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Development of a liquid chromatographic method for bioanalytical applications with sildenafil

Ming-Thau Sheu[a], An-Bang Wu[a], Geng-Cheng Yeh[b], Angel Hsia[a], Hsiu-O Ho[a,*]

[a]*Graduate Institute of Pharmaceutical Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, PR China*
[b]*Department of Pediatrics, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan, PR China*

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Abstract

An improved HPLC method was developed for the determination of sildenafil concentrations in plasma. Analysis of sildenafil in plasma samples was simplified by utilizing a one-step liquid–liquid extraction after alkaline treatment of only 1 ml of plasma. The lower limit of quantitation was 10 ng/ml with a coefficient of variation of less than 20%. A linear range was found to exist from 10 to 1000 ng/ml. This HPLC method was validated with precisions (coefficient of variation, C.V.) for inter- and intra-day runs of 0.41–11.15% and 0.36–8.05%, respectively, and accuracies (the relative error of the mean, REM) for inter- and intra-day runs of −8.72–6.81% and 0.41–11.15%, respectively. A bioavailability study of sildenafil was performed on one normal healthy male volunteer by analyzing sildenafil plasma concentrations with this validated HPLC method. Results demonstrated that this HPLC method is appropriate for applications to bioavailability studies of sildenafil. In addition, an example of the influence of the co-administration of grapefruit juice on sildenafil pharmacokinetics in a healthy volunteer is presented.

Keywords: Sildenafil

1. Introduction

Sildenafil, ((1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pyrazolo-[4,3-*d*] pyrimidin-5-yl)-phenylsulphonyl]-4-methylpiperazine) (Fig. 1), is a potent and selective inhibitor of cGMP (type V)-specific phosphodiesterase which is capable of enhancing relaxation of the penile corpus cavernosum and therefore has the potential to improve penile erectile function [1]. Sildenafil is rapidly absorbed after oral administration, with absolute bioavailability of about 40%. Its pharmacokinetics is dose-proportional over the recommended dose range. It is eliminated predominantly by hepatic metabolism (mainly by cytochrome P450 3A4) after absorption and is converted to an active metabolite with properties similar to itself. Sildenafil and this metabolite have terminal half-lives of approximately 4–5 h. The maximum observed plasma concentration of sildenafil is reached within 30–120 (median, 60) min of an oral dosing in a fasting state. Less than 2% of the administered dose is excreted in the urine as sildenafil or its active metabolite [2,3].

CYP 3A is the largest subfamily of CYP enzymes

*Corresponding author. Tel.: +886-2-2377-1942; fax: +886-2-2377-1942.
E-mail address: hsiuoho@tmu.edu.tw (H.-O Ho).

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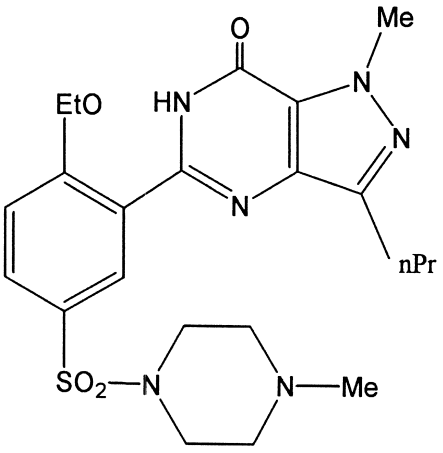

Fig. 1. Structure of sildenafil.

expressed in the human liver and gastrointestinal tract and is involved in the metabolism of many clinically used drugs and other chemicals. Concomitant intake of grapefruit juice causes inhibition of CYP3A4-mediated first-pass metabolism of many drugs in the gut wall and thereby increases the oral bioavailability of these agents [4–6]. Recently many lines of evidence point to the intestine, rather than the liver, as the major site of this interaction in vivo [7]. Lee and Min [8] observed that grapefruit juice appeared to increase the C_{max} of sildenafil by 42% without a significant change in the AUC from a single elderly male subject. Jetter et al. [9] reported that the $AUC_{0-\infty}$ of sildenafil increased 1.23-fold, and a trend toward prolongation of T_{max} after grapefruit juice intake was observed. C_{max} did not differ significantly. Pharmacokinetic profiling of volunteers taking sildenafil with grapefruit juice was inconsistent. The interaction of grapefruit juice with sildenafil is still worth examining.

