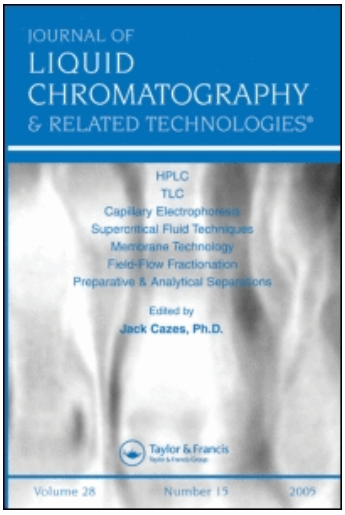

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Abstract: A simple HPLC analysis for the identification and quantification of vardenafil in a pharmaceutical tablet formulation was performed on a conventional C_{18} and Chromolith Performance RP-18e monolithic columns with acetonitrile–phosphate buffer mixtures as mobile phases. The effects of the proportion of organic solvent (from 20% to 90%), phosphate buffer pH (from 2 to 7.5) and flow rate (from 1 to 5 mL/min) were studied. The best chromatographic conditions were 20:80 (v/v) acetonitrile–10 mM phosphate buffer, pH 3.0, as mobile phase at 1 mL/min flow rate for the C_{18} column, whereas 30:70 (v/v) acetonitrile–10 mM phosphate buffer, pH 3.0, as mobile phase at 2 mL/min flow rate was best for the monolithic column. Methanol was found to be a suitable solvent for extraction of the active substance from tablets. For the C_{18} and monolithic column, the calibration plots were linear ($R^2 = 0.9996$ and 0.9997, respectively) in the concentration range 10–1000 μg/mL. Limit of detection (LOD) and limit of quantification (LOQ) values were 0.10 and 0.31 μg/mL for the C_{18} and 0.11 mL and 0.32 μg/mL for the monolithic column. Intra-assay and inter-assay precision studies reflected a high level of reliability and reproducibility of the method. The proposed method is selective, precise (RSD = 0.45%), and accurate (recovery = 103–107%) in both columns used.

Keywords: Liquid chromatography, monolithic column, pharmaceutical preparation, Vardenafil

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INTRODUCTION

Impotence, sexual, or erectile dysfunction (ED), defined as the inability to achieve or maintain a penile erection sufficient for vaginal penetration, is considered as one of the most serious problems affecting men worldwide. Besides its psychogenic origin, it may be caused by organic syndromes including adverse drug reactions such as diuretics and antihypertensives, antidepressants, antipsychotics, anticonvulsants, and sedative/hypnotics; chronic disease such as diabetes, heart, liver, and kidney disease; endocrine disorders such as altered testosterone or prolactin levels; vascular disease such as atherosclerosis; primary neurologic disorders such as spinal cord injury, multiple sclerosis, Alzheimer's, and parkinsonism. Surgical procedures that might damage parasympathetic nerve fibers including prostatectomy, cystectomy, and kidney transplantation are not excluded.[1–5]

Penile erection is a hemodynamic process based on the relaxation of smooth muscle in the corpus cavernosum and its associated arterioles. Thus, in sexual stimulation, the nitric oxide (NO) released from the nerve ends in the corpus cavernosum activates the enzyme guanylate cyclase leading to the increase of cyclic guanosine monophosphate (cGMP) level in the corpus cavernosum. This, in turn, triggers smooth muscle relaxation leading to an increased inflow of blood into the penis causing an erection. The actual cGMP level is regulated by the rate of synthesis via the guanylate cyclase and by the rate of degradation via cGMP hydrolyzing phosphodiesterases (PDEs). The most prominent PDE in the human corpus cavernosum is the cGMP specific phosphodiesterase type 5 (PDE5).

The treatment of ED is based on the inhibition of PDE5, the enzyme responsible for cGMP degradation in the corpus cavernosum, and the enhancement of endogenous NO effect, locally released in corpus cavernosum upon sexual stimulation.

Among the drugs used in the treatment of ED, sildenafil (Viagra) launched in 1998, showed promising results and opened the door for the development of other drugs. Recently, tadalafil (Cialis)[4] and vardenafil (Levitra©)[5] were introduced into the market.

