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Determination of transdermal sildenafil in nude mouse skin by reversed-phase high-performance liquid chromatography

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Abstract

A simple and sensitive high-performance liquid chromatographic method was developed for the determination of sildenafil transdermal permeation of nude mouse skin. A reversed-phase column with UV detection at 224 nm was used for chromatographic separation. The mobile phase consisted of 32% acetonitrile with 0.2% phosphoric acid in water at pH 5.3 adjusted with 10 *M* NaOH with the flow-rate set at 1.0 ml/min. The limit of quantitation achieved was 5 ng/ml, and the calibration curve showed good linearity over the concentration range of 5–500 ng/ml. The relative standard deviations of within- and between-day analyses were all within 15%. Sildenafil was found to be stable between pH 3 and 12 during 24-h incubation with skin. After transdermal administration of 15.8 μg/ml of sildenafil to nude mouse skin, it was detected as early as 15 min. The transport amount of sildenafil could be quantitated and, at pH 8–11, had the highest permeation rate in nude mouse skin.

Keywords: Transdermal; Permeability; Sildenafil

1. Introduction

Sildenafil is used for the treatment of male penile erectile dysfunction through selective inhibition of phosphodiesterase type 5 to decrease the metabolism of cGMP, which induces smooth muscle relaxation in the corpus cavernosum with onset around 30–45 min [1]. However, blood pressure is transiently reduced by oral administration of 100 mg of sildenafil followed by the possible adverse effect of color (blue/green) discrimination, headaches, flushing, and nasal congestion [2]. In the meantime, repeated doses are required to sustain plasma levels because of its short duration of action ($t_{1/2}$=1 h) with high liver metabolism [3]. Thus, topical delivery through a local tissue area could be considered for alternative administration, instead of orally, to avoid these adverse effects, to shorter onset time, and to sustain effect for longer periods. Furthermore, transdermal delivery of sildenafil can offer several advantages over conventional dosage forms and the pulsed nature of delivery from discrete dosages can be eliminated. However, transdermal permeation of compounds in the local skin, in general, is slow due to low permeability for reasons including the physicochemical properties of the compound, low

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partition ability, and the tissue barrier from the stratum corneum creating a low diffusion coefficient [4]. In addition, the structure of sildenafil has basic functional groups whose pK_a value is 6.5 [5] and under different pH environments, sildenafil will have different levels of ionization following the influence of the partition coefficient as well as its permeation ability in the skin. Therefore, modification of the permeation parameters of sildenafil is a major task to develop an optimal drug for topical delivery formulation such as improvement of onset time, and maintenance the tissues or plasma level to decrease adverse effect, etc.

Although, high-performance liquid chromatography (HPLC) with an automated dialysate (ASTED) system [6], or a triple column switching [3], has been used to measure sildenafil concentrations in human plasma, the extraction and dialysis procedures are complex, time-consuming, or both. In addition, the sample pre-treatment is based exclusively on column switching [3,6] or solid-phase extraction [7]. Unfortunately a two-stage cleaning process with different cartridges seems to be necessary to obtain clean chromatograms. The limit of quantitation (LOQ) of published procedures varies from 1 to 10 ng/ml, which reflects the complexity and length of the sample pre-treatment. Radiolabeled sildenafil has also been used to monitor the pharmacokinetics of plasma and urine in mouse, rat, rabbit, dog, and man [7]. However, a radiolabeled compound renders these methods more complex and is not very suitable for a clinical laboratory set-up.

Thus, in order to understand the properties of transdermal permeability of sildenafil in fresh mouse skin, a specific and precise analytical method for measuring transdermal biological constituents is needed to determine the sildenafil dose formulation and to make adjustments. This paper presents a rapid, simple, and inexpensive reversed-phase HPLC method for the evaluation of sildenafil in transdermal biological samples with pharmaceutical formulations of various pH values to influence permeation of sildenafil in nude mouse skin. We present a simple method based on a one-step liquid–liquid extraction with commercially available internal standard, ethylparaben. The sensitivity of the method is sufficient for transdermal permeation studies.

