%) was a gift from Bayer AG Pharma (Leverkusen, Germany). Methanol (HPLC Supra Gradient) was obtained from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate (AR grade reagent) and Formic Acid (AR grade reagent) were purchased from Merck (Darmstadt, Germany). Throughout the procedure water was used which was purified with the Synergy UV water purification system from Millipore (Billerica, MA, USA).

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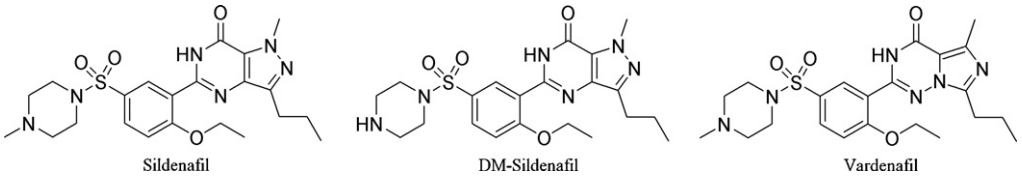

Fig. 1. Structures of sildenafil, its metabolite and the internal standard.

2.2. Calibration standards and QC samples

Calibration standards and QC samples were made by adding known amounts of sildenafil and desmethylsildenafil to blank serum. Sildenafil and desmethylsildenafil stock solutions were prepared at concentrations of 0.15 mg/ml in methanol. A working solution was prepared by diluting the stock solutions with methanol to a concentration of 6 ng/μl for sildenafil and 3 ng/μl for desmethylsildenafil. Blank serum was spiked to yield a concentrations of 15, 30, 75, 150, 300, 600 ng/ml for sildenafil and 7.5, 15, 38, 78, 150, 300 ng/ml for desmethylsildenafil and stored at −20 °C. QCs were made in the same way at four different concentrations: 1.0, 32.7, 81.8 and 205 ng/ml for sildenafil and 1.0, 19.3, 48.1 and 120 ng/ml for desmethylsildenafil (LLOQ, Low, Medium, and High). QC samples remained stable at −20 °C for at least 6 months, showing less then 10% decline in concentration.

2.3. LC

Chromatography was carried out on an Xbridge Shield RP18, 100 mm × 4.6 mm, i.d.; 3.5 μm (Waters, Milford, MA, USA) using a 2795 Alliance HPLC system (Waters). Mobile phase A consisted of 2 mM ammonium acetate in water, containing 0.1% formic acid and mobile phase B was 2 mM ammonium acetate in methanol, containing 0.1% formic acid. A gradient was carried out at a flow rate of 0.4 ml/min starting from 50% B to 60% B in 2 min. Between 2 and 4 min B was subsequently decreased to 50%. Separation was performed at 55 °C. Samples in the autosampler were kept at 10 °C, which is standard in our laboratory.

2.4. MS/MS

A Waters Micromass Quattro micro API was used for all analysis. The instrument was operated in positive ESI ionization mode. Control of the instruments and calculations were made by MassLynx V4.1 software (Waters). Optimization of the operating conditions were made by constantly introducing a standard solution of the compounds (5 μg/ml) to the HPLC flow via a T-connector in the infusion mode. The signal was optimized on the total ion current in MS mode by changing cone-, capillary-, extractor- and RF lens voltages in the source and resolution and ion-energy in the analyser. On fragmentation, sildenafil, desmethylsildenafil and vardenafil (Internal Standard) specific daughter ions were formed, which were used to develop a SRM method for quantification of the compounds. At the same time the collision voltages and resolution in the second quadrupole were optimized.

2.5. Sample preparation

To 50 μl of the calibration standards, QC's or serum samples, 200 μl of internal standard solution (0.01 μg/ml vardenafil in methanol) was added and vortex-mixed for 30 s. After centrifugation for 10 min at 10.900 rpm the clear upper layer was transferred to autosampler vials. A 40 μl aliquot was injected into the system.

