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ORIGINAL RESEARCH

Validation and pharmacokinetic application of a method for determination of doxazosin in human plasma by high-performance liquid chromatography

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ABSTRACT: A simple high-performance liquid chromatographic method for the determination of doxazosin in human plasma was developed and validated. Prazosin was used as internal standard. After extraction twice with ethyl acetate, chromatographic separation of doxazosin in human plasma was carried out using a reversed-phase Apollo C_{18} column (250 × 4.6 mm, 5 μm) with mobile phase of methanol–acetonitrile–0.04 M disodium hydrogen orthophosphate (22:22:56, v/v/v) adjusted to pH 4.9 with 0.9 M phosphoric acid and quantified by fluorescence detection operated with an excitation wavelength of 246 nm and an emission wavelength of 389 nm. The lower limit of quantification (LLOQ) of this assay was 1 ng/mL using 500 μL human plasma. Linearity was established over the range 1–25 ng/mL ($r^2 > 0.9994$). The intra- and inter-day accuracy ranged from 90.5 to 104.4% and the coefficient of variation were not more than 8.6% for both intra- and inter-day precision, over the range of the calibration curve. The absolute recoveries of doxazosin and prazosin from human plasma were more than 91%. Doxazosin demonstrated acceptable short-term, long-term and freeze–thaw stability in human plasma. The assay has been successfully applied to plasma sample analysis for pharmacokinetic study. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: doxazosin; HPLC; fluorescence detector; pharmacokinetics; quantification; validation

INTRODUCTION

Doxazosin mesylate (4-amino-2-[4-(1,4-benzodiaxan-2-carbonyl)-piperazin-1-yl]-6,7-dimethoxyquinazoline mesylate, Fig. 1) is an α_1-adrenoreceptor antagonist used for the treatment of patients with symptomatic benign prostatic hyperplasia and hypertension (Fulton *et al.*, 1995; Lepor *et al.*, 1997; Chung *et al.*, 1999). Several methods have been described for the determination of doxazosin in human plasma (Fouda *et al.*, 1988; Cowlishaw and Sharman, 1985; Jackman *et al.*, 1991). Owing to the quinazoline structure, most of published assays for doxazosin and its members, prazosin, terazosin alfuzosin and tamsulosin, have been based on a high-performance liquid chromatography (HPLC) system with fluorescence detector (Cowlishaw and Sharman, 1985; Guinebault *et al.*, 1986; Fouda *et al.*, 1988; Jackman *et al.*, 1991; Sekhar *et al.*, 1998; Cheah *et al.*, 2000; Macek *et al.*, 2004). Although the method using special stationary phase, alumina-based column and glass-bead guard column with high-pH mobile phase was reported (Fouda *et al.*, 1988), such columns may not be available in most laboratories and the mobile phase may deteriorate the columns and HPLC instrument. For separation of the doxazosin from plasma, the use of solid-phase extraction has also been reported (Jackman *et al.*, 1991), but the method was too expensive and lengthy compared with the liquid–liquid extraction procedure (Cowlishaw and

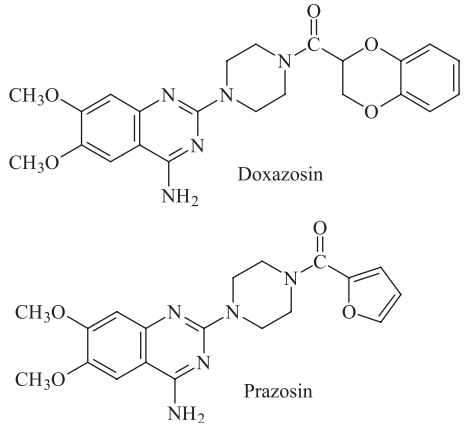

Figure 1. Chemical structures of doxazosin and prazosin.

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Sharman, 1985). The quantification limit of doxazosin was found to be higher than 1 ng/mL (Cowlishaw and Sharman, 1985; Jackman *et al.*, 1991).

