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ORIGINAL RESEARCH

High-performance liquid chromatographic determination of doxazosin in human plasma for bioequivalence study of controlled release doxazosin tablets

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ABSTRACT: A highly sensitive high-performance liquid chromatographic quantification method with fluorescence detection was developed and validated for the determination of doxazosin in human plasma. The developed method employed one-step extraction of doxazosin from plasma matrix with ethyl acetate using propranolol as an internal standard. Chromatographic separation was obtained within 8.0 min using a reverse-phase Capcell-Pak C_{18} column (150 × 4.6 mm i.d., 5 μm) and the mobile phase consisted of methanol–water containing 10 mM perchloric acid and 1.8 mM sodium heptane sulfonic acid (50:50, v/v) and was set at a flow rate of 1.5 mL/min. The calibration curve constructed was linear in the range of 0.3–50.0 ng/mL. The proposed method achieved a lower limit of quantification of 0.3 ng/mL, better than the reported HPLC methods. Average recoveries of doxazosin and the internal standard from human plasma matrix were 87.0 and 85.9%, respectively. The present method was validated by evaluating the precision and accuracy for inter- and intraday variation in the concentration range 0.3–50 ng/mL. The precision values expressed as relative standard deviations in the inter- and intraday validation were 1.17–6.29 and 0.84–5.94%, respectively. This method was successfully applied to the bioequivalence study of two doxazosin controlled release tablets in healthy, male human subjects. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: HPLC; doxazosin, controlled release; bioequivalence; human plasma

INTRODUCTION

Doxazosin mesylate (Fig. 1), a quinazoline derivative, is a long-acting selective α_1-adrenoceptor antagonist that is efficacious in the treatment of hypertension and benign prostatic hyperplasia (Chung *et al*., 1999). In hypertensive patients, doxazosin lowers blood pressure by selectively inhibiting postjunctional α_1-adrenergic receptors and antagonizing stimulant activity of noradrenaline, thereby decreasing systemic vascular stress (Brown and Dickerson, 1991; Englert and Barlage, 1991; Fulton *et al*., 1995). Doxazosin has also been shown to have a beneficial effect for patients with benign prostatic hyperplasia by effectively blocking α_1-adrenoceptors that exist in the prostate and bladder neck, resulting in reduced prostatic tone and alleviating obstruction (Black *et al*., 2000; Fawzy *et al*., 1999).

Oral controlled release formulations of doxazosin have demonstrated an enhanced pharmacokinetic profile and optimum drug delivery rate, compared with the standard formulation and eliminated the need for slow dose titration to reduce the risk of adverse first-dose effects (Andersson *et al*., 1994; Os and Stokke, 1999). The controlled release dosage form has led to therapeutically effective levels being reached more rapidly without excessive plasma levels, and more uniform plasma concentrations can also be obtained with minimal peak-to-trough fluctuation (Chung *et al*., 1999).

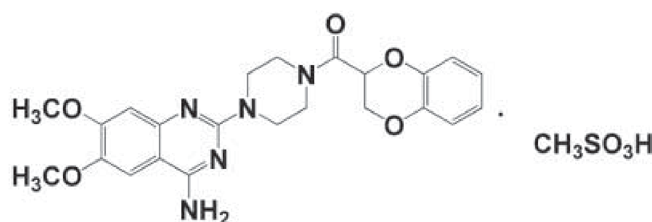

Figure 1. Structure of doxazosin mesylate.

Several analytical techniques utilizing HPLC with fluorescence detection have been reported to quantify doxazosin in biological fluids. For instance, methods with a sensitivity of 1 ng/mL having sample preparation using solid-phase extraction (Jackman *et al*., 1991), a sensitivity of 0.5 ng/mL having sample preparation using liquid–liquid extraction (Chung *et al*., 1999; Cho *et al*., 2000) and a sensitivity of 0.5 ng/mL having sample preparation using protein precipitation

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(Sripalakit *et al.*, 2005) are available. However, methods for the determination of doxazosin in human plasma with lower limit of quantification less than 0.5 ng/mL are not available for use for bioavailability and bioequivalence studies. The primary aim of this study was, therefore, to develop an HPLC method for the measurement of doxazosin in human plasma, with lower limit of quantification than 0.5 ng/mL. The developed method was validated and used for a bioequivalence study of two controlled release tablet formulations of doxazosin in 26 healthy male volunteers.

