Medicinal Chemistry Department, Venezuelan Scientist Research Institute, Caracas, Venezuela

Validation of an HPLC method for sildenafil citrate analysis in human plasma samples

A. QUINTERO, A. CALDERA, B. MILANO, I. ODREMAN, A. HURTADO, L. MANZANARES, J. VILLAMIZAR

Received June 4, 2009, accepted June 12, 2009

Dr. Alberto Quintero, IVIC, Departamento de Química Medicinal, Carretera Panamericana, K11, 2010 A, Caracas, Venezuela
ajquinte@ivic.ve

Pharmazie 64: 796–799 (2009) *doi: 10.1691/ph.2009.9180*

A simple, precise, rapid and accurate HPLC method for the determination of sildenafil citrate (SLD) in human plasma has been developed based on previous reports, to offer an alternative way to detect and quantify SLD and also to improve some characteristics of the validation process. Chromatography was carried out on a C18 reversed-phase column, using a mixture of acetonitrile: ammonium acetate (0.3 M pH 6.8) (1:1 v/v) as the mobile phase at a flow of 0.750 mL/min. Diazepam was used as an internal standard (IS) and detection was by UV at 240 nm. Samples were extracted with 200 μl of 0.1 M Na_2CO_3 and 5 mL of diethyl ether: dichloromethane (60:40). Retention times of SLD and IS were 4.8 and 7.1 min, respectively; total run time was 10 min. The linear range of SLD was found to be 20 – 1000 ng/mL. Limit of quantitation (LOQ) and limit of detection (LOD) were calculated to be 10 and 20 ng/mL, respectively. An improved percentage recovery of analyte is reported, showing a fast and reproducible approach to detect SLD in plasma human sample. The method was validated for its linearity, precision, accuracy, recovery, stability and specificity.

1. Introduction

Sildenafi citrate (SLD) 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo[4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl] sulfonyl]-4-methyl piperazine citrate is a active inhibitor of cyclic guanosine monophosphate (cGMP)-specifi phosphodiesterase type 5 (PDE5) and is used to improve penile erectile activity in patients with erectile dysfunction (Boolell et al. 1996). Various analytical methods have been reported for the determination of SLD including HPLC or LC-MS (Daragmeh et al. 2001; Dinesh et al. 2002; Liaw et al. 2001; Shim et al. 2002; Flores et al. 2004; Berzas et al. 2002). Some of them use C18 or monolithic columns. There are some articles in the literature reporting the determination of sildenafi in pharmaceuticals (Qin et al. 2002) or in serum samples using micellar electrokinetic methods (Pelander et al. 2003), capillary zone electrophoresis (Lee et al. 2005), gas chromatography (Ergun et al. 2005) or HPLC (Sheu et al. 2003). In procedures to detect and quantify metabolites in human fluids it is very important to achieve methodologies that could give accuracy and precision good enough to undergo a complete validation process. In this work, we developed a single, rapid, precise, and accurate method using reverse-phase HPLC combined with UV detection which can be applied to pharmaceutical forms and human plasma. The methodology used in this project incorporates different features reported in other papers about the mobile phase, internal standard and extraction process in order to improve the conditions to determine SLD from human plasma samples. The method proposed here offers an alternative approach that can be validated for its precision, accuracy, recovery, stability, sensitivity and specificit and it is highly reproducible for SLD from standard or tablets.