The plasma protein precipitation technique for determining doxazosin (Sripalakit *et al.*, 2005) cannot be applied to the pharmacokinetic study, since it might be the protein–drug binding characteristic differences between the fresh plasma directly collected from volunteers and the thawed pooled plasma. The aim of this study was to develop a simple and inexpensive liquid–liquid extraction method for quantification of doxazosin in human plasma using reversed-phase HPLC with fluorescence detector. This assay method was validated to provide enough selectivity, sensitivity and stability for pharmacokinetic study according to the bioanalytical guidelines (Thailand FDA, 2001; US FDA, 2001).

EXPERIMENTAL

Chemicals and reagents

The reference standards, doxazosin mesylate (99.5%) and prazosin hydrochloride (>99%) as internal standard were obtained from the United States Pharmacopeial Convention, Inc. (Rockville, MD, USA). Analytical-grade disodium hydrogen orthophosphate was purchased from Fisher Chemicals (Fairlawn, NJ, USA). All solvents for analysis were of HPLC-grade and obtained from various sources, and the other chemicals were analytical grade.

Instrumentations and conditions

The HPLC system comprised a pump (LC-10ATVP, Shimadzu, Kyoto, Japan), a fluorescence detector (RF-10AXL, Shimadzu) equipped with system controller (SCL-10AVP, Shimadzu) and a Rheodyne (7725) sample injector (Rohnert Park, CA, USA) fitted with a 100 µL sample loop. The separation was performed on an Apollo C_{18} (250 × 4.6 mm i.d., 5 µm, 250 Å) column (Alltech, Deerfield, IL, USA) protected with a refillable guard cartridge packed with Apollo C_{18} (7.5 × 4.6 mm i.d., 5 µm, Alltech). The column was maintained at room temperature. The mobile phase was composed of methanol–acetonitrile–0.04 M disodium hydrogen orthophosphate (22:22:56 v/v/v) adjusted to pH 4.9 with 0.9 M phosphoric acid, which was isocratically pumped at a flow rate of 1.2 mL/min. The detector was operated at 246 nm, wavelength of excitation, and 389 nm, wavelength of emission.

Standard solutions

Stock solutions of doxazosin mesylate and the internal standard, prazosin hydrochloride, were prepared separately in methanol to yield primary standard solutions with a concentration of 1 mg/mL as the base. Secondary standard solutions were prepared by dilution with methanol. Working standard solutions of doxazosin in plasma at 25, 20, 10, 5, 2 and 1 ng/mL were prepared by dilution of the secondary standard solution with drug-free human plasma. Working internal standard solution giving a concentration of 50 ng/mL was prepared in methanol. The quality control samples at concentrations of 1 (low quality control, LQC sample) and 25 (high quality control, HQC sample) ng/mL were also prepared for the stability tests.

Sample preparation

A 500 µL volume of various concentrations of working doxazosin solution in plasma was accurately transferred to a 10 mL screw-capped test tube, followed by addition of 50 µL of 50 ng/mL prazosin internal standard solution, and 200 µL of 1 M NaOH. After vigorously mixing for 5 s, 2 mL ethyl acetate was added. The mixture was stepwise vortex-mixed for 20 s and was then centrifuged for 5 min at 700***g***. The organic layer was transferred to a conical glass tube. To ensure complete extraction, the residue was repeatedly extracted with 1 mL of ethyl acetate and the sample was then vortex-mixed and centrifuged in the same conditions as above. The collections of organic layer were combined and evaporated to dryness using speed evaporator for 2 h at 45°C. Then, the dried extract was reconstituted in 400 µL of mobile phase, filtered through a 0.45 µm nylon disposable filter (Alltech) and a 100 µL aliquot was injected into chromatographic system. Samples were quantified using the peak area ratio of doxazosin over the internal standard.