EXPERIMENTAL

Chemicals and reagents. Doxazosin mesylate and test tablets were purchased from Hyun Dai Pharmaceutical Company (Seoul, Korea). Cardura XL® tablets were obtained from Pfizer Pharmaceutical Company (Seoul, Korea). Propranolol HCl and sodium heptane sulfonic acid were provided by Sigma-Aldrich Company (MO, USA). Methanol, ethyl acetate and perchloric acid of HPLC grade were supplied by Fisher Scientific Korea Limited (Seoul, Korea). All other reagents were of analytical grade. Drug-free human plasma (blank plasma) was obtained from healthy male volunteers by centrifugation of whole blood treated with the anticoagulant, heparin, and stored at approximately −20°C until needed.

Chromatographic conditions. Chromatographic separation was carried out on a Hitachi HPLC system equipped with an L-7200 autosampler, L-7100 pump and L-7485 fluorescence detector (Tokyo, Japan). The data acquisition was performed by Analyst® (version 1.3) data system from Hitachi Company. A 50 µL of reconstituted sample was injected into a Capcell Pak C_{18} column (150 × 4.6 mm i.d., 5 µm, Shiseido, Tokyo, Japan), maintained at 40°C with a mobile phase consisting of methanol–water containing 10 mM perchloric acid and 1.8 mM sodium heptane sulfonic acid (50:50, v/v). The flow rate was set at 1.5 mL/min. Doxazosin and internal standard were detected at an excitation wavelength of 330 nm and emission wavelength of 395 nm.

Preparation of standard solutions and plasma samples. Stock solutions (1.0 mg/mL) of doxazosin were prepared by dissolving 10 mg of doxazosin in 10 mL of a mixture of water and methanol (50:50, v/v); these were then stored at −20°C. Working solutions of doxazosin were prepared by serial dilutions with a mixture of water and methanol (50:50, v/v) from primary stock solution. Spiked plasma samples, used as calibration standards, were prepared daily by the addition of 30 µL of the working solutions to 270 µL of drug-free human plasma, resulting in calibration standards with concentrations of 0.3, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 ng/mL.

Sample preparation. After cooling down at room temperature, an aliquot of each sample (300 µL) was pipetted into a polypropylene 1.5 mL conical-bottom centrifuge tube (Eppendorf, Hamburg, Germany) and 50 µL of internal standard solution (propranolol 10.0 µg/mL) were added. After vortexing for 0.5 min, doxazosin and propranolol were extracted with ethyl acetate (1.1 mL) for 10 min on a shaker and the mixture was centrifuged at 12,000 rpm for 10 min. The organic layer was transferred to another clean tube and evaporated with vacuum concentrator for 50 min. The residue was reconstituted with 120 µL of the mobile phase. A 50 µL volume was injected into the HPLC system.

Extraction recovery. Extraction recoveries were evaluated for doxazosin at three concentrations of 1.0, 5.0 and 10.0 ng/mL and for the internal standard at a concentration of 500 ng/mL. Six replicate samples for each concentration were extracted and chromatographed. The extraction recoveries were determined by comparing the peak heights obtained from quality control samples in human plasma with those obtained by direct injection of the working standard solutions in 50% methanol at the identical concentration.

Assay validation for doxazosin. For method validation and linearity studies, blank plasma samples obtained from healthy volunteers were used. To examine the possible interferences of endogenous compounds, 26 human plasma samples from different volunteers were extracted and analyzed during method validation. These plasma samples were pretreated according to the sample preparation procedure except for the addition of the IS. After plotting of peak-height ratios (doxazosin/IS) against the drug concentrations, calibration curves were constructed by linear least-squares regression analysis. The lower limit of quantification (LLOQ) was determined as the lowest plasma concentration of doxazosin obtained with a coefficient of variation of less than 20% and estimated to be 0.3 ng/mL. For the determination of the intra-day accuracy and precision, replicate analysis of spiked plasma samples was performed on the same day. The run consisted of five replicates of each 0.3, 1.0, 5.0, 10.0, 20.0 and 50.0 ng/mL quality control samples. Inter-day accuracy and precision were assessed by analysis of five batches of six different quality control samples on five different days. The evaluation of precision (i.e. the deviation of each concentration level) should not be more than ±15.0% from the nominal concentration except for the LLOQ, for which it should not be more than ±20.0%. Similarly, for accuracy assessment, the mean value should not deviate by more than ±15.0% from the nominal concentration except for the LLOQ, where it should not deviate by more than ±20.0% from the nominal concentration. To test the short- and long-term stability of extracted doxazosin, quality control samples of 1.0, 5.0 and 10 ng/mL were kept at ambient temperature (25 ± 5°C) and 4°C for 24 h and at −70°C for 90 days. After these periods, the samples were processed, analyzed and compared with the theoretical values and the samples were considered stable if the deviation from the nominal concentration was not more than ±15.0%.