2. Investigations, results and discussion

An alternative procedure is proposed here as a suitable method for the analysis of SLD from human plasma. The chromatographic conditions, internal standard (Wang et al. 2003a) and extraction procedure have been already published by different authors. In this project we developed a different way to determine SLD in human plasma samples by combining different features of studies already reported to give a better validation process. A mixture of acetonitrile:ammoniun acetate (0.3 M pH 6.8) (1:1 v/v) at a f ow rate of 0.750 mL/min was found to be an appropriate mobile phase instead of 0.2 M as reported by Daraghmeh et al. (2001), allowing adequate and faster separation of SLD and diazepam as IS. Under these conditions, the retention times obtained were 4.8 min for SLD and 7.1 for IS. The IS was clearly separated from the drug and the retention times for both of them were highly precise. In human plasma samples both retention times were the same as those seen in mobile phase and non–interference from excipients was observed in chrogmratograms using placebo. This suggests that the mobile phase and extraction method employed here allow detection and quantificatio of SLD. A typical chrogmratogram for analysis of SLD and IS in human plasma is shown in the Fig. The calibration curve was linear over the concentration range of 20–1000 ng/mL for the analyte. The eight-point calibration curve gave acceptable results and was used for all the calculations. The mean correlation coefficien of the weighted calibration generated during the validation was 0.998 for the analyte. The precision and accuracy for SLD covering these concentrations ranged from 2.04 to 10.77% and 95.97 to 110.29%, respectively. Limit of quantificatio was 20 ng/mL, corresponding to the lowest value of the calibration curve. Limit of detection for

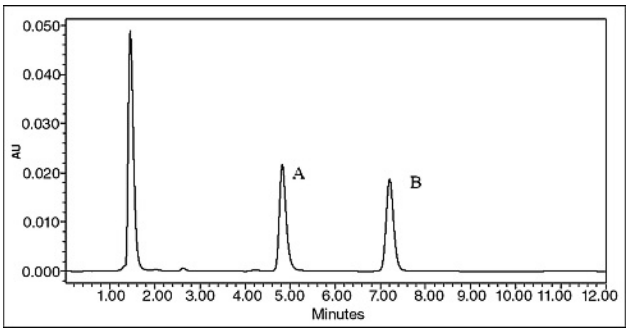

Fig.: Typical chromatogram for SLD 1 μg/mL (A) and IS 0,5 ug/mL (B) in human plasma

this system was 10 ng/mL which was calculated to be the lowest concentration detected with reproducible results (precision and accuracy for this value were 2.01 and 105.28%, respectively).

The method was validated for its intra and inter-day precision and accuracy. As seen in Table 1, at concentrations of 60, 150 and 800 ng/mL of SLD in plasma, intra-day precision and accuracy were in the range of 3.77–11.55% and 93.6–112.71%, respectively. In the inter-day experiment the values were between 3.10–6.81% and 92.64–107.63% for precision and accuracy, respectively. In this work, inter-analyst precision and accuracy were evaluated to examine whether the method was robust enough to support variation due to results coming from two different people. The data in inter-analyst assays showed values ranging from 3.84–6.20% and 92.47–110.20% for precision and accuracy, respectively (data not shown). These assays did not give significan error dispersion and the values found here were within the acceptance criteria.

SLD recovery was from the standard solution and from 50 mg tablets. The quantities of SLD and IS in the extraction process were improved when 5 ml of diethyl ether:dichloromethane (60:40) was added. The percentage recoveries of SLD at concentration of 60, 150 and 800 ng/mL were higher than 93.23% ± 9.62 (mean ± S.D., n = 6) and from the tablets were not less than 91.35% ± 11.90 (Table 2). The recovery of IS was assayed at the same three concentrations and the values ranged from 89.99 ± 3.97% to 97.36% ± 4.10 (data not shown). The data showed higher percentage recovery values than those reported by Wang et al. (2005) for SLD citrate. These results demonstrate that the extraction process of SLD and diazepam from human plasma is highly efficien and robust; indicating that tablet excipients did not interfere in the recovery of SLD either.

Stability of SLD and its IS after processing in the autosampler (post-extraction stability) is advantageous a larger number of plasma samples to be determined. QC samples were analyzed at once (controls), and 24 h and 48 h later. The results indicated that the analyte and IS were stable for at least 48 h. It took less than 18 h to run 100 samples. The precision and accuracy for the analyte ranged from 1.99 to 6.81% and 91.07 to 98.57%, and this suggests that samples can be analyzed by this system up to 48 h after the processing phase.

The extraction process can last up to 6 h as there are several stages and with a quite large number of samples, this does mean that the analyte contained in each sample might suffer degradation. In order to determine whether SLD and IS are affected by this lengthy treatment (and also the stability of the extraction process), QC samples were left at room temperature on the bench for 6 h and processed later. The values of precision and accuracy were between 1.48 and 8.66% and 89.39 and 99.85, respectively. The values found here established that samples were not degraded or affected by leaving them at room temperature for 6 h.