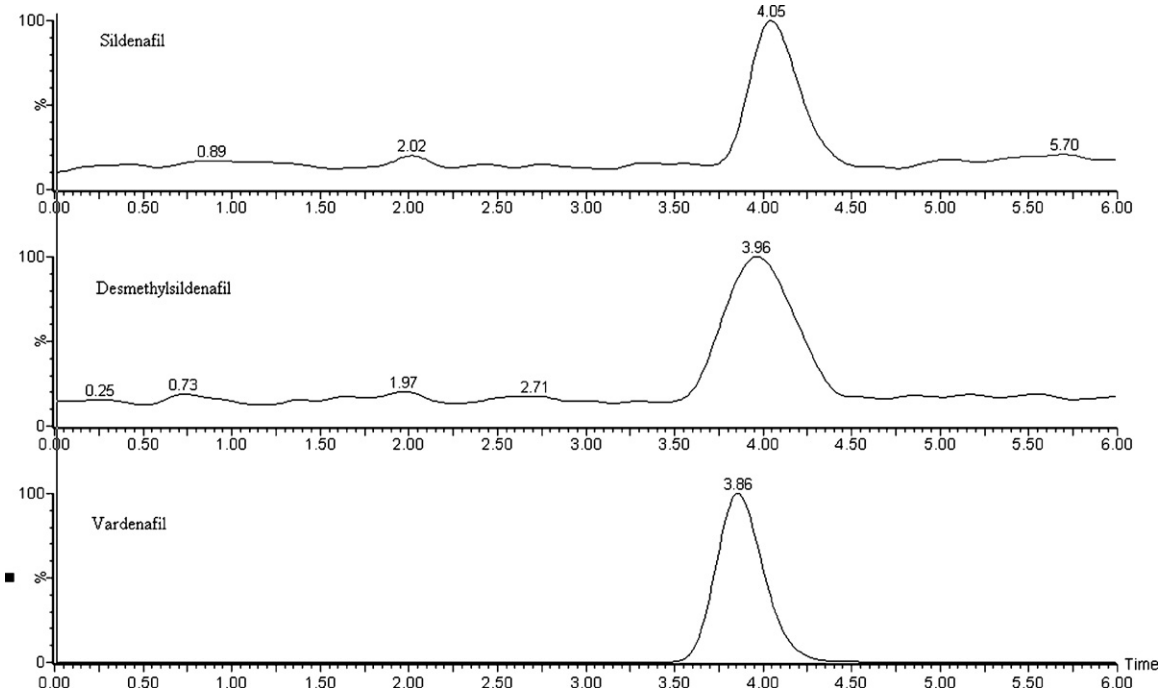

Fig. 2. SRM-chromatograms of a QC sample at LOQ level (sildenafil and DM-sildenafil 1.0 ng/ml).

