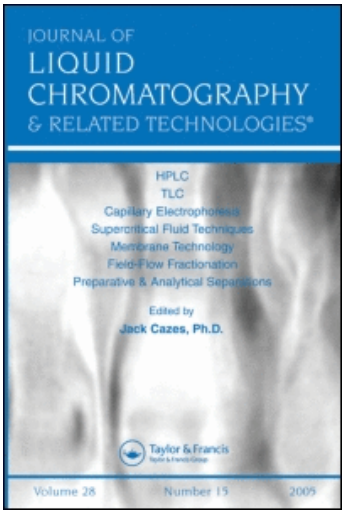

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Development of a Liquid Chromatographic Method for the Determination of Sildenafil in Seminal Plasma

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ABSTRACT

A sensitive high performance liquid chromatographic (HPLC) method with ultraviolet absorption detection (230 nm) was developed and validated for the determination of a phosphodiesterase V inhibitor, Sildenafil, in seminal plasma. A single step liquid–liquid extraction procedure using ethyl acetate was performed to recover sildenafil from 1.0 mL of seminal plasma combined with 200 μL of NaOH 0.1 M. A symmetry C_{18} column

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(150 × 4.6 mm I.D. 5 μm) was used as a stationary phase and the mobile phase consisted of 32% acetonitrile and 68% phosphoric acid (0.016 M; pH 5.3) at a flow rate of 1.0 mL/min. The quantitation limit was 5 ng/mL. Intra- and inter-day relative standard deviation (RSD) did not exceed 6.6%. This HPLC method has been successfully used in medical laboratories to assay seminal plasma samples for studies on the treatment with sildenafil.

Key Words: Sildenafil; HPLC analysis; Seminal plasma.

INTRODUCTION

Sildenafil citrate or 1-[[3-(4,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo-[4,3-*d*]pyrimidin-5-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine (Fig. 1), is an orally active inhibitor of cyclic guanosine monophosphate (cGMP)-specific phosphodiesterase type V (PDE 5), and has been shown to be an effective treatment for male erectile dysfunction.[1–3]

Several methods for the determination of sildenafil citrate concentrations have been developed in pharmacokinetic studies after single intravenous and oral doses to mouse, rat, dog, and man,[4,5] involving high performance liquid chromatography (HPLC) in combination with liquid–liquid extraction.[6,7] An automated sequential trace enrichment of a dialysate (ASTED) system, incorporating a dialysis system for on-line dialysis, as well as a switching valve for trace enrichment, has also been used.[8] The determination of sildenafil in biological fluids has been carried out by liquid chromatography coupled with electrospray ionization tandem mass spectrometry.[9–14] An automated HPLC method using narrow-bore column switching has also been employed for the simultaneous determination of sildenafil and its active metabolite from human plasma samples.[15] The determination of sildenafil has also recently been achieved in pharmaceutical formulation by adsorptive stripping voltammetry[16] FIA[17] and RP-HPLC.[18–20] A simple and reliable HPLC assay was developed by Lee and Min[21] that illustrates the potential interaction of grapefruit juice with sildenafil. Berzas Nevado[22] have developed a new micellar electrokinetic capillary electrophoresis method for the simultaneous determination of sildenafil and its metabolite in human plasma. A reversed-phase HPLC system was recently developed for the determination of sildenafil concentrations in transdermal permeation study.[23] Recently, a gas-chromatographic (GC) method to measure sildenafil in human serum and urine and its metabolite in serum after an extraction and preconcentration process in a C_{18} cartridge, and without previous derivatization has been developed.[24]