The ways in which efficiency and practicality of the development of new drugs are defined is dependent on a large number of factors. Among these factors are suitable analytical methods for the determination of the drugs in samples. Thus, the modern and most sensitive methods used in the determination of biologically active substances in pharmaceutical preparations are gas chromatographic (GC) and high performance liquid (HPLC) chromatographic methods. To the best of our knowledge, the validation of a chromatographic analysis of vardenafil in pharmaceutical formulation has not yet been reported. Accordingly, this report describes the direct HPLC

determination of vardenafil, chemically known as {2-[2-ethoxy-5-(4-ethyl-piperazine-1-sulfonyl)-phenyl]-5-methyl-7-propyl-3*H*-imidazo[5,1-*f*][1,2,4]-triazin-4-one hydrochloride trihydrate} (Figure 1) in a pharmaceutical preparation using a conventional C_{18} and monolithic column.

EXPERIMENTAL

Chemicals and Reagents

Vardenafil hydrochloride trihydrate (Art. Nr. 02847837) and the vardenafil tablets (Levitra®) (containing 10 mg of vardenafil hydrochloride trihydrate per tablet) were obtained from Bayer AG (Leverkusen, Germany). Both standard solutions (50 mg/mL) of standard vardenafil and the vardenafil tablets (Levitra®) were prepared in methanol. Deionized water was prepared using a Millipore Milli-Q (Bedford, MA) water purification system. HPLC-grade methanol, acetonitrile, and *o*-phosphoric acid were purchased from Fluka (Buchs, Switzerland). Sodium dihydrogen orthophosphate ($NaH_2PO_4 \cdot 2H_2O$) of AR grade was purchased from BDH Ltd. (Poole, England). The phosphate buffers were prepared according to standard procedure and adjusted using *o*-phosphoric acid. Acrodisc 4CR syringe filter, PTFE, 4 mm diameter and 0.45 μm pore size were purchased from Aldrich (Milwaukee, WI).

Calibration samples were prepared using the following concentration levels: 10, 50, 100, 500, and 1000 μg/mL.

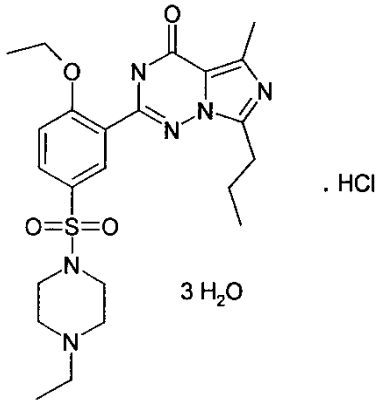

Figure 1. Chemical structure of vardenafil hydrochloride trihydrate.

Instrumentation

The mobile phase was filtered through a Millipore membrane filter (0.2 μm) from Nihon Millipore (Yonezawa, Japan) and degassed before use. The buffer was adjusted using a pH-meter from Orion Research (model 611) from Thermo Orion Research Inc., (Westmont, IL). The HPLC system consisted of a Waters binary pump, Model 1525, (Milford, MA), equipped with a dual λ absorbance UV detector Model 2487 and an autosampler Model 717plus operating at room temperature. The UV detector was set at 230 nm. The C_{18} column was a Supelcosil LC-18 (250 × 4 mm, 5 μm) purchased from Supelco (Bellefonte PA) and the monolithic column was Chromolith Performance, RP-18e (100 × 4.6 mm i.d.) obtained from Merck (Darmstadt, Germany). Collection of data was performed using Breeze Software from Waters.

Extraction

Two tablets of Levitra containing 10 mg each of vardenafil hydrochloride trihydrate were ground in a mortar and extracted three times with methanol. The extract is centrifuged and the supernatant is filtered through a syringe filter 0.45 mm pore size, and 20 μL was used for the chromatographic analysis.

Validation

The feasibility of method transfer from a conventional C_{18} column to a monolithic column was investigated. Thus, the analysis of a set of standard samples (pure vardenafil hydrochloride) in different concentrations 10, 50, 100, 500, and 1000 μg/mL, together with the active substance extracted from the commercially available tablets (vardenafil in Levitra as hydrochloride trihydrate), was performed. The validation of the method was ascertained by carrying out seven sets ($n = 7$) of the chromatographic and extraction procedures under identical conditions. The regression analysis was carried out using a Microsoft Excel program.