Bioanalytical method validation

Calibration curve. A calibration curve was constructed from working standard solutions of doxazosin at concentrations range 1–25 ng/mL, including lower limit of quantification (LLOQ). The ratios of the peak area of doxazosin to the peak area of prazosin were plotted against doxazosin concentrations. Linearity was assessed by a weighted least-squares regression analysis. The calibration curve requires a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration should be within 15% deviation of standards other than LLOQ from the nominal value (Thailand FDA, 2001; US FDA, 2001).

Accuracy and precision. To assess the accuracy and precision of the method, intra- and inter-day (five consecutive days) measurements of doxazosin were determined by analyzing six and 30 (six sets each consecutive day) sets, respectively, of each working standard plasma sample. For the acceptance criteria of inter- and intra-day accuracy and precision, accuracy values should be within 85–115% over the calibration range, except at the LLOQ, where it should be between 80 and 120%, and coefficient of variation (CV) values should be <15% over the calibration range, except at the LLOQ, where it should not exceed 20% (Thailand FDA, 2001; US FDA, 2001).

Recovery. The recoveries of doxazosin and prazosin in the extraction procedure were determined by comparing the peak area obtained from six extracted sample spiked with known amounts of doxazosin and prazosin with those obtained from

the pure compounds of the same concentrations in the mobile phase. The recoveries were determined at 1, 2, 5, 10, 20 and 25 ng/mL for doxazosin and 50 ng/mL for prazosin. The acceptance criterion of recovery should be consistent, precise and reproducible with near 100% or not lower than 50–60% (US FDA, 2001; Thailand FDA, 2001).

Stability. Analyte stability was tested using low and high quality control samples for three freeze–thaws, short-term, long-term and post-preparative stabilities. After completion of the storage time for stability testing, the samples (n = 3) were analyzed and the results compared with that of freshly prepared samples. For the acceptance criteria of stabilities, the deviation compared with the freshly prepared standard should be within ±15% (Thailand FDA, 2001; US FDA, 2001).

Pharmacokinetic application

A single 2 mg dose of doxazosin (Dozozin-2®, Umeda Co. Ltd, Bangkok, Thailand) was administered orally to 24 healthy male volunteers after an overnight fast. Eight milliliter blood samples were collected into lithium heparin-coated plastic tubes by catheterized venipuncture of the subjects' forearm 0.5 h before dosing and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 18, 24, 48 and 72 h after the administration. The blood sample tubes were centrifuged at 1000***g*** for 15 min at 4°C. The plasma samples were separated and kept in the cryovial tube at –70°C until drug analysis. The study was approved by Naresuan University Ethical Committee. The pharmacokinetic parameters were determined by WinNonlin Professional version 4.0.1 (Pharsight Corporation, Mountain View, CA, USA).

RESULTS AND DISCUSSION

Chromatograms and specificity

The specificity/selectivity of the method was investigated by analyzing blank human plasma extract and an extract of the plasma spiked with 25 ng/mL of doxazosin and 50 ng/mL of internal standard. As shown in Fig. 2, no significant interfering peaks from endogenous substances in the blank plasma were seen in drug-free human plasma at the retention time of the analytes. The retention times of prazosin and doxazosin were 4.4 and 13.4 min, respectively, and the total run time for each sample was 16 min. The stationary phase could be equilibrated with mobile phase for 30 min before analyzing the samples. For long-term column efficiency and reproducibility of drug peaks, the overnight cleansing of the column after everyday use with methanol should be performed. The extracting solvent, ethyl acetate, was safer and easier than the composition of dichloromethane and diethyl ether used in the previous report (Cowlishaw and Sharman, 1985). After reconstitution with mobile phase, a high-viscosity liquid was found from the plasma extracted with dichloromethane and diethyl ether, which seemed to be difficult to inject into the HPLC instrument. Furthermore, silanizing all glasswares before use (Cowlishaw and Sharman, 1985) was not necessary in this study. The advantage of this alternative liquid–liquid extraction method was that it was economical and faster than using solid phase extraction (Jackman *et al.*, 1991).