2. Experimental

2.1. Chemicals

Sildenafil citrate was obtained from Orchid Chemicals & Pharmaceuticals (Chennai, India). Acetonitrile was purchased from BDH (Poole, UK), 85% phosphoric acid was purchased from Riedel-de Haën (Seelze, Germany), and the internal standard, ethylparaben, was obtained from Sigma (Milwaukee, WI, USA). All other reagents were of analytical grade from E. Merck (Darmstadt, Germany).

2.2. Apparatus

The HPLC system consisted of an LC-10AD Shimadzu pump (Kyoto, Japan), an LC-10 Shimadzu automatic sample injector, and a Spectra 100 Spectra-Physics UV detector (Mountain View, CA, USA) with an integrator (EZ Chrom chromatography data system version 6; San Roman, CA, USA). The separation was performed on a LUNA $C_{18}(2)$ particle size 5 μm, 150×4.60 mm I.D. column (Phenomenex, Torrance, CA, USA).

2.3. Optimal HPLC conditions

The mobile phase consisted of 32% acetonitrile in water with 0.2% phosphoric acid. The pH of the mobile phase was adjusted to 5.3 by 10 *M* sodium hydroxide. The rate of the mobile phase delivery through the HPLC system was 1 ml/min. Sildenafil was monitored at 224 nm with a time constant of 2 s. The analytical and guard columns and the mobile phase were all maintained at room temperature (25±2°C). Sildenafil in samples (nude mouse skin constituents) was quantitated by comparing the peak height of sildenafil in samples with a standard calibration curve of sildenafil in nude mouse skin constituents.

2.4. Preparation of calibration standards

A stock solution of sildenafil (citrate salt) was prepared by dissolving approximately 10 mg in 10 ml of deionized Milli-Q filter water. Further solutions were obtained by serial dilutions of the stock

solution with deionized Milli-Q filter water. Both a 20-μl aliquot of a 50-ng/ml sildenafil solution and a 25-μl aliquot of a 50-ng/ml ethylparaben were injected into the HPLC system to determine the retention time of sildenafil under the experimental chromatographic conditions. These solutions were added to drug-free transdermal phosphate-buffered saline (PBS) solution sample constituents, which were collected from the receiver chamber of transdermal nude mouse skin under a 10-h, 37°C incubation (described in Section 2.6) in volumes not exceeding 1% of the transdermal samples. After adding 0.8 ml of acetonitrile to 0.4 ml of nude mouse skin constituents, the mixture was placed in a vortex mixer for at least 30 s, and centrifuged at 3000 rpm for 10 min. The supernatant was decanted into another fresh borosilicate culture test tube, and dried under a nitrogen stream, and resuspended in a 100-μl mobile phase. An aliquot of this solution was injected directly into the chromatographic system. Standard concentrations of sildenafil ranged between 5 and 500 ng/ml. The calibration curves were obtained by linear regression: the ratio of sildenafil peak height to ethylparaben peak height was plotted vs. sildenafil concentration in ng/ml. The suitability of the calibration model was confirmed by back-calculating the concentration of the calibration standards.

2.5. Method validation

Drug-free transdermal PBS sample constituents with 7, 15, 150, and 400 ng/ml of sildenafil, prepared for quality control, were for extra-low, low, medium, and high-level transdermal PBS sample constituents, respectively. The precision and accuracy of the method were evaluated by testing six replicates of four concentrations (7–400 ng/ml) of transdermal PBS sample constituents for the within-day and by assaying two replicates of four concentrations sample constituents over 6 different days for inter-day. The precision and accuracy were defined as the relative standard deviation (RSD) and as the error from the theoretical nominal concentration, respectively. The linearity data were obtained by means of calibration curves ($n=6$). The limit of quantitation (LOQ) was defined as the lowest concentration at which the precision expressed by the RSD was lower than 15% and the accuracy expressed by the relative difference of the measured and true value was also lower than 15%. Six calibration curves were analyzed for RSDs and the determination of LOQ.