RESULTS AND DISCUSSION

The conventional method of analysis consisted of a straightforward chromatographic separation using a 250 × 4 mm i.d. octadecylsilica (ODS) C_{18} column. Because of the hydrophobic nature of the analyte (Figure 1), the mobile phase consisted of 20% acetonitrile and 80% sodium phosphate buffer, pH 3, flow rate of 1 mL/min, at room temperature. Under these

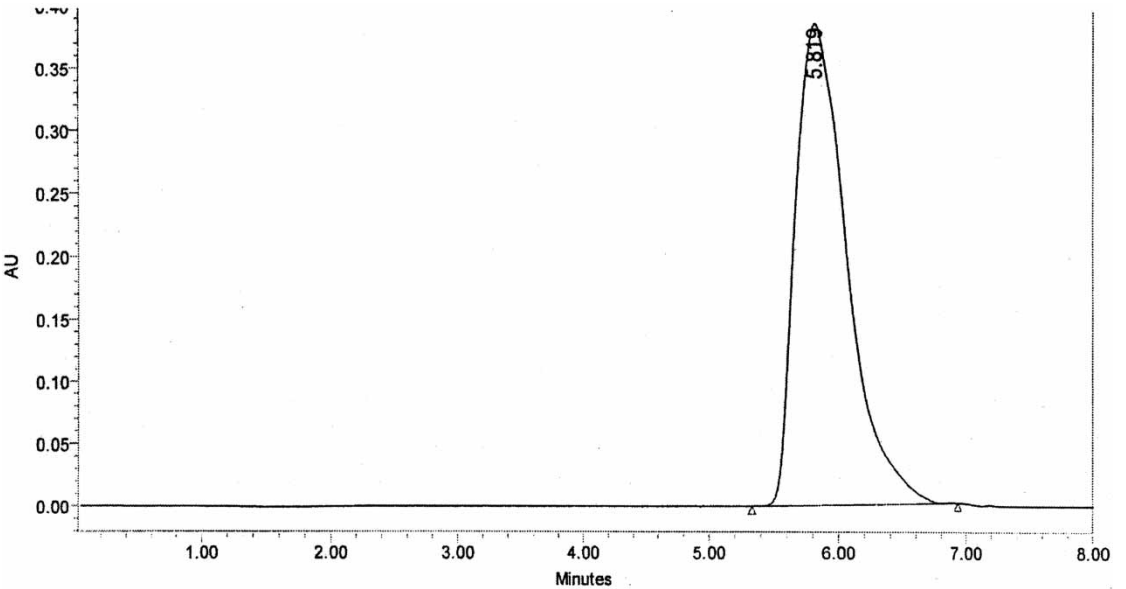

Figure 2. A typical chromatogram of the HPLC analysis of vardenafil hydrochloride trihydrate (standard) dissolved in acetonitrile/buffer pH 3 (20:80 v/v) on a conventional C_{18} column and flow rate of 1 mL/min.

conditions, vardenafil (standard) eluted at 5.8 min (Figure 2). Attempts to vary the proportion of the organic solvent versus the buffer resulted in a large tailing with poor shaped peaks. Increasing the flow rate, results in a high back-pressure and poor shaped peaks appearing at an earlier time (e.g., 2.9 min at flow rate 2 mL/min).

The method is easily transferred to a monolithic LC phase, with 100 × 4.6 mm i.d.; a comparable separation was obtained using the same conditions. However, peak tailing was observed when using the same conditions applied for the C_{18} column. These results prompted us to optimize the chromatographic conditions for the monolithic column. Thus, the effects of the proportion of organic solvent acetonitrile (from 20% to 90%), the concentration of the phosphate buffer (10 or 100 mM) and its pH (from 2 to 7.5), and the flow rate (from 1 to 5 mL/min) were studied. The results revealed that increasing the percentage of the acetonitrile versus phosphate buffer from 20:80 (v/v) (Figure 3), results in a faster elution time for the vardenafil (0.82 sec at 80:20 (v/v) acetonitrile–phosphate buffer pH 3) (chromatogram is not shown), however, the high throughput analysis was at the expense of the peak shape. Thus, poor shaped peaks were observed when the proportion of acetonitrile exceeds that of the buffer. The best well-shaped peak was observed when using 30:70 (v/v) acetonitrile/buffer pH 3 (retention time is 1.7 min) (Figure 3 and 4 for comparison).