The storage time in a long-term stability evaluation spans the time between the firs sample collection and the last sample analysis. The long-term storage stability of samples at −70 °C was evaluated to establish acceptable storage conditions. Aliquots of human plasma at QC concentration were analyzed at day 1. Then samples from the same pool were analyzed against calibration curves from freshly prepared standards after storage at −70 °C for 1 month. The results showed that samples were stable for that time, precision and accuracy values being within acceptance criteria.

The freeze-thaw stability of the analyte was determined by measuring the assay precision and accuracy for samples which underwent three freeze-thaw cycles. In each freeze-thaw cycle, the frozen plasma samples were thawed at room temperature for 2–3 h and refrozen for 24 h. After completion of each cycle the samples were analyzed and results were compared with those after zero cycles. The results showed that SLD was stable in human plasma through three freeze-thaw cycles; from 1.48 to 8.66% for precision and from 89.39 to 99.95 for accuracy.

The stock solutions were evaluated at 1 month in order to observe changes in drug concentration. Precision and accuracy for QC concentrations of SLD ranged from 2.69 to 4.24% and from 91.80 to 106.67%, respectively. The IS values for precision and accuracy at 500 ng/mL were 2.58 and 97.71, respectively (data not shown), which suggests that the drug was stable when stored at 4 °C for the time over which the validation process was done.

Human plasma from four different subjects was hemolyzed and lipemic samples were processed using placebo components in order to visualize any interference. The chromatogram showed no interference peaks corresponding to the retention times for SLD and IS, (data not shown).

All these results indicate that the proposed method, which is based on different reports, is an alternative way to detect and quantify SLD in human plasma samples as it is suitable, simple, precise, accurate, and rapid, and it can support a complete validation process for the determination of SLD in human plasma.

3. Experimental

The HPLC system consisted of an Alliance Separations Module 2695, Waters (Germany). The UV detector was a Dual λ Absorbance Detector 2487, Waters, (Germany). The type of column used was a Symmetry® C18 3.9 × 150 nm 5 μm, Waters (Germany). Pre-column was a C18 Symmetry®, Waters (Germany). HPLC double distilled water from a Milli-Q system

Table 1: Intra- and inter-day precision and accuracy assay

Nominal concentration (ng/mL)	Observed concentration of SLD {ng/mL}					
	Intra-day			Inter-day*		
	Mean (n = 6)±SD	CV (%)	Accuracy (%)	Mean (n = 6)±SD	CV (%)	Accuracy (%)
60	56.16 ± 2.58	4.6	93.6	55.65 ± 5.56	3.10	92.64
150	153.27 ± 17.71	11.55	102.18	154.10 ± 10.49	6.81	102.73
800	902.46 ± 34.03	3.77	112.71	858.65 ± 47.28	5.51	107.33

* On three differents days

Table 2: Recovery observed of SLD from standard solution and tablets

Nominal concentration (ng/mL)	Concentration of SLD (ng/mL)			
	From standard solution		From tablets	
	Mean (%)*(n = 6)±SD	CV (%)	Mean(%)*(n = 6)±SD	CV (%)
60	96.94 ± 3.62	3.64	94,44 ± 4.97	5.26
150	93.23 ± 9.62	10.33	98.37 ± 1.68	1.71
800	92.66 ± 7.14	7.71	91.35 ± 11.90	13.03

(Millipore, Bedford, MA, USA) was used. Data were collected with Enpower II software (Waters).

Standard SLD and product (tablets containing 50 mg of active compound) were kindly supplied by INTERCAPS, C.A. (Caracas, Venezuela). Diazepam (as internal standard (IS) 99,8%) was kindly provided by Roche, C.A. (Caracas, Venezuela). Acetonitrile for chromatography and dichloromethane were purchased from Burdick & Dickson, CA (USA). Diethyl ether was purchased from Riedel-de-Häen (Germany). Na_2CO_3 was purchased from Merck (Germany). The water used was double distilled.