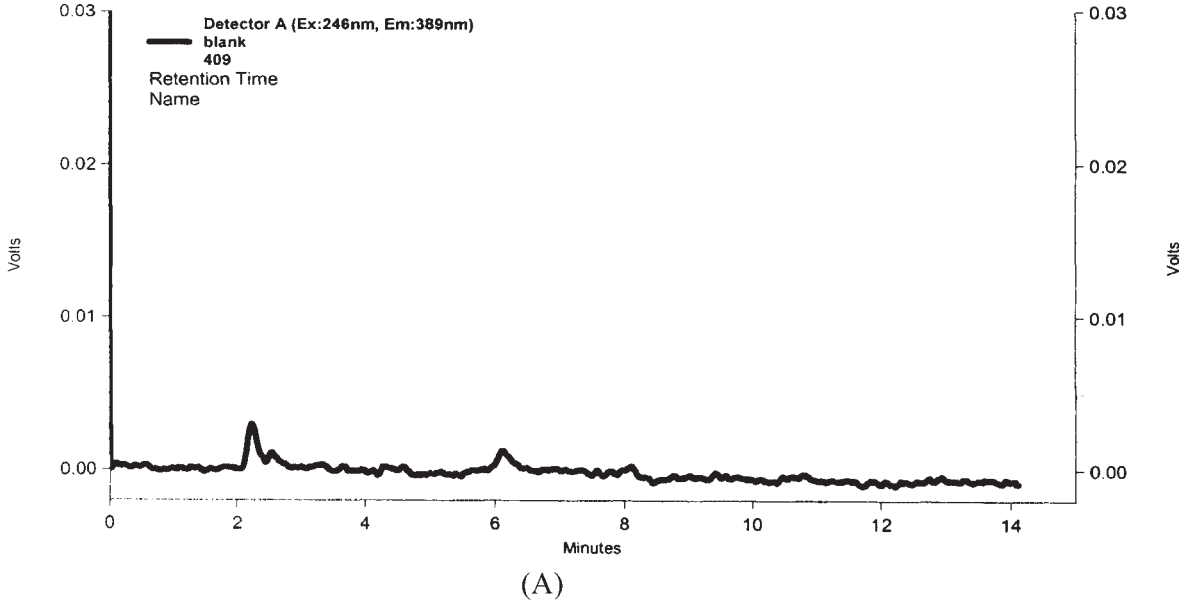
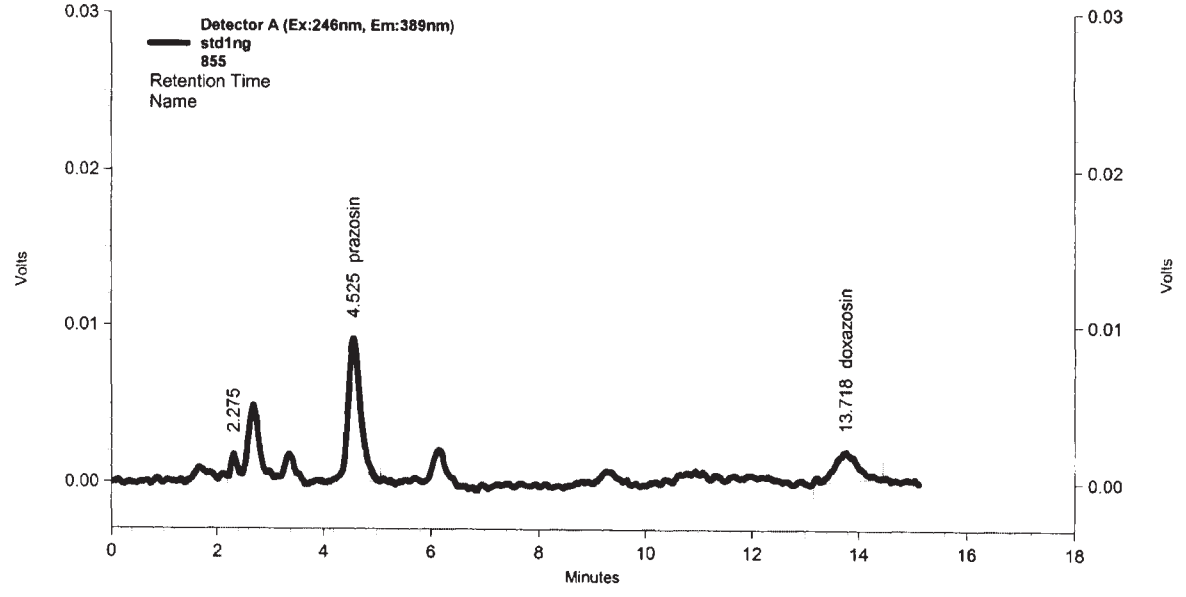