Bioequivalence study design. The developed method was used to evaluate the bioequivalence of two controlled-release tablet formulations of doxazosin mesylate in healthy volunteers: Cadoxil™ controlled release 4 mg tablets (test formulation from Hyun Dai Pharmaceutical Company, Seoul, Korea; lot no. 58001) and Cardura XL™ 4 mg tablets (reference formulation from Pfizer Pharmaceutical Company, Seoul, Korea; lot no. 340005107).

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The bioequivalence protocol used was approved by the Korean Food and Drug Administration. Twenty-six healthy male volunteers, aged between 19 and 27 years, were selected for this study after clinically assessing their health status by evaluation of hematology, biochemistry and electrolytes, and urinalysis testing. The volunteers had the following clinical characteristics: age, 22.54 ± 2.52 years; height, 175.77 ± 4.81 cm; body weight, 70.15 ± 8.89 kg.

The study was based on a double-dose, randomized, two-period crossover design. During phase 1 period, the volunteers were hospitalized at 18:00 h and had a normal evening meal, and then after an overnight fast they were administered (at 08:00 h) a double dose of doxazosin (i.e. doxazosin 8 mg) with 240 mL water. Food and drinks were not allowed until 4 h after administration. Lunch and dinner were served at 4 and 10 h after administration. After a wash-out period of 7 days, the study was repeated in the same manner (phase 2) to complete the crossover design.

Heparinized blood samples (10 mL) were collected from a suitable forearm vein using an indwelling catheter into heparin-containing tubes before (0 h) and 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 24, 48, 72 and 96 h after dosing. The blood samples were centrifuged at 3000 rpm for 10 min, and plasma samples were separated and stored at −70°C until required for analysis.

Pharmacokinetic parameters including AUC_{96h} (the area under the plasma concentration vs time curve from time 0 to 96 h), C_{max} (peak plasma concentration), T_{max} (time to C_{max}), and $t_{1/2}$ (elimination half-life) were calculated using K-BE TEST 2002 supplied by the Korean Food and Drug Administration.

RESULTS AND DISCUSSION

Assay specificity, selectivity, linearity and lower limit of quantification (LLOQ)

An assay specificity was assessed by extracting samples of six different batches of blank plasma and then comparing the resulting chromatograms for plasma samples spiked with propranolol (IS) and doxazosin at the lowest (0.3 ng/mL) or the highest concentration (50 ng/mL) of the calibration standards. The chromatograms were also inspected visually for interfering chromatographic peaks caused by endogenous substances. Figure 2 shows representative chromatograms of doxazosin and propranolol. Figure 3 also demonstrates two peaks of doxazosin with a measured concentration of 17.3 ng/mL and propranolol (IS), obtained from a healthy volunteer's plasma sample at 6 h after the oral administration. There were no interferences with either doxazosin or the internal standard, propranolol, in the chromatogram. Calibration curves were prepared using eight calibration standards in the 0.3–50 ng/mL range. The slope and intercept values of three replicate calibration curves gave the equation $y = 0.0999x + 0.0071$. The calibration curves were linear over the concentration range employed with the average regression coefficient (r) of 0.9999. The LLOQ achieved was 0.3 ng/mL, which is better than the reported HPLC assays.