There are several studies in the literature reporting the determination of sildenafil in plasma samples using an HPLC method [10,11]. 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 [12]. In addition, the 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 [13]. Plasma concentrations of sildenafil and *N*-demethylsildenafil can be determined up to 24 h post-administration by use of liquid chromatography in tandem with mass spectrometry [9]. Radiolabeled sildenafil has also been used to monitor the pharmacokinetics of plasma and urine in the mouse, rat, rabbit, dog, and human [14]. However, normal laboratories with limited financial resources are less likely to have access to special techniques and complicated procedures, so a simpler assay method must be developed.

A reversed-phase HPLC system was recently developed by Liaw et al. [15] for the determination of sildenafil concentrations in an in vitro transdermal permeation study. The lower limit of a quantifiable concentration was reported to be 5 ng/ml for analyzing sildenafil samples collected in transdermal permeation studies. But difficulties may arise in analyzing biological samples such as plasma. Losses may occur especially during treatments of extracts for various sample preparation procedures. Therefore, there is still a demand for development of a simpler and more-sensitive HPLC method for analysis of sildenafil in biological fluids such as plasma. The purpose of the present study was to compare and improve the assay method for sildenafil in plasma using a reversed-phase HPLC system. This validated HPLC method was then applied in a bioavailability study of sildenafil in a human following a single oral administration of sildenafil with water or grapefruit juice.

2. Experimental

2.1. Materials and methods

2.1.1. Drug and methods

Standard sildenafil citrate was obtained from Trans American Chemicals (San Diego, CA, USA). The internal standard, butylparaben, was purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile for liquid chromatography were HPLC grade and were obtained from Merck (Darmstadt, Germany). All other reagents used were reagent grade or better.

2.2. Instrumentation

A high-performance liquid chromatographic (HPLC) system equipped with a pump (Jasco PU-980 Intelligent HPLC pump; Jasco, Tokyo, Japan) and an autosampler (Jasco AS-950-10 Intelligent Sampler) were used. The eluent was detected with a Jasco UV-975 UV–Vis detector at 230 nm. A 4.6× 150-mm (I.D.) Inertsil 5 ODS-2 column (Veracopak®) with a particle size of 5 μm and a mobile phase consisting of acetonitrile and a 30-m*M* potassium dihydrogen phosphate buffer solution (pH 6.0 adjusted with 1 N NaOH) at a 55:45 (v/v) ratio was used. The flow-rate was set at 0.5 ml/min. This method was found to be selective, precise, and linear over a concentration range of 10–1000 ng/ml. All mobile phase solutions were filtered and degassed ultrasonically before use. The HPLC system was controlled by a PC workstation using Chromatography Data Station software (SISC, Taiwan) installed on it.

2.3. Internal standard solution and sample preparation

Plasma sample preparation and the extraction method are described step by step as follows. The plasma sample (1 ml) was spiked with 0.1 ml of the internal standard (butylparaben, 20 μg/ml in methanol) solution and 0.1 ml of a NaOH solution (1 N). After vortex-mixing thoroughly for 5 s, the mixture was extracted with 3 ml of ethyl acetate by vortex-mixing for 5 min, and then centrifuged at 2950 *g* for another 10 min. The supernatant (organic phase) was transferred to another clean glass tube and evaporated under a stream of nitrogen gas at 40 °C until completely dry. Then, 0.2 ml of the mobile phase was added to dissolve the residue, and 0.1 ml was automatically injected into the HPLC system for analysis.

2.4. Quantification and calibration curve preparation

To examine the linearity of the assay, calibration curves for sildenafil at concentrations ranging from 10 to 1000 ng/ml in plasma were prepared. Standard plasma samples containing sildenafil at concentrations of 10, 20, 50, 100, 200, 500, and 1000 ng/ml were supplemented with 20 μg butylparaben and extracted and analyzed as described above. The peak area ratio (PAR) of sildenafil to butylparaben was measured, and a calibration curve was obtained from the least-squares linear regression of the PAR versus spiked concentrations. The regression line was used to calculate concentrations of sildenafil in the unknown plasma samples based on the PAR.