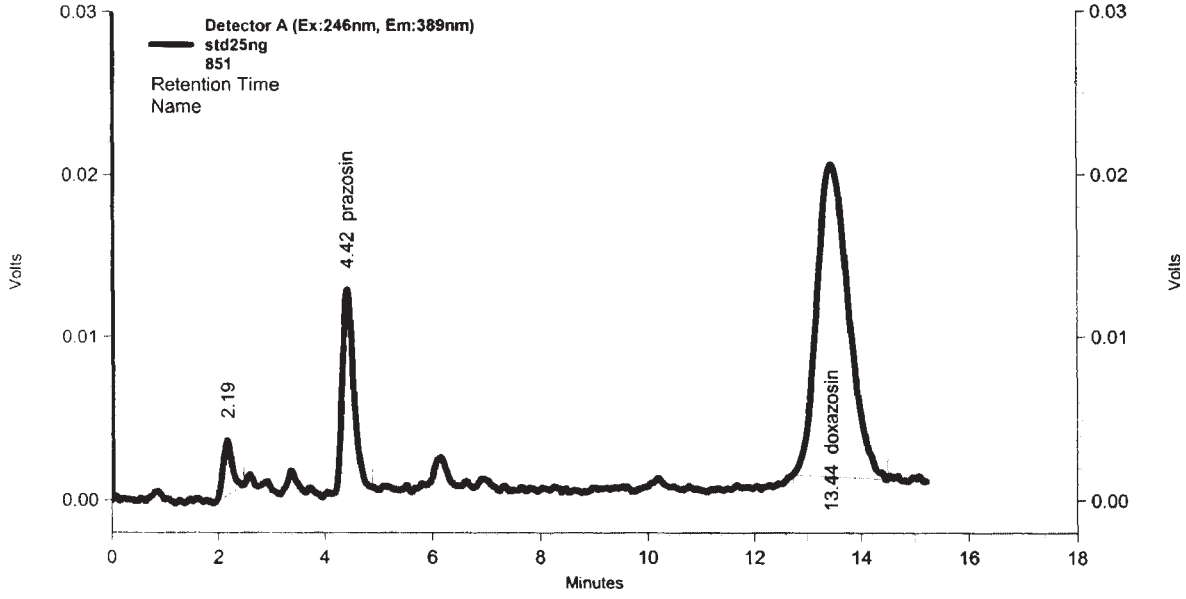