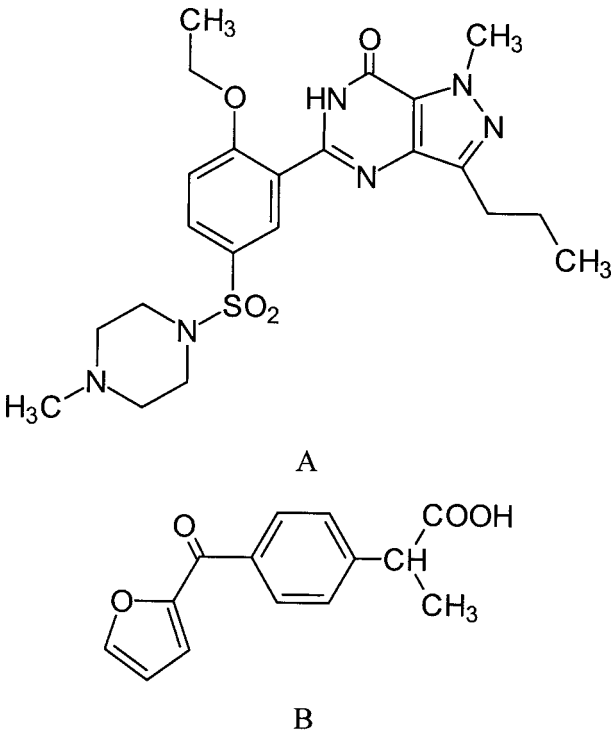

Figure 1. Chemical structures of sildenafil (A) and internal standard furprofen (B).

Plasma concentrations of sildenafil and *N*-demethylsildenafil can be determined up to 24 hr post-administration by use of liquid chromatography in tandem with mass spectrometry.[14] A procedure has also been described for the estimation by HPLC of sildenafil in human erythrocytes in vitro.[15] So far, no method for the determination of sildenafil in seminal plasma has been reported. In this work, we have developed and validated a sensitive and precise analytical method for sildenafil concentrations in seminal plasma samples.

EXPERIMENTAL

Chemicals and Reagents

Sildenafil citrate and furprofen [internal standard; Fig. 1(B)] were kindly supplied by the Department of Pharmacology, University of L'Aquila

(L'Aquila, Italy). The purity of the supplied sildenafil was confirmed to be 99.5% by HPLC analysis. Acetonitrile (HPLC grade) was obtained from Carlo Erba Reagenti (Milan, Italy). Ethyl acetate, trichloroacetic acid, orthophosphoric acid, and sodium hydroxide, all analytical-grade reagents, were purchased from Fluka Chemika-BioChemika (Buchs, Switzerland). Water (HPLC grade) was obtained by passage through the ELIX 3 and Milli-Q Academic water purification systems (Millipore, Bedford, MA).

Apparatus and Chromatographic Conditions

HPLC analysis was carried out using a chromatographic system composed of the following: a Model 515 pump and a Model 996 diode array detector (Waters, Milford, MA). A Model 7725i sample injector (Rheodyne, Cotati, CA) equipped with a 100 μL loop was used. Chromatographic data management was automated using software Millennium[32] (Waters, Milford, MA).

The analysis was performed on an analytical 150 × 4.6 mm i.d. reversed-phase symmetry C_{18} (5 μm particle size) column (Waters, Milford, MA), protected by a 20 × 4.6 mm i.d. (40 μm particle size) disposable Pelliguard precolumn (Supelco, Bellefonte, PA). The assay was performed at ambient temperature. The mobile phase consisted of a mixture of acetonitrile and phosphoric acid (0.016 M; pH 5.3 with NaOH) (32 : 68, v/v). Phosphoric acid, prior to use, was filtered through a WCN 0.45 μm filter, while acetonitrile through WTP 0.5 μm filter (Whatmann, Ltd., Maidstone UK). The mobile phase was prepared daily, degassed using an in-line degasser (Waters, Milford, MA), and delivered at a flow rate of 1.0 mL/min. Column eluate was monitored at 230 nm.

Standard Solutions and Samples

Stock solutions of sildenafil and internal standard were prepared by dissolving 10 mg of each compound in 100 mL of acetonitrile. These solutions could be stored at −20°C for over 1 month with no evidence of decomposition. Standard solutions, each containing the two drugs, were obtained by diluting the stock solutions with drug-free seminal plasma in the range 10–1500 ng/mL. For each solution, the internal standard was added at a constant level of 100 μL of a 2.5 μg/mL acetonitrile solution. These standards were treated concurrently in the same manner as the samples to be analyzed. Calibration graphs were obtained by plotting the peak-area ratios of sildenafil to internal standard against the drug concentrations obtained after extraction.

Extraction and Isolation Procedures

For verifying the extraction procedure, specimens of sperm were obtained from volunteer patients. All the samples were rapidly centrifuged at 3000*g* for 10 min and the supernatant frozen at −20°C until extraction. Sildenafil was extracted from seminal plasma by liquid–liquid extraction. Briefly, 100 μL of internal standard, 400 μL of trichloroacetic acid, and 150 μL of NaOH 0.1 M were added to 1 mL of seminal plasma in a 10 mL glass test tube. The samples were mixed and 1 mL of ethyl acetate was added. The mixture was wortex-mixed for 60 sec and then centrifuged at 3000*g* for 10 min. The organic phase was then transferred into a clean tube. Fresh ethyl acetate was added to the first tube and the same extraction procedure was repeated. The organic phases collected from the two extractions were pooled, and the solvent evaporated to dryness with a nitrogen stream under vacuum, by using the Supelco drying attachment. The sample was subsequently reconstituted with 200 μL of mobile phase. Aliquots (50 μL) were used for chemical analysis.