At a constant proportion of mobile phase [30:70 (v/v)] acetonitrile: 10 mM buffer at flow rate of 2 mL/min, the effect of pH was studied in the range from pH 2 to pH 7.5. The results revealed that increasing the pH from 2 to 7.5 delays the elution of vardenafil (e.g., at pH 2, RT = 1.46 min and at pH 6.5, RT = 1.8 min) (Table 1). Varying the concentration of the buffer used (pH 3) from 10 to 100 mM results in the appearance of a large negative peak interfering with the peak of the standard. Thus, higher buffer concentration is not recommended.

An almost 3.5-fold reduction in run time could be achieved by simply increasing the flow rate from 1 to 5 mL/min when using a mobile phase consisting of 20:80 (v/v) acetonitrile:10 mM phosphate buffer pH 3, while an almost 5-fold reduction in run time could be achieved using similar flow rate and a mobile phase consisting of 30:70 (v/v) acetonitrile:10 mM phosphate buffer pH 3 (Tables 2 and 3).

The pressure did not exceed, in both cases, 117.2 bar. Increasing the flow rate up to 2 mL/min had no effect on the peak shape (no tailing); however, a further increase in the flow rate results in a peak tailing and a poor shaped peak. Thus, 1 and 2 mL/min were the best flow rates for the analysis of vardenafil hydrochloride trihydrate on the monolithic column. However, for a shorter retention time, 2 mL/min was applied in the method of choice. Indeed, the high throughput analysis is also obviously caused by the reduced length of the monolithic column, when compared with the conventional C_{18} column. Accordingly, the best chromatographic conditions