The following chromatographic conditions were optimized during the method development phase. The mobile phase consisted of ammonium acetate 0.3 M pH 6,8: acetonitrile (50:50, v/v); derived from a mobile phase developed by Daraghmeh et al. (2001). Different compounds were evaluated as Internal Standard; only diazepam (Wang et al. 2005a) was shown to be a good IS due to its retention time and good recovery. A fl w rate of 0.750 mL/min was employed with a total running time of 10 minutes at 25 °C. The volume of injection was 30 µl. Quantization was at 240 nm. Under these conditions, retention times were typically 4.8 min for SLD and 7.1 min for diazepam.

Stock solutions of SLD (100 ug/ml) and diazepam (100 ug/ml) were prepared in mobile phase. IS was prepared at 500 ng/mL in blank plasma. Standard solutions of SLD were prepared with mobile phase in the range of 1×10^{-4}–2×10^{-7} gs/mL. These solutions were diluted 1/100 to obtain a fina concentration of 1×10^{-6}–2×10^{-9} gs/mL. Calibration curves were prepared using blank plasma at concentrations of 20, 50, 75, 100, 250, 500, 750 and 1000 ng/mL. The calibration curves for HPLC analysis were constructed by plotting the ratio of peak normalization of SLD to internal standard against concentration. The calibration curve had to have a correlation coefficien (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except the lower value which was set at 20%. Quality control (QC) samples were prepared in blank plasma at concentrations of 60, 150 and 800 ng/mL.

Frozen plasma samples were thawed at ambient temperature and centrifuged at 3000 × g, 4 °C for 10 min. An aliquot of plasma (460 µl) was placed in a glass tube with 20 µl of SLD and 20 µl of IS and mixed by vortex for 30 s. Then, 200 µl of 0.1 M Na_2CO_3 and 5 mL diethyl ether:dichloromethane (60:40) were added and the mixture was vortex-mixed for 5 min, followed by centrifugation at 3500×g for 15 min at 4 °C. This procedure was reported by Wang et al. (2005b), but in this case we added 5 mL diethyl ether:dichloromethane (60:40) instead of 3 ml, to improve recovery percent and also sensitivity. The tubes were frozen for 20 min and the organic layer was transferred to another clean glass tube and evaporated at 40 °C with nitrogen. The dry residues were reconstituted with 1 mL of mobile phase and vortex mixed for 1 min. A 30 µl aliquot of reconstituted sample was injected into the HPLC system.

The detection limit (LOD) of SLD was estimated as the drug amount in serum which corresponded to fi e times the baseline noise. The limit of quantificatio (LOQ) was determined as the lowest concentration of the calibration curve. Linearity was done by analyzing six sets of samples at concentrations from 20 to 1000 ng/mL. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value. The calibration curve had to have a correlation coefficien (r^2) of 0.99 or better.

Stability under thawing conditions was assessed using QC samples stored for 4 h at room temperature and subjected to three freeze-thaw cycles (−20° to −25 °C). Long-term stability was assayed by storing QC samples at −70 °C for 1 month. Stability post-extraction was evaluated by leaving QC samples in the HPLC system for 24 and 48 h. Stability of samples through processing time was examined by leaving QC samples at room temperature for 6 h followed by the extraction procedure. SLD and diazepam stock solutions were stored at 4 °C and measured every day for 1 month to determine changes in drug concentration. The acceptance criteria of all stability tests were less than 15% or precision and 100 ± 15% of better for accuracy.

The intra-day precision and accuracy were determined by analyzing six sets of QC samples in a batch. The inter-day precision and accuracy was found by analyzing six sets of QC samples on three different days. The inter-analyst precision and accuracy ware done also studied to measure the effect of this condition on the results. The QC samples were randomized daily, processed and analyzed immediately following the standard curve, in the middle of the batch, or at the end of the batch. The acceptance criteria were less than15% for precision and 100 ± 15% for accuracy.