2.6. Transdermal permeation of sildenafil in nude mouse skin

Fresh samples of whole nude mouse skin were removed from the abdomen of cadavers immediately after postmortem and mounted carefully between the two compartments of a Franz cell [8] with rigid clamps. The initial concentration of sildenafil in the donor compartments was held at 15.8 μg/ml, while the pH of formulation was varied (pH 4, 5, 6, 7, 8, 9, 10, 11, and 12). The receiver compartments were filled with PBS solution (pH 7.4) and with stirring throughout the permeation studies. Transdermal PBS sample constituents (0.6 ml) were taken from the receiver compartments at fixed intervals and replaced with an equal volume of previously warmed PBS at 37°C. To the fresh samples was added a 25-μl aliquot of 50 ng/ml ethylparaben and then assayed by HPLC–UV [8,9]. The in vitro apparent permeability coefficients (P_{coeff}) were calculated from the following equation [10]:

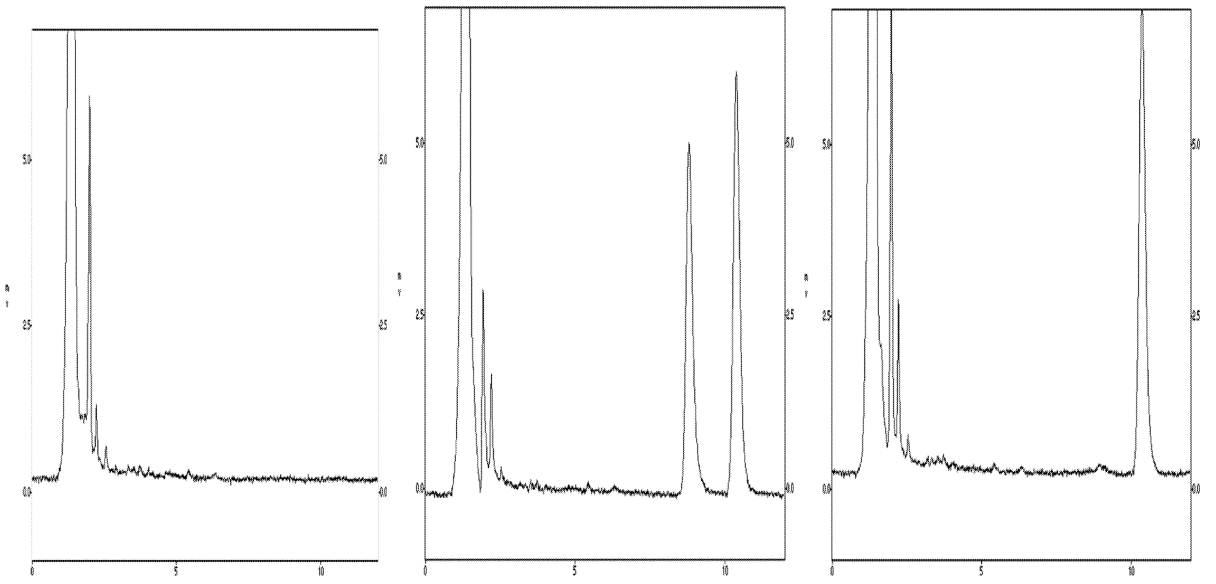
$$P_{\mathrm{coeff}} = \left(\frac{V}{A}\right) \cdot \left(\frac{\mathrm{d}(\%\ \mathrm{transported})}{\mathrm{d}t}\right)$$

in which the percent transport of the dose through the skin tissues can be calculated after correction for sampling and solution replacement at each time point. These values were then plotted versus time. Therefore, the apparent permeability coefficient, P_{coeff} (cm/s), can be calculated from the slope using the receiver compartment volume V (7 ml) and surface area of tissue A (0.627 cm^2).