2.5. Validation of the assay method

Several pre-dose human plasma samples from different subjects were tested for the presence of interfering compounds. The coefficients of variation and relative errors of the mean were used to validate the precision and accuracy of the intra- and inter-day assays by determining standard samples of sildenafil in plasma. For inter-day validation, five sets of control samples at seven different concentrations (10–1000 ng/ml) were evaluated on five different days (with seven standard curves being constructed). For intra-day validation, five sets of controls at seven different drug concentrations were assayed with one standard curve on the same run.

2.6. Blood sample collection and processing

A bioavailability study (approved by the Ethics Committee of Taipei Medical University Hospital) was conducted on one subject who received a single 25-mg dose of sildenafil citrate with 250 ml of either water or grapefruit juice. After dosing, heparinized venous blood samples (~10 ml) were collected by means of an indwelling venous cannula of the cubital vein according to a predetermined time schedule, which included a blank sample just prior to dosing and then at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, and 8 h after dosing. Plasma was separated immediately by centrifugation at 1690 *g* for 10 min, then transferred to suitably labeled tubes, and stored at −25 °C until the assay.

2.7. Analysis of pharmacokinetic data

The following parameters were assessed for the period of 0–8 h: the area under the plasma con-

centration–time curves from time zero to the last measurable sildenafil sample time and to infinity (AUC_{0-last} and $AUC_{0-\infty}$), maximum concentration (C_{max}), and time to maximum concentration (T_{max}). All pharmacokinetic variables were calculated by non-compartmental methods. C_{max} and T_{max} were obtained directly from the concentration–time curve data. The area under the concentration–time curve from time zero (predose) to time of last quantifiable concentration (AUC_{0-last}) was calculated using the linear trapezoidal method.

3. Results and discussion

The HPLC method carried out in this study was an attempt at developing a simple chromatographic system capable of eluting and resolving sildenafil from human plasma and which complies with general requirements for system suitability. Preliminary investigations were directed towards the effect of certain variables on the suitability of the method. The parameters assessed include the type and quantity of the organic modifier, the column, the salt concentration, and the pH of the mobile phase. The results show that the Inertsil 5 ODS-2 column system was suitable for the determination of sildenafil from human plasma because of its excellent resolution, appropriate retention time, and good sensitivity (Fig. 2).

After several trials in this study, it was found that the present method using one-step liquid–liquid extraction of sildenafil in plasma after alkaline treatment was satisfactory. Using the peak area of the sildenafil sample at the same injection amount without extraction as 100%, it was found that the recovery of sildenafil added to human plasma was almost quantitative (at least 75%) with minimal interference. This indicates that the method is reproducible and suitable for the analysis of plasma samples. The method provides a simple and practical way to process plasma samples containing sildenafil with almost quantitative recovery.

Fig. 3 shows typical HPLC chromatograms of sample analysis. No interfering peaks were observed for drug-free human plasma. The retention times of sildenafil and butylparaben were around 7.6 and 11.3 min, respectively. Good separation and baselines with low background were observed. The peaks of interest were well resolved, and there was no interference from endogenous plasma substances. Also, the symmetry of both peaks (sildenafil and butylparaben) was clearly indicated.

The intra- and inter-day validations for assaying sildenafil in plasma samples are shown in Tables 1 and 2. These data show good precision and high accuracy of the analysis. The linearity of the calibration curve of sildenafil was well correlated ($r^2 > 0.999$) within a range of 10–1000 ng/ml for intra- and inter-day assays. All data show the excellent reproducibility of the sample analysis. Since the coefficient of variation for 10 ng/ml was less than 20%, it was set as the lower limit of quantitation (LLOQ). The coefficients of variation for the remaining standard concentrations were all less than 15%, which complies with the requirements of assay validation. Preferably, only 1 ml of plasma sample is necessary to achieve such a low limit of quantitation. As expected, the limit of detection (LOD) was even lower than one-third of the LLOQ.