Sample Analysis

Seminal samples were stored at −20°C after taking, were thawed just before the extraction procedure, and centrifuged at 3000*g* for 10 min. Of the sample, 1.0 mL were then rapidly extracted and analyzed as previously described.

RESULTS AND DISCUSSION

Extraction Efficiency

Recoveries of sildenafil and internal standard from spiked samples were calculated by comparing the peak area of seminal plasma with standards at low, medium, and high concentration levels, and submitted to the sample preparation procedure; with those obtained from the analysis of corresponding directly-injected standards ($n = 5$). The extraction recoveries of sildenafil and internal standard in seminal plasma were 87 ± 2.5% and 93 ± 1.6%.

Selectivity of Assay

The selectivity of the assay was determined by analysis of blank seminal plasma from seven different subjects, with and without internal standard.

Under these chromatographic conditions, no endogenous sources of interference were observed in seminal plasma, and the resolution between sildenafil and internal standard was satisfactory (Fig. 2).

Sensitivity of Assay

Under the experimental conditions used, the lower limit of detection was 5 ng/mL at a signal-to-noise ratio of 3. The limit of quantification was

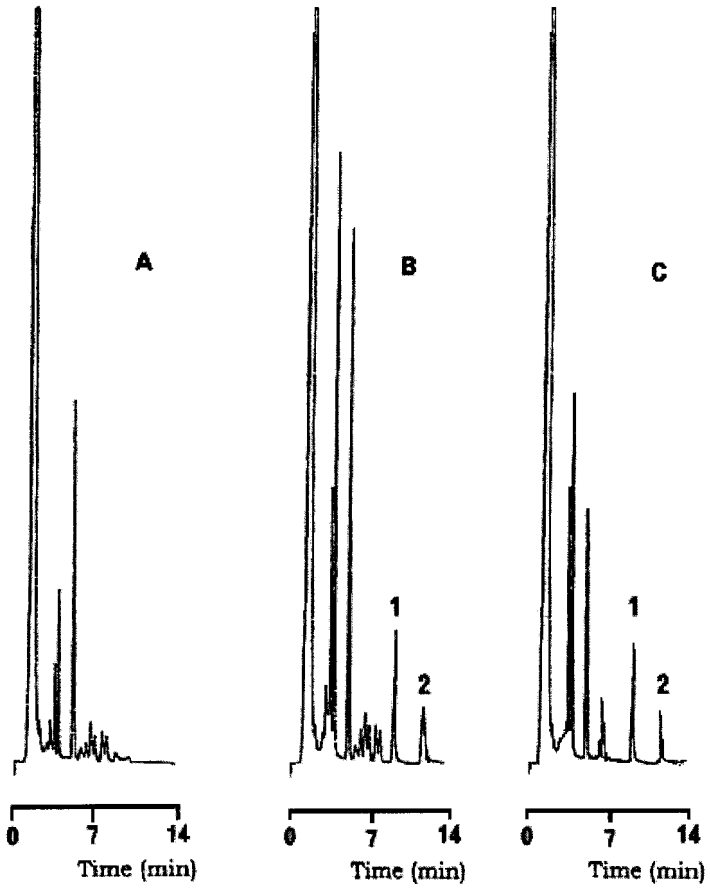

Figure 2. Typical chromatograms of seminal plasma samples after extraction. (A) Blank seminal plasma; (B) Blank seminal plasma spiked with internal standard (1) and sildenafil (2); (C) Sample obtained from a volunteer treated with sildenafil.

defined as the lowest amount detectable with a precision of less than 15% ($n = 3$) and an accuracy of $\pm 15\%$ ($n = 3$). The limit of quantification was to be 10 ng/mL.

Linearity of Assay

Calibration curves for HPLC analysis were determined by linear regression. The assays exhibited linearity between the response (y) (peak-area ratio of sildenafil over the internal standard) and the corresponding concentration of sildenafil (x), over the 10–1500 ng/mL range in seminal plasma (typical equation: $y = 0.015x + 0.003$). The results of linear regression analysis show that the correlation coefficients of all standard curves are ≥ 0.9993. In addition, calculated standard points compared to nominal ones evaluated the quality of fit. The assays exhibited linearity ($r > 0.9995$), with a slope near to unity (0.9992) and an intercept not statistically different from zero.