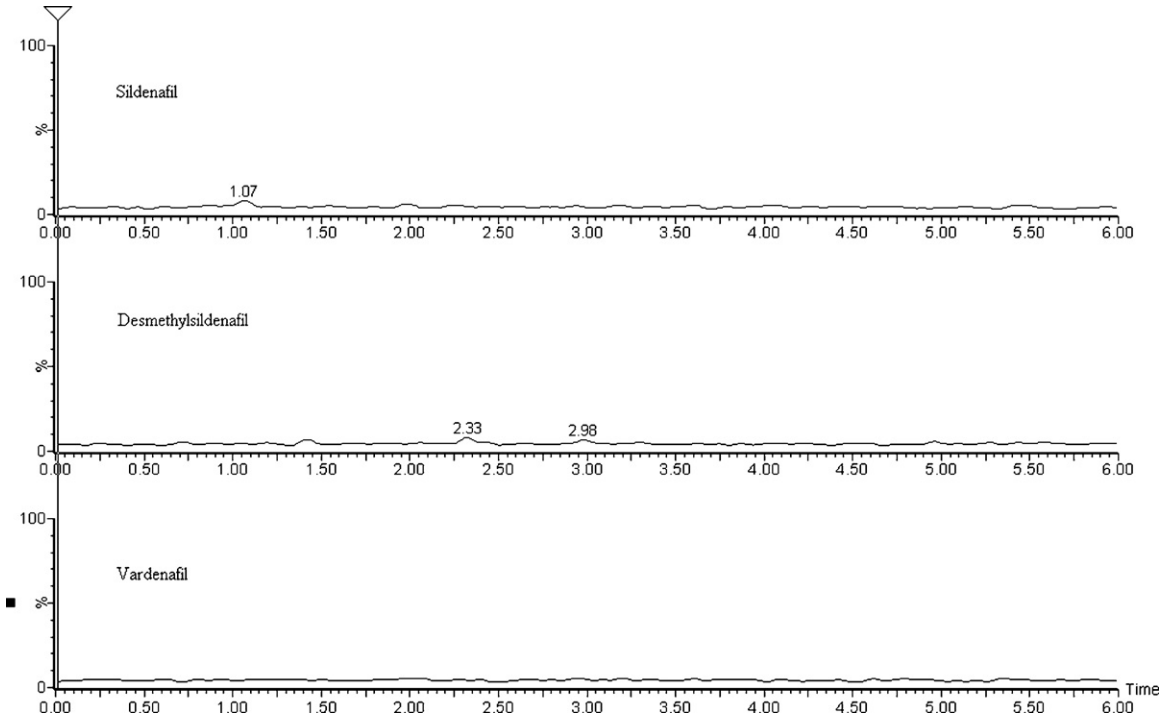

Fig. 3. SRM-chromatograms of a blank human serum sample.

2.6. Method validation

2.6.1. Matrix effects

To determine if any matrix-effect occurred blank samples ($n = 6$) were treated as samples. The aliquot was injected into the system while a solution of analytes (100 ng/ml) was added to the column outlet by a Tee-in. Interpretation was made if any increase or decrease occurred in the baseline at the retention time of the analytes. Thereafter solvent standards (SS) and post-extraction spikes (PES) were made to quantify the amount of matrix effects ($n = 6$). The difference between these standards was calculated as the difference between the areas.

$$\%\ \text{matrix effects} = ((\text{area PES}/\text{area SS}) - 1) \times 100$$

The subtraction of 1 was added so that a negative result indicates suppression and a positive result indicates enhancement of the analyte signal.

2.6.2. Linearity

Calibration curves of 6 concentrations sildenafil and desmethylsildenafil in serum were analysed in threefold. Linear regression curves were constructed of the peak ratios of analyte/internal standard versus concentration (ng/ml) and linearity was checked by ANOVA Lack of Fit.

2.6.3. Precision and accuracy

Precision and accuracy were carried out using four different concentrations of QC samples on the same day (intra-day; $n = 6$) and over 6 days (inter-day; $n = 6$). The concentrations of the analytes were determined from the calibration curves. Mean value and relative standard deviation were calculated and used to estimate the intra- and inter-day precision, Accuracy is expressed as the percent difference between the calculated determined mean concentration and the nominal concentration.

2.6.4. Clinical application

In a clinical study 50 mg of sildenafil was administered to 10 intensive care in patients with ARDS via nasogastric tube. Blood samples were collected before administration end every 30 min thereafter until 300 min for the measurement of sildenafil and metabolite levels. Serum samples were stored at −20 °C until analysis.

3. Results

3.1. LC–MS/MS analysis

In the MS/MS experiments the protonated precursor molecular ions $[M+H]^+$ were selected for fragmentation. The detector was operated in selected reaction monitoring (SRM) mode using the transitions of sildenafil at m/z 475.10 → 283.15, desmethylsildenafil at 461.10 → 283.15 and vardenafil at 489.10 → 151.1, respectively.

The optimum values for the cone voltage was 45 (V) and for the collision energy 35 (eV) for all analytes. The source temperature was set to 120 °C and the desolvation temperature to 350 °C. The desolvation gas flow was 800 l/h.