The method was applied to a bioavailability study for examining the influence of the co-administration of grapefruit juice on the absorption of sildenafil. The absolute relative errors of the mean in each sample analysis run for QC samples were between 0.09 and 4.45%, indicating that the stability of sildenafil in plasma during storage periods was acceptable. Typical chromatographs of analyzed plasma samples are shown in Fig. 4. The suitability of this method was proven in this bioavailability study of sildenafil after oral administration of sildenafil (25 mg) in one healthy male volunteer with either water or grapefruit juice.

Fig. 5 displays the sildenafil plasma concentration–time profiles in the volunteer after orally administration of the sildenafil formulation with 250 ml of either water or grapefruit juice. Grapefruit juice changed the area under the sildenafil plasma concentration–time curve from time zero to infinity [$AUC_{0-\infty}$] from 63.06 to 169.14 ng·h/ml, corresponding to a 168% increase. A trend toward prolongation of the T_{max} of sildenafil after grapefruit juice intake from 0.50 to 0.75 h was observed. Grapefruit juice increased the C_{max} of sildenafil by approximately 35% from 50.53 to 68.02 ng/ml. These results indicate that co-administration of sil-

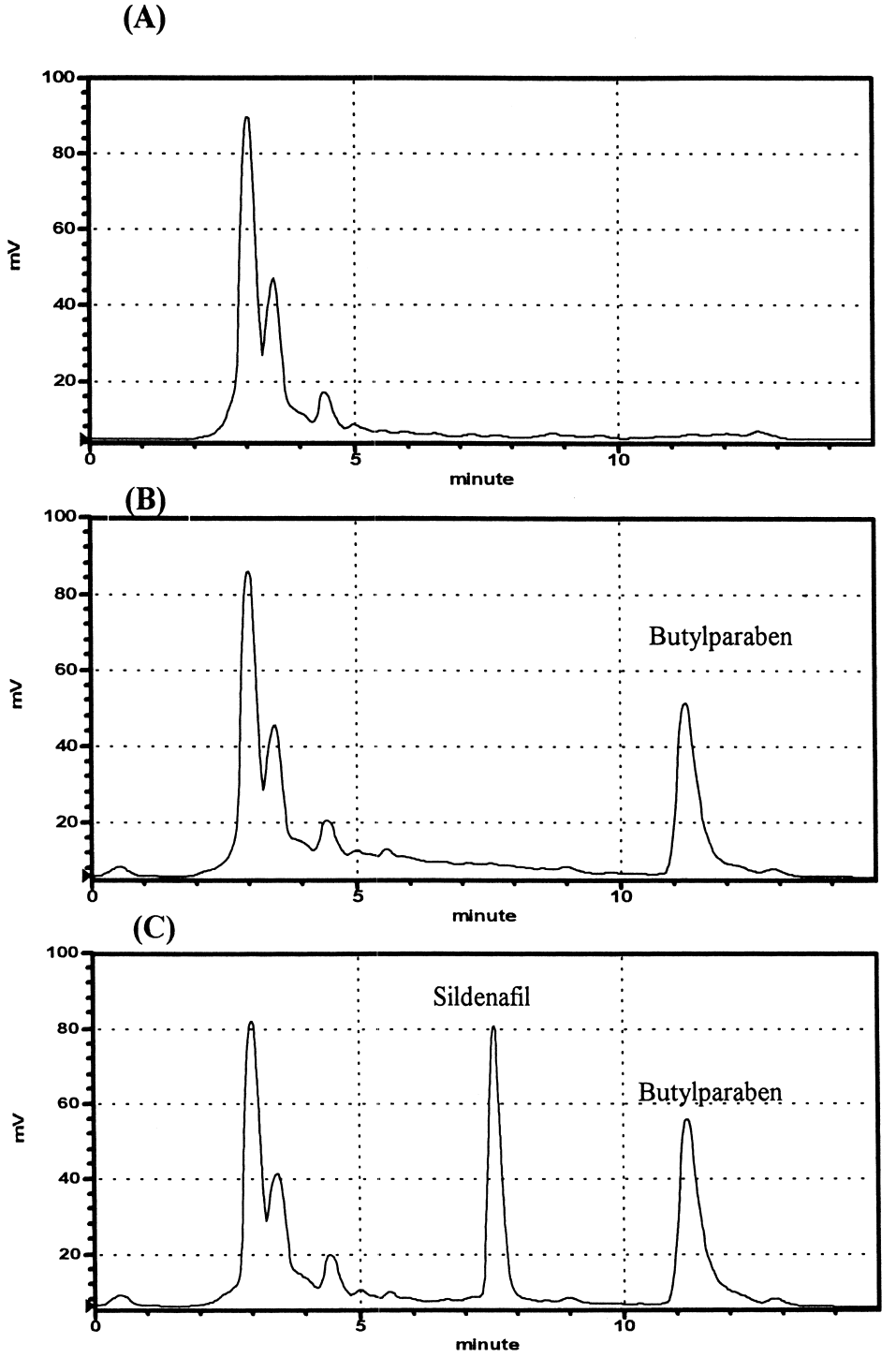

Fig. 2. HPLC chromatograms using the Inertsil ODS column. (A) Blank plasma; (B) the internal standard, butylparaben; (C) sildenafil (1000 ng/ml) and butylparaben (2 μg/ml).

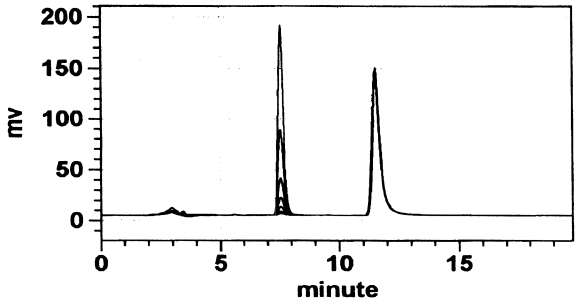

Fig. 3. HPLC chromatograms of standard plasma concentrations of sildenafil (from 10 to 1000 ng/ml) and butylparaben (2 μg/ml).

denafil with grapefruit juice seems to increase the T_{max}, C_{max}, and AUC. The grapefruit juice effect has been postulated to be the result of competitive inhibition of CYP3A4 in the enterocytes lining the small intestine, because grapefruit juice has been shown to contain 6′,7′-dihydroxybergamottin (DHB) which is capable of competitively inhibiting this enzyme [7]. Therefore grapefruit juice can increase sildenafil bioavailability and thus tends to delay sildenafil absorption.