Table 1. Intra- and inter-day coefficient of variation and accuracy for determination of doxazosin in human plasma (*n* = 5)

Theoretical concentration (ng/mL)	RSD (%)		Accuracy (%)	
	Intraday	Inter-day	Intraday	Inter-day
0.3	5.94	6.29	112.23	116.29
1	3.37	2.14	95.59	96.31
5	1.27	1.17	94.11	94.23
10	1.98	2.46	93.71	92.30
20	1.13	2.20	94.58	93.58
50	0.84	1.42	95.75	95.10

Table 2. Extraction recoveries of doxazosin and the internal standard from human plasma

Analyte	Concentration (ng/mL)	Mean recovery ± SD (%)	*n*
Doxazosin	1	90.5 ± 2.0	6
Doxazosin	5	88.0 ± 2.9	6
Doxazosin	10	82.3 ± 7.9	6
Propranolol	500	85.9 ± 6.1	6

Precision and accuracy

The intraday precision and accuracy were determined by repeating the analysis of the spiked standards five times in a single day at the concentrations of 0.3, 1, 5, 10, 20 and 50 ng/mL (Table 1). The intraday precision values of the doxazosin level obtained as a relative standard deviation (RSD) ranged from 0.84 to 5.94%, while the accuracy values were between 93.71 and 112.23%. Precision and accuracy values were determined on five different days at six different concentration levels. At the LLOQ, accuracy was 116.29%, but the precision and accuracy values at other concentration levels did not exceed 15%.

Extraction recovery

The results obtained from the recovery study are shown in Table 2. The overall recovery of doxazosin and the internal standard were found to be 86.9 ± 4.3 and 85.9 ± 6.1% (mean ± SD), respectively. The recovery values obtained with the current method are considered to be satisfactory for a sensitive measurement of doxazosin in human plasma.

Stability

To evaluate doxazosin stability in human plasma, drug-free plasma samples were spiked at 1.0, 5.0 and 10.0 ng/mL. In the short-term stability study for 24 h, doxazosin

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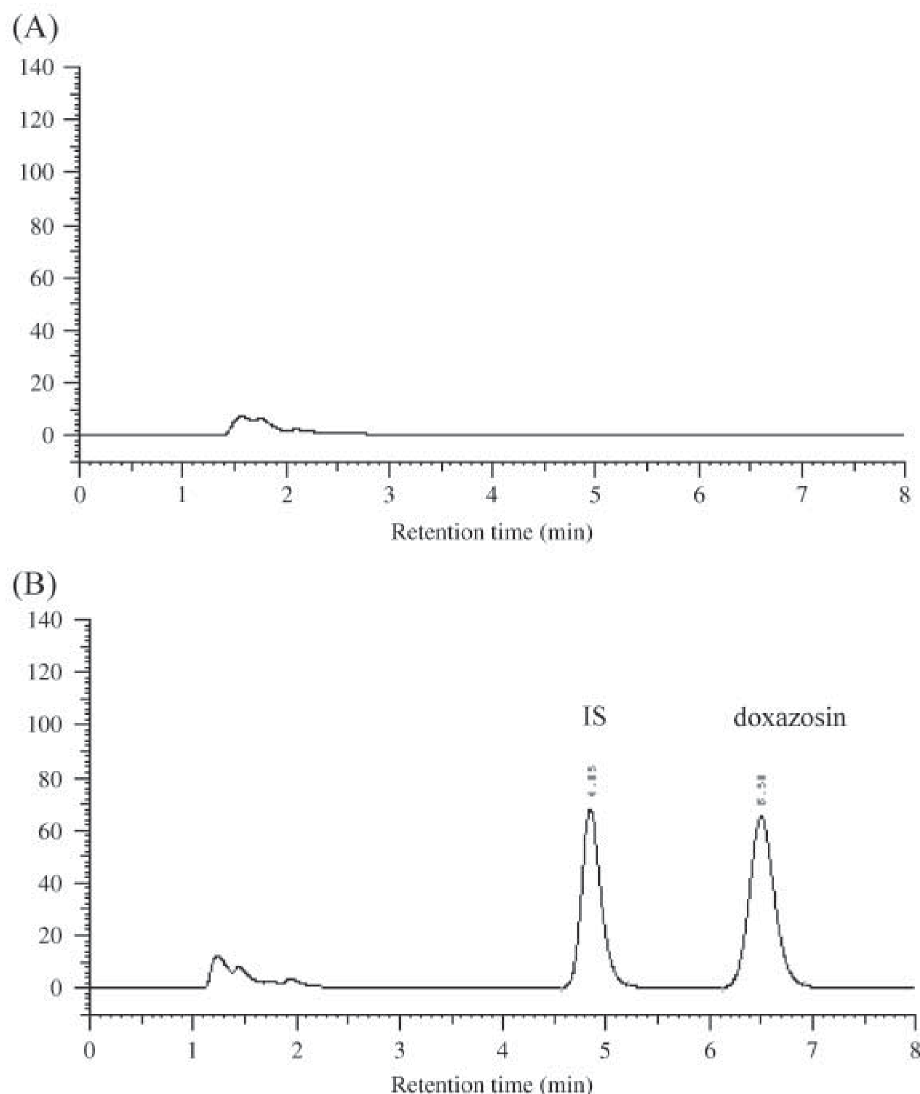