Figure 2. Chromatograms for the analysis of doxazosin in drug-free human plasma. (A) Blank plasma. (B) Human plasma spiked with 50 ng/mL prazosin and 1 ng/mL doxazosin (LOQ). (C) Human plasma spiked with 50 ng/mL prazosin and 25 ng/mL doxazosin.



(B)



(C)

Figure 2. (*continued*)

Linearity and limit of quantification

Calibration curves were obtained by plotting the peak area ratio of doxazosin to that of prazosin vs plasma doxazosin concentrations. The standard calibration curve was linear in the concentration range 1–25 ng/mL, with correlation coefficient (r^2) ≥0.9994, slope 0.1742 and intercept 0.1759. It was found that, below 1 ng/mL, the back-calculated values failed to meet the acceptance criteria. Based on a signal-to-noise ratio, the lower limit of quantification (LLOQ) was 1 ng/mL (at least five times the peak height response compared to blank response) with 9% or better coefficient of variation and 90% or better accuracy. The lower limit of detection (LOD) was 0.5 ng/mL. Although doxazosin molecule was highly sensitive to fluorescence detection and the limitation of the instrument program, the obtained peak response value was incorrect at the concentration of 30 ng/mL or higher.

Accuracy and precision

The accuracy and precision for the intra- and inter-day assay of doxazosin were determined at concentration of

Table 1. Accuracy and precision of the method for determining doxazosin concentration in plasma samples

Targeted concentration (ng/mL)	Measured concentration (mean ± SD; ng/mL)	Accuracy (%)	Precision
Intra-day (n = 6)			
1	0.90 ± 0.07	90.5	8.2
2	2.05 ± 0.11	102.3	5.2
5	5.17 ± 0.23	103.4	4.4
10	9.31 ± 0.69	93.1	7.5
20	18.96 ± 0.64	94.8	3.4
25	25.08 ± 0.45	100.3	1.8
Inter-day (n = 30)			
1	0.94 ± 0.08	94.3	8.1
2	2.09 ± 0.12	104.4	5.7
5	5.02 ± 0.31	100.4	6.2
10	9.66 ± 0.83	96.6	8.6
20	19.23 ± 1.05	96.1	5.5
25	24.93 ± 1.63	99.7	6.5

1–25 ng/mL (Table 1). Accuracy was the percentage of the concentration found compared with the theoretical concentration. Precision was based on calculation of the CV. The inter-day assay was calculated using data accumulated over a period of 5 days. To improve the accuracy and precision of this method, plasma samples were added with 1 M NaOH and extracted twice with ethyl acetate, which is different from the previous study (Fouda *et al.*, 1988) using single liquid–liquid extraction without buffer being added. The accuracy values were between 90.5 and 104.4% both intra- and inter-day. The CV ranged from 1.8 to 8.2% for intra-day precision and from 5.5 to 8.6% for inter-day precision.

Recovery

The percentage of doxazosin recovered from plasma was calculated by comparison of the drug peak area in the extracted plasma samples (n = 6) with the mean peak area obtained from direct injection of the corresponding unextracted standard solutions. The recovery was measured at six different concentrations (1, 2, 5, 10, 20 and 25 ng/mL) over the calibration range used. Regarding the internal standard, recovery was only calculated at the working concentration (50 ng/mL). Table 2 shows the recovery, expressed as percentage, obtained form both doxazosin and internal standard. Regardless of the drug concentration, the absolute recovery found ranged from 91.4 to 94.8% with a CV between 2.1 and 12.0%. The CV value of recovery was highest at the concentration of 1 ng/mL. For internal standard, a recovery of 92.6% was obtained. In addition, single extraction of plasma samples with ethyl acetate gave 60% or below recovery in some concentrations.

Table 2. Absolute recovery of the method for determining doxazosin concentration in plasma samples (n = 6)

Concentration (ng/mL)		Absolute recovery (mean ± SD; %)	CV (%)
Doxazosin	1	92.9 ± 11.2	12.0
	2	94.8 ± 10.5	11.1
	5	92.8 ± 2.0	2.1
	10	92.4 ± 7.6	8.2
	20	91.5 ± 6.3	6.9
	25	91.4 ± 5.4	5.9
Prazosin	50	92.6 ± 6.4	6.9

Stability

The stability tests of the analyte were designed to cover anticipated conditions that clinical samples may experience. Plasma samples at two doxazosin concentrations (1 and 25 ng/mL) were used for stability experiments. Stability data were summarized in Table 3. The deviation of the mean test responses was within ±15% of appropriate controls in all stability tests of doxazosin in human plasma. Three freeze–thaw cycles and ambient temperature storage up to 6 h prior to sample analysis of the quality control samples appeared to have no effect on the quantification of analyte. Quality control samples stored in a freezer at −70°C remained stable for at least 2 months. For short-term stability for the quality control samples kept in the freezer for 12 h, no effect on quantification was observed.