Accuracy of Assay

The accuracy of the assay method was defined as the percentage of the systematic error, which is calculated as the standardized agreement between the measured value and the true value. To be acceptable, the measures should be within $\pm 15\%$ at all concentrations. The accuracy values in intra-day variation studies at low, medium, and high concentrations of sildenafil in seminal plasma were in acceptable limits (Table 1).

Precision of Methods

The precision of a method is expressed as the relative standard deviation (RSD) of replicate measurements. To be acceptable, the measures should be

Table 1. Accuracy of HPLC method for determining sildenafil in seminal plasma samples.

Concentration added (ng/mL)	Concentration found (mean ± SD) (ng/mL)	Accuracy (%)
20	19.6 ± 0.3	−2.0
50	48.7 ± 0.6	−2.6
100	99.4 ± 0.3	−0.6

Note: $n = 5$; SD, standard deviation.

within $\pm 15\%$ at all concentrations. In this work, precision of the method was tested by both intra-day and inter-day reproducibilities in seminal plasma.

Intra-Day Variability of the Assay

The intra-day variability of the assay was determined by repeated analysis of quality control samples at low, medium, and high concentrations on the same day. Results are shown in Table 2. These data indicate that the assay method is reproducible within the same day.

Inter-Day Variability of the Assay

The inter-day variability of the assay was determined by repeated analysis of quality control samples at low, medium, and high concentrations on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at $-20^{\circ}C$ until analysis. Results are shown in Table 3. These data indicate that the assay method is reproducible within different days.

Figure 2 illustrates representative chromatograms of a drug-free seminal plasma, of a blank seminal plasma spiked with sildenafil and internal standard, and of a seminal plasma of a volunteer treated with the drug. The elution peaks are lacking in interferences deriving from other seminal plasma components, and are characterized by retention times of 10.8 (internal standard) and 13.4 (sildenafil) min, respectively.

The optimization of the analytical procedure has been carried out by varying the following: reversed-phase column used, mobile phase composition, flow rate, pH, and wavelength. The degree of reproducibility of the results obtained through small deliberate variations in method parameters and by changing instruments and operators has been very satisfactory. Table 4 shows that the percent of recoveries of sildenafil were good under

Table 2. Intra-day variability of HPLC method for determining sildenafil in seminal plasma samples.

Concentration added (ng/mL)	Concentration found (mean $\pm$ SD) (ng/mL)	RSD (%)
20	19.4 ± 0.3	1.5
50	49.2 ± 0.4	0.8
100	98.6 ± 0.4	0.4

Note: $n = 5$; RSD, relative standard deviation.

Table 3. Inter-day variability of HPLC method for determining sildenafil in seminal plasma samples.

Concentration added (ng/mL)	Concentration found (mean ± SD) (ng/mL)	RSD (%)
20	18.9 ± 1.3	6.6
50	48.8 ± 0.6	1.3
100	98.0 ± 0.7	0.7

Note: $n = 5$.

most conditions, and did not show a significant change when the critical parameters were modified. The tailing factor for sildenafil and the internal standard was always less than 1.4 and the components were well separated under all the changes carried out.

Considering the modifications in the system suitability parameters and the specificity of the method, as well as carrying the experimental at room temperature, would conclude that the method conditions are robust. The liquid–liquid extraction procedure eliminates endogenous interference, which is frequently present in a biological sample. The filtration of extracts before injection onto chromatographic column avoids rapid obstruction

Table 4. Effect of Experimental parameters on the percent recoveries of sildenafil.

Parameter	Modification	Recovery (%)
Mobile phase ratio (v/v)	H_3PO_4 : ACN	
	18 : 82	99.5
	20 : 80	100.0
	22 : 78	99.3
pH	5.1	98.8
	5.3	100.0
	5.5	100.0
Flow rate (mL/min)	0.8	99.4
	1.0	100.0
	1.2	100.0
Column type	Used column	99.8
	New column	100.0

Note: ACN, acetonitrile.

of the precolumn, increasing its life. The HPLC assay method presented here is rapid, sensitive, specific, and robust, and should be of value for the quantification of sildenafil in seminal plasma of volunteers treated with this drug.

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