3. Results and discussion

3.1. Chromatography

Typical chromatograms with blank transdermal PBS constituents, spiked with 1 μg/ml sildenafil



A B C

Fig. 1. Chromatograms of blank transdermal PBS biological samples (A), a transdermal biological sample spiked with 1 μg/ml of sildenafil (B), and the limit of quantitation of sildenafil for transdermal PBS constituents (C). Retention times for sildenafil and ethylparaben are 8.8 and 10.5 min, respectively.

from transdermal constituents and spiked at the limit of quantitation, are shown in Fig. 1. These assay methods were selective as demonstrated by the lack of interfering peaks in the blank transdermal PBS constituents following a visual inspection of the chromatograms. The retention times for sildenafil and ethylparaben are 8.8 and 10.5 min, respectively. The total time consumed for sample preparation and chromatography was less than 15 min for one sample. The pK_a of sildenafil is 6.5. The best peak shape and resolution were obtained when the mobile phase pH was between 5.0 and 6.0. A mobile phase pH of greater than 6.5 gave an irreproducible peak height and poor peak shape for sildenafil. The capacity factor (k') of sildenafil under the chromatographic conditions of this experiment was sensitive to the ionic strength of the mobile phase. The retention time decreased with an increase in the mobile phase ionic strength. Use of phosphate buffer in the mobile phase improved the capacity factor of sildenafil in the system and decreased the variation between the retention time of sildenafil and the injection volume (20–100 μl).

3.2. Method validation

The transdermal PBS constituent's standard curve (5.0–500.0 ng/ml) was found to be linear with a correlation coefficient of more than 0.999 (Fig. 2). The accuracy and precision of the calibration curve concentrations are shown in Table 1. The relative standard deviations were less than 15% with relative errors of −5.2 to 3.7%. The quality control samples of intra-day and inter-day accuracy and precision are shown in Tables 2 and 3. All of the relative standard deviations for intra-day and inter-day assays were less than 14% with relative error −11.4 to 6.5%. The limit of quantitation for transdermal PBS constituents was 5.0 ng/ml for sildenafil, where the relative standard deviations for inter-, and intra-day

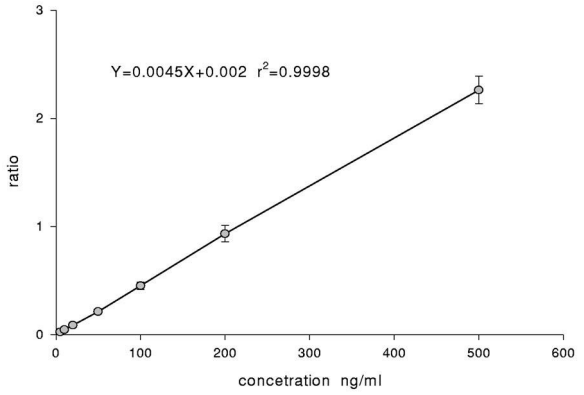


Fig. 2. Linearity of the calibration standard curve of sildenafil.

Table 1
Precision and accuracy of the standard curve of sildenafil in blank transdermal PBS constituents

Spiked concentration (ng/ml)	Calculated concentration (mean±SD, $n=6$) (ng/ml)	RSD (%)	Error (%)
5	5.1±0.8	15.0	2.0
10	10.2±0.5	5.0	2.0
20	19.3±1.6	8.0	−3.5
50	47.4±3.4	6.8	−5.2
100	100.5±7.5	7.5	0.5
200	207.4±17.0	8.5	3.7
500	502.1±28.2	5.6	0.4

assays were less than 15% with acceptably low relative errors. Although the LOQ was a little higher compared with the automated sequential trace enrichment of dialysates (1 ng/ml) in human plasma samples [6], this method has easy preparation and low cost for transdermal biological samples. In addition, the LOQ results (5 ng/ml) are similar to those of analysis of plasma sildenafil concentrations in mice and dog except the authors used a solid-phase extraction [7].

3.3. Stability of sildenafil in pH range from 3 to 12 for 24 h

In order to ensure that the changing permeability coefficient of sildenafil does not result from its degradation, the stability of sildenafil (200 ng/ml) in different pH values is shown in Fig. 3. We found that sildenafil was stable in various pH values at room temperature for 24 h. In addition, we observed no degradation of sildenafil after applying the transdermal biological sample solution to nude mouse skin contents in a 12-h incubation. It will help with modifying partition and penetration properties of sildenafil by pH formulations to know that sildenafil is stable over a wide range of pH values.