4. Conclusions

In conclusion, the HPLC assay method developed in this study using a reversed-phase system was proven to be acceptable for assaying sildenafil concentrations in plasma. High precision and accuracy with minimal interference and peaks with high symmetry were demonstrated. A one-step liquid–liquid extraction further provides a simple and practical way to process plasma samples containing sildenafil with almost quantitative recovery. This method is appropriate for applications to bioavailability studies of the oral administration of sildenafil and to examine the influence of grapefruit juice on the pharmacokinetic profiling of sildenafil.

Table 1
Precision and accuracy of intra-day validation

Concentration (ng/ml)	Y1[a]	Y2	Y3	Y4	Y5	Mean	SD	C.V. (%)	REM (%)
10	9.74	11.13	11.07	9.30	9.93	10.23	0.82	8.05	2.33
20	20.37	19.19	20.63	22.92	22.11	21.04	1.48	7.03	5.21
50	51.95	48.21	51.43	57.91	56.82	53.26	4.03	7.56	6.53
100	107.07	114.63	93.61	102.83	103.63	104.35	7.60	7.29	4.35
200	198.05	192.59	203.91	194.46	182.15	194.23	8.02	4.13	2.88
500	486.99	489.15	499.30	485.18	505.92	493.31	8.92	1.81	1.34
1000	1005.83	1005.13	1000.05	1007.41	999.45	1003.57	3.59	0.36	0.36

SD, standard deviation; C.V., coefficient of variation; REM, relative error of the mean.
[a] Number of the replication.

Table 2
Precision and accuracy of inter-day validation

Concentration (ng/ml)	Y1[a]	Y2	Y3	Y4	Y5	Mean	SD	C.V. (%)	REM (%)
10	8.39	10.49	8.62	8.21	9.93	9.13	1.02	11.15	−8.72
20	19.00	18.43	19.16	23.47	22.11	20.43	2.22	10.86	2.17
50	51.35	49.75	54.95	49.07	56.82	52.39	3.36	6.42	4.78
100	106.67	104.99	109.99	108.79	103.63	106.81	2.62	2.45	6.81
200	188.51	192.07	196.04	186.66	182.15	189.09	5.28	2.79	−5.46
500	509.51	506.56	484.20	504.63	505.92	502.16	10.20	2.03	0.43
1000	996.56	997.71	1007.03	999.18	999.45	999.99	4.11	0.41	0.00

SD, standard deviation; C.V., coefficient of variation; REM, relative error of the mean.
[a] Number of the replication.