Under the described conditions, sildenafil, desmethylsildenafil and vardenafil gave retention times of 4.1, 4.0 and 3.9 min, respectively. Representative SRM chromatograms are shown in Figs. 2 and 3.

3.2. Method validation

3.2.1. Matrix effects

Fig. 4 shows SRM-chromatograms of a blank sample, pre-treated as described, under continuous infusion of analyte solution. An relatively small increase of response is observed in the SRM-chromatogram of vardenafil.

The quantification of the matrix effect, calculated from the post-extraction spikes versus the solvent standard was ($n = 6$)—3.3% for sildenafil, 7.9% for desmethylsildenafil and 0.4% for vardenafil.

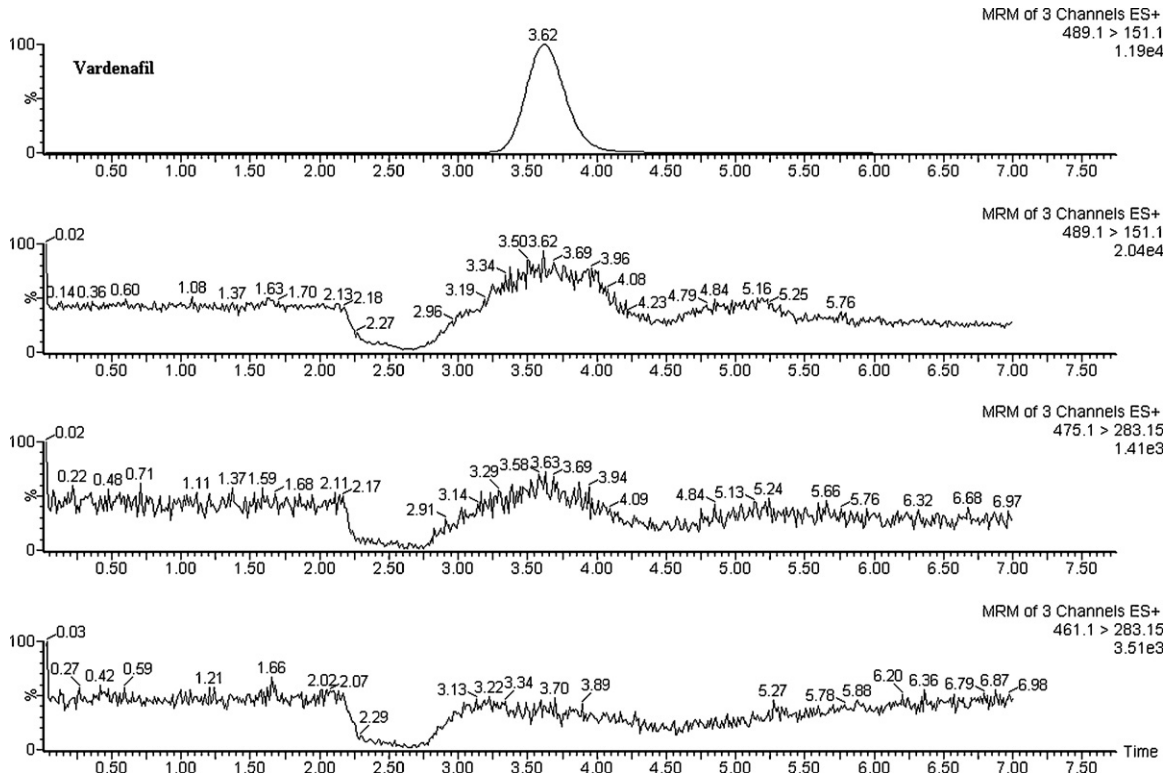

Fig. 4. Matrix effects qualitative.

3.2.2. Linearity

The assay was found to be linear in the concentration range 1–600 ng/ml for sildenafil and 1–300 ng/ml for desmethylsildenafil. Correlation coefficients were 0.9957 and 0.9966, respectively. A weighting factor of $1/x$ was used.

The corresponding equations were $y = -0.016 + 4.26x$ for sildenafil and $y = -0.022 + 1.09x$ for desmethylsildenafil.