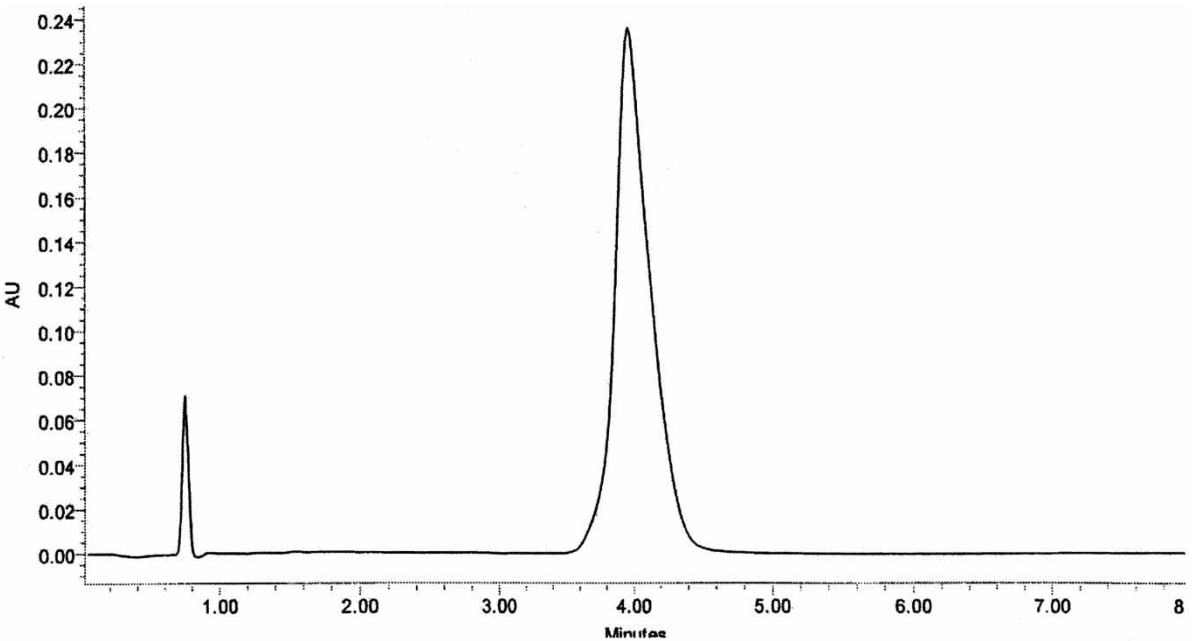

Figure 3. A typical chromatogram of the HPLC analysis of vardenafil hydrochloride trihydrate (standard) dissolved in acetonitrile/buffer pH 3 (20:80 v/v) on the monolithic column and flow rate of 2 mL/min (RT = 3.9 min).

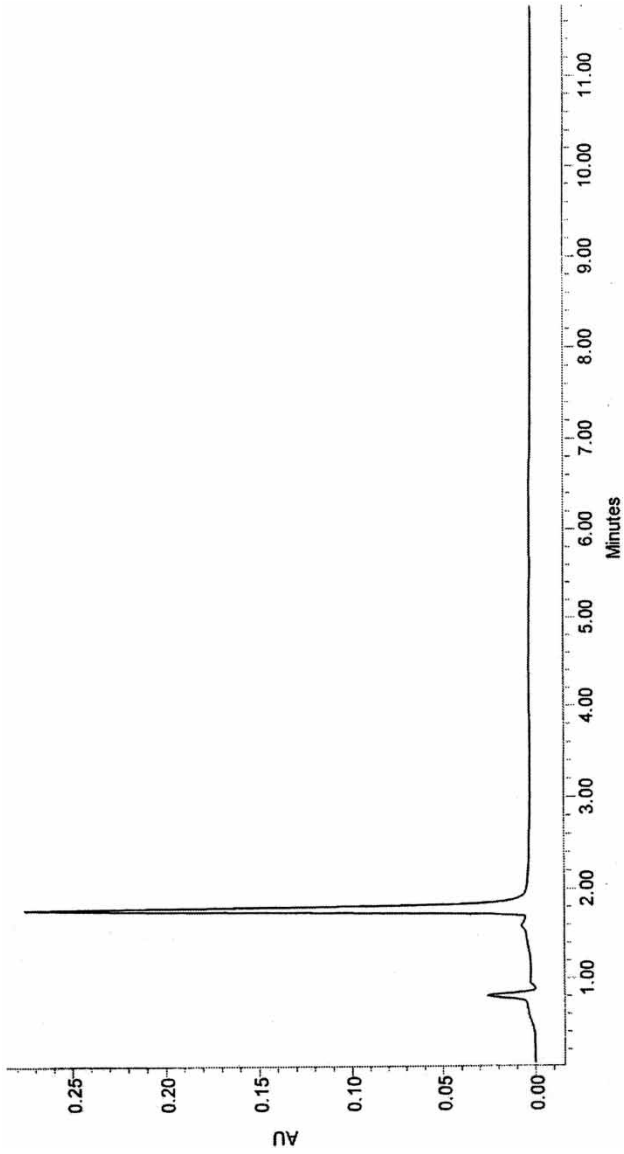

Figure 4. A typical chromatogram of the HPLC analysis of vardenafil hydrochloride trihydrate (standard) dissolved in acetonitrile/buffer pH 3 (30:70 v/v) on the monolithic column and flow rate of 2 mL/min (RT = 1.7 min).

Table 1. Effect of pH on the retention time analysis of vardenafil hydrochloride trihydrate (standard) dissolved in acetonitrile/buffer pH 3 (30:70 v/v) on the monolithic column

pH	Run time (min)
2.0	1.4
3.0	1.7
4.5	1.8
6.5	1.9
7.5	2.0

for the monolithic column consisted of 30:70 (v/v) acetonitrile–10 mM phosphate buffer, pH 3.0, as mobile phase, at 2 mL/min flow rate (Figure 4).

The quantitative analysis of vardenafil hydrochloride trihydrate in the pharmaceutical tablet formulations of Levitra® was determined by comparing the peak area of vardenafil in Levitra® tablets and that of the standard (vardenafil hydrochloride trihydrate). Results revealed the recovery of 103–107% of the active substance in the tablets.