Pharmacokinetic application

Doxazosin was reported to bind well to plasma proteins (98.3%) [Cardura®, (doxazosin mesylate),

Table 3. Stability of doxazosin in human plasma (n = 3)

	Concentration (ng/mL; mean ± SD)	
Stability	1[a]	25[b]
Three Freeze–thaw cycle stability (–70°C)		
Initial	0.87 ± 0.20	26.54 ± 1.92
Measured	0.98 ± 0.20	24.02 ± 1.08
Deviation (%)	+13.4	–9.5
Long-term stability (2 months at –70°C)		
Initial	0.92 ± 0.05	25.73 ± 1.20
Measured	0.80 ± 0.03	22.14 ± 1.70
Deviation (%)	–13.1	–14.0
Short-term stability (12 h at –70°C)		
Initial	0.93 ± 0.31	25.73 ± 1.20
Measured	0.81 ± 0.11	26.54 ± 1.92
Deviation (%)	–13.1	+3.1
Post-preparative stability (6 h at room temperature)		
Initial	0.98 ± 0.10	25.56 ± 1.10
Measured	1.01 ± 0.08	24.75 ± 0.25
Deviation (%)	+2.3	–3.2

[a] Low quality control samples.
[b] High quality control samples.

full US prescribing information from Pfizer Inc., New York, April 2002; Elliott *et al.*, 2004]. In pharmacokinetic study, the precipitation of plasma protein with methanol could not be applied to determine doxazosin in human plasma from volunteers, because a higher concentration of doxazosin was found in plasma protein precipitate compared with the supernatant sample. The binding interactions between doxazosin and plasma proteins in human body were higher than their interactions in drug-spiked plasma sample.

The developed liquid–liquid extraction method has been successfully used to quantify the doxazosin concentration in the human plasma samples after the administration of a 2 mg doxazosin tablet. The mean plasma concentration vs time profile of 24 subjects receiving a single dose of doxazosin is presented in Fig. 3. The observed mean C_{max} (maximum concentration) of doxazosin in these volunteers was 18.39 ± 5.16 ng/mL at T_{max} (time required to reach the maximum concentration) of 2.1 ± 1.2 h. The other pharmacokinetic parameters are shown in Table 4.

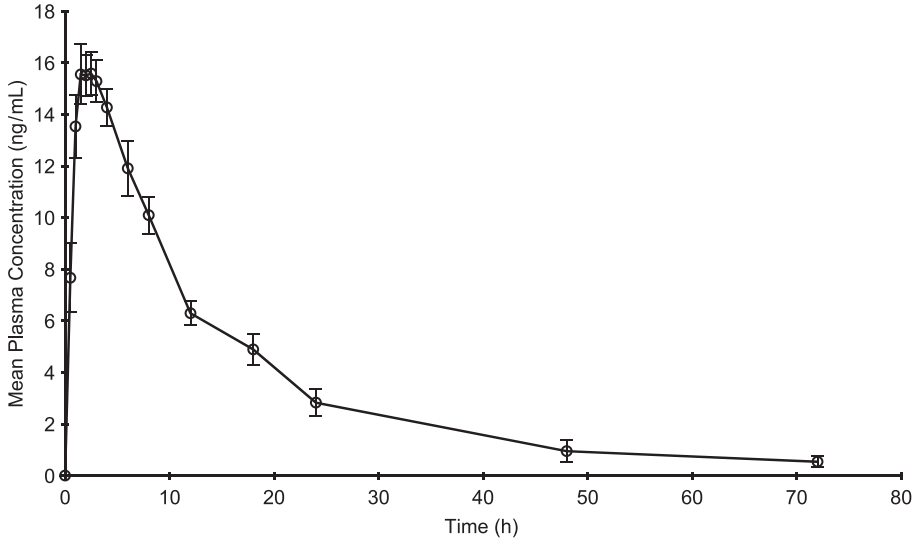


Figure 3. Mean plasma concentration-time curve over 72 h of doxazosin in human plasma from 24 volunteers receiving a single 2 mg oral dose of doxazosin tablet. Each point represents the mean ± SE (n = 24).