Figure 2. Representative HPLC chromatograms of blank human plasma sample (A) and blank human plasma spiked with doxazosin (10 ng/mL) and the internal standard, propranolol (500 ng/mL) (B).

and propranolol were found to be stable at 4°C and room temperature for at least 24 h (Table 3). In the long-term stablility study at −70°C, the plasma samples spiked with doxazosin and propranolol also showed no loss of analytes when they were stored for 90 days.

Application to bioequivalence study

The developed method was successfully used for the bioequivalence study involved in 26 healthy male volunteers and pharmacokinetic parameters were determined up to 96 h after the oral administration. Plasma drug concentration–time curves are shown in Fig. 4. Calculated kinetic parameters were listed in Table 4. All pharmacokinetic parameters were similar to those reported previously.

There were no significant differences for the kinetic parameters between the two formulations. Using data obtained with logarithmically transformed, the 90% confidence intervals of test to reference ratio of the $AUC_{0-96\,h}$ were within the bioequivalence range of 80–125% set by the Korean Food and Drug Administration, and those of C_{max} were also within 80–125%. Therefore, two products were concluded to be bioequivalent (Table 5).

CONCLUSIONS

We have successfully developed and validated an HPLC method with fluorescence detection for the determination of doxazosin in human plasma. The method uses liquid–liquid extraction of the drug

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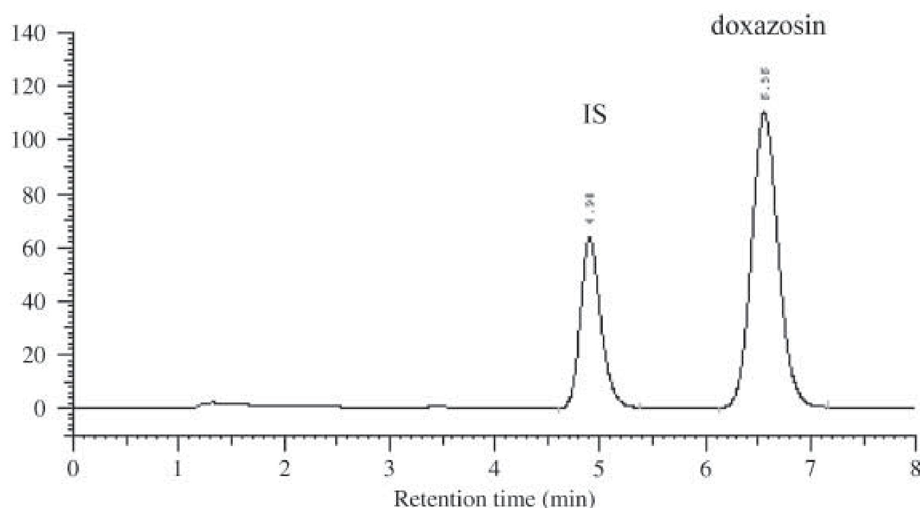

Figure 3. HPLC chromatogram of a volunteer's plasma sample taken 6 h after the oral administration of 8 mg (two tablets) of controlled release reference tablet (Cardura XL™ 4 mg). The plasma concentration of doxazosin corresponds to 17.3 ng/mL.