Recovery of SLD and diazepam were evaluated by comparing the mean peak areas of six extracted low, medium and high QC samples (A) to mean peak areas of six extracted blank plasma samples with SLD and diazepam (also low, medium and high QC concentrations) added after the extraction process (B). Recovery percentage was determined by calculating the ratio of A to B and the acceptance criterion was 80% or better.

In order to establish whether tablet components give interference and dispersion in the results, precision, accuracy and recovery were determined in QC samples with SLD from tablets. The acceptance criteria for precision and accuracy were less than 15% and 100 ± 15% or better, respectively. Recovery percentage was determined by calculating the ratio of A to B and the acceptance criterion was 80% or better.

To evaluate possible endogenous interferences, four human serum blanks with placebo were analyzed. Hemolyzed and lipemic serums were also analyzed in order to establish any interference with the method.

References

Boolell M, Gepi-Atte S, Gingell JC, Allen J (1996) Sildenafil a novel effective oral therapy for male erectile dysfunction. Br J Urol 78: 257–261.

Daragmeh N, Al-Omari M, Badman AA, Jaber AMY (2001) Determination of sildenafi citrate and related substances in the commercial products and tablets dosage form using HPLC. J Pharm Biomed Anal 25: 483–492.

Dinesh ND, Nagaraja P, Made Gowda NM, Rangappa KS (2002) Extractive spectophotometric methods fot the assay of sildenafi citrate (Viagra) in pure form and in pharmaceutical formulations. Talanta 57: 757–764.

Liaw J, Chang TW (2001) Determination of transdermal sildenafi in nude mouse skin by reversed-phase high-performance liquid chromatography. J Chromatogr B 765:161–166.

Shim HJ, Lee EJ, Lee EJ, Jung HY, Kim SH, Yoo M, Kwon JW, Kim WB, Lee MG (2002) Determination of a new phosphodiesterase V inhibitor, DA-8159, in plasma and urine by high-performance liquid chromatography. J Pharm Biomed Anal 30: 527–533.

Flores R, Berzas Nevado J, Peñalvo J, Mora Diez N (2004) Development of a micellar electrokinetic capillary chromatography method for the determination of 3 drugs employed in the erectile dysfunction therapy. J Chromatogr B 811: 231–236.

Berzas Nevado J, Rodríguez Flores J, Castañeda Peñalvo G, Mora Diez N (2002) Determination of sildenafi citrate and its main metabolite by sample stacking with polarity switching using micellar electrokinetic chromatography. J Chromatogr A 953: 279–286.

Qin W, Li SFY (2002) An ionic liquid coating for determination of sildenafi and UK-103, 320 in human serum by capillary zone electrophoresis-ion trap mass spectrometry. Electrophoresis 22: 2004–2009.

Pelander A, Ojanpera I, Sistonen J, Rasanen I, Vuori E (2003) Screening for basic drugs in 2-ml urine samples by dual-plate overpressured layer chromatography and comparison with gas chromatography-mass spectrometry. J Anal Toxicol 27: 226–232.

Lee M, Min D I (2001) Determination of sildenafi citrate in plasma by high-performance liquid chromatography and a case for the potential interaction of grapefruit juice with sildenafi citrate. Ther Drug Monit 23: 21–26.

Ergun B, Saracoglu A, Sinem I, Atkosar Z, Kircali K, Altiokka G (2005) Validation of a reversed-phase HPLC method for the

sildenafi citrate in pharmaceutical preparations and in spiked human plasma. J Liq Chromatogr Relat Technol 28: 1539–1548.

Sheu Wu, Yeh G, Hsia A, Ho H (2003) Development of a liquid chromatographic method for bioanalytical application of sildenafil J Chromatogr B 791: 255–262.

Wang J, Wang Y, Fawcett P, Wang Y (2005a) Determination of omeprazole in human plasma by liquid chromatography-electrospray quadrupole linear ion trap mass spectrometry. J Pharm Biomed Anal 39: 631–635.

Wang Y, Wang J, Cui Y, Fawcett J, Gu J (2005b) Liquid chromatographic-tandem mass spectrometric method for the quantitation of sildenafi in human plasma. J Chromatogr B 828: 118–121.