Table 4. The pharmacokinetic parameters of doxazosin in 24 volunteers after oral administration of a 2-mg doxazosin tablet

Parameters	Mean ± SD
C_{max} (ng/mL)	18.39 ± 5.16
T_{max} (h)	2.1 ± 1.2
AUC_t (ng h/mL)	229.49 ± 116.61
AUC_∞ (ng h/mL)	311.48 ± 186.95
$T_{1/2}$ (h)	16.7 ± 9.1

CONCLUSION

A high-performance liquid chromatographic method for determination of doxazosin in human plasma has been developed and validated. It has been shown to be accurate, precise and sensitive. There was no evidence of instability of doxazosin in human plasma under various stability testing conditions. The method has certain advantages over the previous reported method and was successfully used to analyze doxazosin concentrations in human plasma samples from a pharmacokinetic study.

Acknowledgements

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REFERENCES

Cheah PY, Yuen KH and Liong ML. Improved high-performace liquid chromatographic analysis of terazosin in human plasma. *Journal of Chromatography B* 2000; **745**: 439.

Chung M, Vashi V, Puente J, Sweeney M and Meredith P. Clinical pharmacokinetics of doxazosin in a controlled-release gastrointestinal therapeutic system (GITS) formulation. *British Journal of Clinical Pharmacology* 1999; **48**, 678.

Cowlishaw MG and Sharman JR. Doxazosin determination by high-performance liquid chromatography using fluorescence detection. *Journal of Chromatography* 1985; **344**: 403.

Elliott HL, Meredith PA and Reid JL. Pharmacokinetic overview of doxazoisn. *American Journal of Cardiology* 2004; **59**: 78.

Fouda HG, Twomey TM and Schneider RP. Liquid chromatography analysis of doxazosin in human serum with manual and robotic sample preparation. *Journal of Chromatographic Science* 1988; **26**: 570.

Fulton B, Wagstaff AJ and Sorkin EM. Doxazosin: an update of its clinical pharmacology and therapeutic applications in hypertension and benign prostatic hyperplasia. *Drugs* 1995; **49**: 295.

Guinebault P, Broquaire M, Colafranceschi C and Thenot JP. High-performance liquid chromatographic determination of alfuzosin in biological fluids with fluorimetric detection and large-volume injection. *Journal of Chromatography A* 1986; **353**: 361.

Jackman GP, Colagrande F and Louis WJ. Validation of a solid-phase extraction high-performance liquid chromatographic assay for doxazosin. *Journal of Chromatography* 1991; **566**: 234.

Lepor H, Kaplan SA and Klimberg I. (1997). Doxazosin for benign prostatic hyperplasia: long-term efficacy and safety in hypertensive and normotensive patients: The multicenter study group. *Journal of Urology* 1997; **157**: 525.

Macek J, Klima J and Ptacek P. Rapid determination of tamsulosin in human plasma by high-performance liquid chromatography using extraction with butyl acetate. *Journal of Chromatography B* 2004; **809**: 307.

Sekhar EC, Rao TRK, Sekhar KR, Naidu MUR, Shobha JC, Rani PU, Kumar TV and Kumar VP. Determination of terazosin in human plasma, using high-performace liquid chromatography with fluorescence detection. *Journal of Chromatography B* 1998; **710**: 137.

Sripalakit P, Nemhorm P and Saraphanchotiwitthaya A. Improvement of doxazosin determination in human plasma by using high-performance liquid chromatography with fluorescence detector. *Journal of Chromatographic Science* 2005; **43**: 63.

Thailand FDA. *Guidelines for the Conduct of Bioavailability and Bioequivalence Studies.* Drug Control Division, Ministry of Public Health: Bangkok, 2001.

US FDA. *Guidance for Industry: Bioanalytical Method Validation.* Center for Drug Evaluation and Research: Rockville, MD, 2001.