3.2.3. Precision and accuracy

Precision (coefficient of variation) and accuracy for LOQ, low, medium and high concentrations of sildenafil and desmethylsildenafil in human serum ($n = 6$) are shown in Table 1.

3.2.4. Clinical application

In a clinical study of sildenafil in patients with ARDS 50 mg of sildenafil was administered. In Fig. 5 a typical concentration–time curve is shown. The concentration range of sildenafil within the group of patients was 0–975 ng/ml. The concentration range of desmethylsildenafil was 0–195 ng/ml.

Table 1
Validation results.

Concentration (ng/ml)		Accuracy (%)		Precision (%)	
Sildenafil	DM-sildenafil	Sildenafil	DM-sildenafil	Sildenafil	DM-sildenafil
Intra-day					
1.0 (LLOQ)	1.0 (LLOQ)	98.9	103.2	6.5	6.5
32.7	19.3	92.8	96.5	4.4	3.2
81.8	48.1	95.7	103.2	4.1	3.8
205	120	96.4	101.1	4.3	5.6
Inter-day					
1.0 (LLOQ)	1.0 (LLOQ)	95.9	100.3	8.5	8.0
32.7	19.3	95.2	97.3	5.6	5.1
81.8	48.1	96.5	102.8	4.6	2.3
205	120	96.6	100.7	1.6	1.2

4. Discussion

The LC/MS/MS system has become the instrument of choice for drug assay in biological fluids due to its selectivity and sensitivity. To minimize sample pretreatment protein precipitation can be applied. However a disadvantage of protein precipitation is that the assay is more susceptible for matrix effects in comparison with other sample pretreatment techniques. In this study it was established that minimal matrix-effects occurred which did not influence the outcome of the analysis. The assay showed to be accurate, precise and sensitive. It can be used for pharmacokinetic

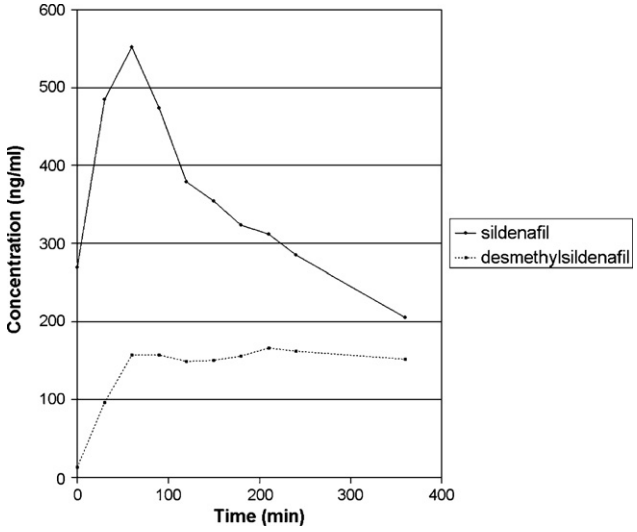

Fig. 5. Concentration–time curve from a patient with ARDS receiving 50 mg of sildenafil.

studies of sildenafil and desmethylsildenafil. It covers the concentration range of samples from the described study.

Pretreatment time was approximately 20 min for 30 samples. Cooper et al. [4] and Eerkes et al. [5] used automated equipment. This requires additional investments. Guermouche and Bensalah [6] used a off-line SPE procedure. It is our experience SPE requires 2–3 times more pretreatment time then the protein precipitation procedure. Kim et al. [7], Tracqui and Ludes [8] and Wang et al. [9] used a liquid–liquid extraction. This is even more time consuming because shaking for up to 15 min is required. After centrifugation, up to 10 min, evaporation is necessary which also costs time. Furthermore protein precipitation requires no large volume of organic solvents needed in liquid–liquid extraction. Fast sample pretreatment becomes a greater issue, because the time of analysis is becoming shorter these days. The introduction of ultra rapid LC (e.g. UPLC) enhances that.

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