Table 3. Stability data for doxazosin (*n* = 3 per test)

Stability	Theoretical concentration (ng/mL)	Mean concentration found (ng/mL)	RSD (%)	Accuracy (%)
Long-term,	1	0.92	2.92	91.58
90 days, −70°C	5	4.79	0.84	95.78
	10	9.33	1.07	93.29
Short-term, 24 h,	1	1.00	1.08	99.72
room temperature	5	4.64	0.33	92.71
	10	9.29	0.48	92.90
Short-term, 24 h,	1	0.97	0.59	97.03
4°C	5	4.72	0.44	94.47
	10	9.02	0.59	90.15

Table 4. Pharmacokinetic parameters of doxazosin after the oral administration of 8 mg (two tablets) of controlled release test (Cadoxil™ 4 mg) and reference (Cardura XL™ 4 mg) formulations to healthy human volunteers (mean ± SD, *n* = 26)

Pharmacokinetic parameters	Test	Reference
T_{max} (h)	14.00 ± 4.60	14.00 ± 2.83
C_{max} (ng/mL)	23.97 ± 8.78	22.49 ± 5.30
$AUC_{0-96\,h}$ (ng h/mL)	773.84 ± 260.60	748.58 ± 217.52
$t_{1/2}$ (h)	18.27 ± 2.99	17.79 ± 3.69

from human plasma with reverse-phase chromatographic separation. This assay is considered to be accurate and reproducible and has a sensitivity of 0.3 ng/mL. Also, this method has been successfully applied in a bioequivalence study of the doxazosin-controlled release tablets. The present method may also be used as a stability-indicating method to analyze doxazosin in pharmaceutical products.

Table 5. Bioequivalence statistics

Dependent variable	Reference	Test	90% confidence limits
Doxazosin			
$\ln(AUC_{0-96\,h})$	6.57 ± 0.32	6.60 ± 0.35	92.59–113.19
$\ln(C_{max})$	3.11 ± 0.25	3.12 ± 0.36	93.04–114.38

Acknowledgments

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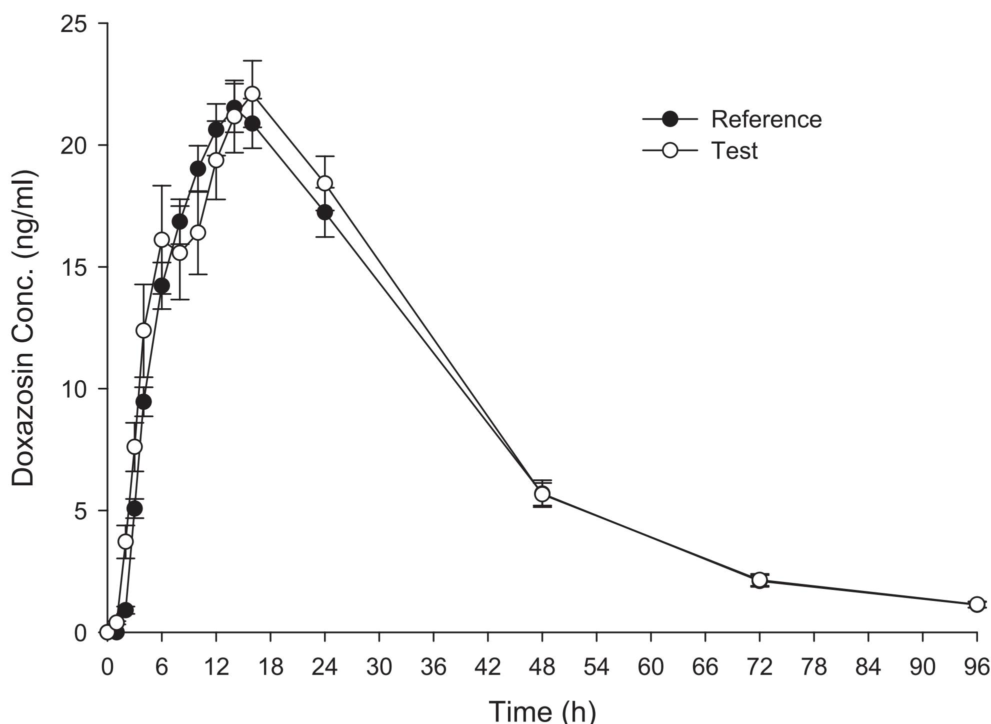

Figure 4. Plasma drug concentration–time curves of doxazosin after the oral administration of 8 mg (two tablets) of controlled release test (Cadoxil™ 4 mg) and reference (Cardura XL™ 4 mg) formulations to healthy human volunteers (mean ± SD, $n = 26$).

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