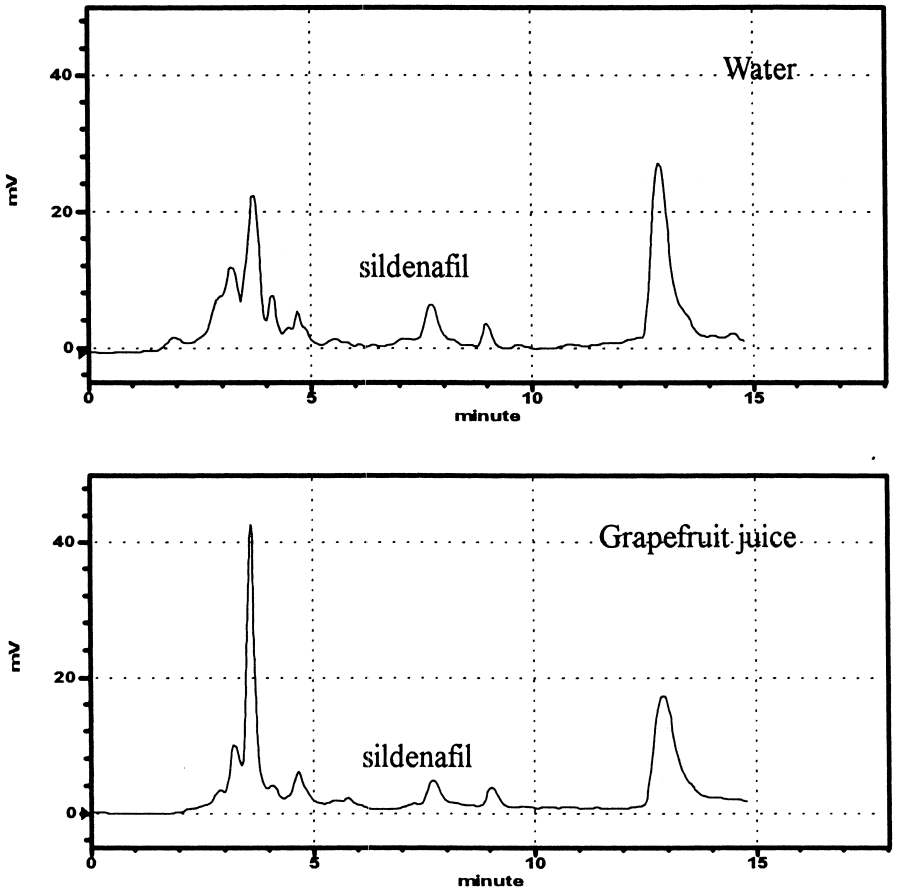

Fig. 4. Representative results of HPLC analysis of plasma samples from the bioavailability study.

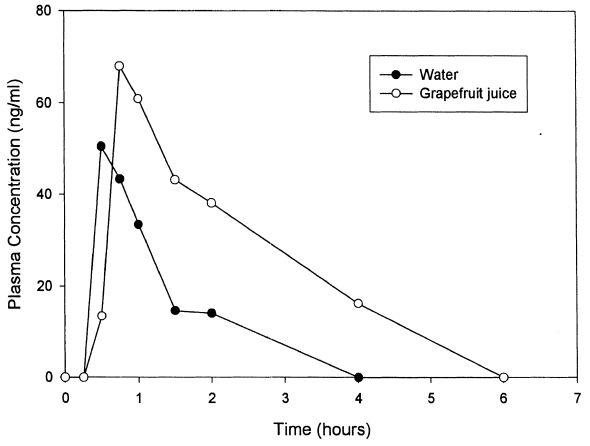

Fig. 5. Sildenafil plasma concentration–time profile of one volunteer.

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