Linearity

The linearity was tested for concentrations from 10 to 1000 μg/mL. Each concentration was analyzed five times. The calibration curve was constructed by plotting the analyte peak area versus the concentration. A good linear relationship between peak area and the concentration of vardenafil was found. The correlation coefficients (R^2) were 0.9996 for the analysis on the C_{18} column and 0.9998 for the monolithic column. [Figure 5(a) for that of the C_{18}

Table 2. Effect of flow rate on the retention time analysis of vardenafil hydrochloride trihydrate (standard) dissolved in acetonitrile/buffer pH 3 (20:80 v/v) on the monolithic column

Mobile phase flow rate (mL/min)	Back-pressure (Bar)	Run time (min)
1.0	27.5	5.6
2.0	48.2	3.9
3.0	75.8	2.6
4.0	96.5	1.9
5.0	117.2	1.6

Table 3. Effect of flow rate on the retention time analysis of vardenafil hydrochloride trihydrate (standard) dissolved in acetonitrile/buffer pH 3 (30:70 v/v) on the monolithic column

Mobile phase flow rate (mL/min)	Back-pressure (Bar)	Run time (min)
1.0	27.5	3.0
2.0	55.1	1.7
3.0	75.8	0.9
4.0	103.4	0.7
5.0	124.1	0.6

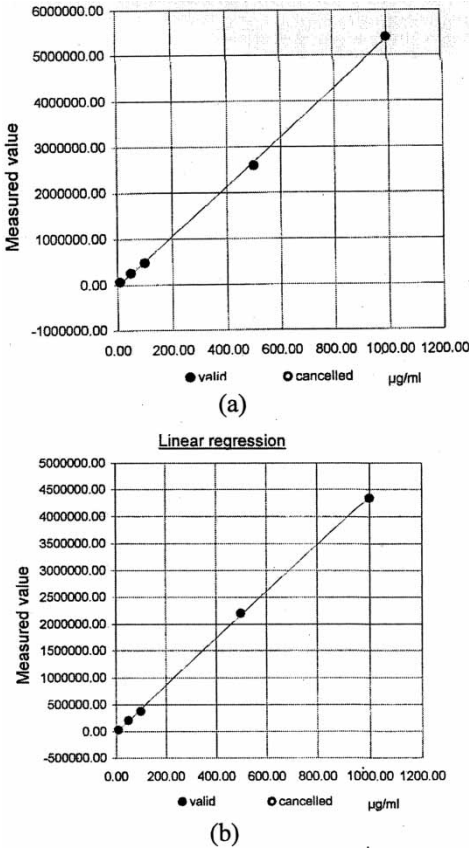

Figure 5. A typical calibration curve of vardenafil hydrochloride trihydrate (standard) dissolved in acetonitrile/buffer pH 3 (30:70 v/v) on a C_{18} column $R^2 = 0.9996$ (a) and on the monolithic column $R^2 = 0.9997$ (b).

column and 5(b) for the monolithic column], while the confidence limit was 99%.

Limit of Detection and Limit of Quantification

The limit of detection (LOD), defined as the minimum amount of analyte that can be detected by the proposed method at three times the signal-to-noise ratio, was found to be 0.10 and 0.11 $\mu g/mL$ for the C_{18} and monolithic column, respectively. The limit of quantification (LOQ) values were 0.31 and 0.32 $\mu g/mL$ for the C_{18} and monolithic column, respectively.

Precision and Accuracy

Intra-assay and inter-assay precision studies reflected a high level of reliability and reproducibility of the method. The proposed method is selective, precise (RSD = 0.45%), and accurate (recovery = 103%–107%) in both columns used.

CONCLUSION

We have described a simple HPLC analytical method for the identification and quantification of vardenafil in its pharmaceutical tablet formulation on a conventional C_{18} and Chromolith Performance RP-18e column, using acetonitrile–phosphate buffer mixtures as mobile phases. The results showed the efficiency of the monolithic column in the analysis of pharmaceutical drugs.

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