Table 2
Intra-day precision and accuracy of sildenafil in transdermal PBS constituents

Spiked concentration (ng/ml)	Calculated concentration (mean±SD, $n=6$) (ng/ml)	RSD (%)	Error (%)
7	6.2±0.7	10.0	−11.4
15	14.2±0.7	4.6	−5.3
150	159.8±7.6	5.0	6.5
400	414.9±18.5	4.6	3.7

Table 3
Inter-day precision and accuracy of sildenafil in transdermal PBS constituents

Spiked concentration (ng/ml)	Calculated concentration (mean±SD, $n=18$) (ng/ml)	RSD (%)	Error (%)
7	6.9±1.0	14.0	−0.1
15	14.8±1.4	9.3	−1.3
150	157.7±9.9	6.6	5.1
400	404.7±25.6	6.4	1.1

Samples were analyzed every day for 6 days.

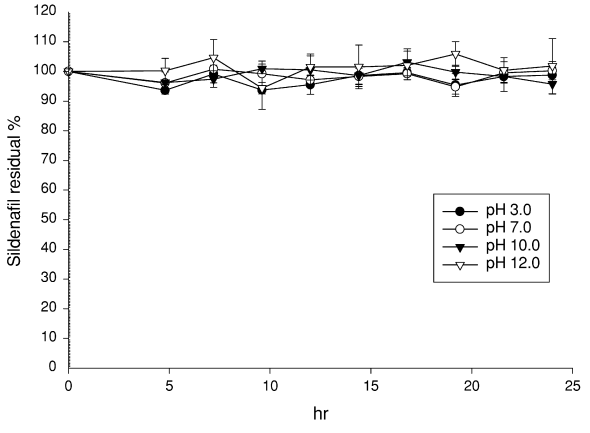


Fig. 3. Stability of sildenafil at different pH values with transdermal PBS constituents (mean±SD, $n=6$).

3.4. Application of the permeability coefficient of transdermal sildenafil in nude mouse skin

Fig. 4 shows the effect of pH on sildenafil permeation through nude mouse skin. We clearly found that the permeability coefficients of sildenafil changed with pH, and the higher permeation rates fell in the pH range of 8–11, at a range of $2–3\times 10^{-6}$ cm/s. Sildenafil is a weakly basic compound ($pK_a=6.5$) [5,7], which is therefore only partially ionized at physiological pH values. It is consistent that the major non-ionized form of sildenafil has higher permeation due to the increasing partition coefficient of sildenafil in mouse skin above pH 7.0. In addition, Gobry et al. [11] recently reported that sildenafil has a very weak acidic moiety ($pK_a=9.6\sim 10.1$), which will be completely anionized above pH 11 following decreased partition coefficient of sildenafil. This observation was similar to our results which observed decreased permeation ability of sildenafil above pH 11. Thus, the physicochemical parameter of partition of sildenafil, moderately lipophilic (log $D_{7.4}=2.7$) resulting in good solubility [12], confers the penetration properties observed for this transdermal agent.

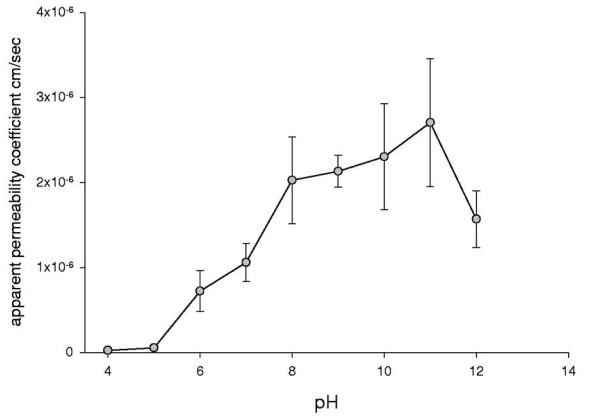


Fig. 4. Effect of pH on the sildenafil permeability coefficient through transdermal nude mouse skin (mean±SD, $n=6$).

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