Simple Method of Extraction and Determination of Doxazosin Mesylate in Human Plasma by High – Performance Liquid Chromatography with Fluorescence Detector

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ABSTRACT

A simple, selective, sensitive and precise reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been developed for the determination of doxazosin mesylate in human plasma. Diazepam was used as an internal standard. The simple extraction method utilized protein precipitation with acetonitrile for sample preparation. Good chromatographic separation was achieved by using ODS hypersil (5 µm, 125 x 4.0 mm) column and a mobile phase consisting of acetonitrile : 10 mM ammonium acetate (40:60) at a flow rate of 1.0 ml/min. Doxazosin mesylate and diazepam were detected with fluorescence detector at Ex = 246 nm, Em = 376 nm. No endogenous substances were found to interfere. Linearity range for doxazosin mesylate was 1.0–50.0 ng/ml. The coefficient of variation (%CV) for intra – day and inter – day precision were less than 4.0 and 5.9 % respectively , at all concentration levels while the intra–day and inter – day accuracy ranged from 97.47–110.67 % at all concentration levels. This analysis method was successfully used in pharmacokinetic and bioequivalence study of doxazosin mesylate in healthy volunteers.

Key words: Doxazosin mesylate, Pharmacokinetics, Plasma analysis, HPLC, Determination

INTRODUCTION

Doxazosin mesylate is a quinazoline-derivative postsynaptic α_1-adrenergic blocking agent.The drug is chemically and pharmacologically related to prazosin and terazosin. On a weight basis, the postsynaptic α_1-adrenergic blocking potency of doxazosin mesylate is half of that of prazosin and the α_1-receptor selectivity is one-fourth of that of terazosin when tested in human postate adenoma.

Doxazosin mesylate reduces peripheral vascular resistance and blood pressure as a result of its vasodilating effects, the drug produces both arterial and venous dilation. Doxazosin mesylate reduces blood pressure in both supine and standing patients, the effect is most pronounced on standing blood pressure and postural

hypotension can occur. Doxazosin mesylate generally causes a change in heart rate of cardiac output in the supine position. Cardiovascular responses to exercise (e.g., increased heart rate and cardiac output) are maintained during doxazosin mesylate therapy.

Effects of doxazosin mesylate on the cardiovascular system are mediated by the drug' s activity at α_1-receptor sites on vascular smooth muscle. α_1-adrenergic receptors also are located in nonvascular smooth muscle (e.g., bladder trigone and sphincters, GI tract and sphincter, prostate adenoma and capsule, ureters, uterus) and in nonmuscular tissues (e.g., CNS, liver, kidneys). Because of the prevalence of α_1- receptors on the prostate capsule, prostate adenoma and the bladder trigone and the relative absence of these receptors on the bladder body, α_1-blocker decreases urinary outflow resistance in men.

Doxazosin mesylate may improve, to a limited extent, the serum lipid profile (e.g., small increases in high-density lipoprotein cholesterol concentrations [HDL] and HDL/total cholesterol ratio, small decreases in low-density lipoprotein cholesterol [LDL], total cholesterol and triglyceride concentrations and can reduce blood glucose and serum insulin concentrations. The drug does not appear to affect plasma renin activity appreciably (McEvoy, 2005).

The chemical name of doxazosin mesylate is 1-(4-amino-6,7-dimethyoxy-2-quinozolinyl)-4-(1,4-benzodioxan-2-ylcarbonyl) piperazine methanesulfonate.The molecular formula for doxazosin mesylate is $C_{23}H_{25}N_5O_5.CH4O3S$ and the molecular weight is 547.6. Itís chemical structure is presented in Figure 1(O' Neil , 2001).

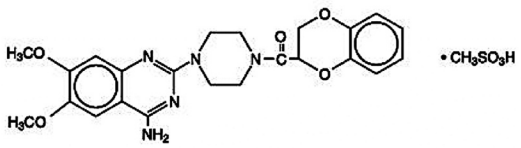

Figure 1. Chemical structure of doxazosin mesylate.

High–performance liquid chromatography has been developed for the determination of doxazosin mesylate in biological fluid by fluorescence detector (Fouda et al., 1988) in which doxazosin mesylate in human plasma was extracted by liquid – liquid extraction with ethyl acetate and prazosin was used as an internal standard. Jackman and his coresearchers used solid-phase extraction to separate doxazosin from plasma samples and Zorbax CN column was used as stationary phase (Jackman et al.,1991). Both methods were of good chromatographic conditions for determination of doxazosin in human plasma but sample preparation was complicated, time-comsuming and costly.

Thus, the purpose of the present study was to develop a simple HPLC analysis method for doxazosin mesylate in human plasma with high specificity / selectivity, sensitivity, accuracy, precision and reproducibility.

MATERIALS AND METHODS

Chemicals and reagents Standard doxazosin mesylate was obtained from Euresian. Diazepam was supplied by Shandong pingyuan pharmaceutical factory. HPLC-grade methanol (Fisher, United Kingdom), HPLC-grade acetonitrile (Merck, Dormstadt Germany), analytical-grade ammonium acetate (Merck, Dormstadt Germany) were used. Deionized water from a Milli Q apparatus was used in this study. Plasma was purchased from a local blood bank to generate a drug-free plasma pool.

Apparatus and chromatographic conditions The HPLC system consisted of a model LC–10ATvp pump (Shimadzu, Kyoto, Japan), a model DGU-14A degasser (Shimadzu, Kyoto, Japan) and a model SIL–10ADvp auto injector (Shimadzu, Kyoto, Japan). Seperation was achieved on ODS hypersil (5 µm) cartridge column (125 x 4.0 mm I.D) (Agilent Tecnologies, U.S.A) and detected with fluorescence detector model RF-10Axl (Shimadzu, Kyoto, Japan) at Ex = 246 nm, Em = 376 nm.

The part of extraction consisted of a model 2601 multi-tube vortexer (Scientific Manufacturing Industries, U.S.A), abbott centrifuge (Abbott Laboratories, Germany).

The mobile phase consisted of acetonitrile–ammonium acetate (10 mM) (40 : 60). It was filtered with 0.25 µm membrane filter (Sartorius, Germany) before use. Chromatography was performed at ambient temperature. Flow rate was 1.0 ml/min and injection volume was 50 µl.

Stock and standard solution Stock solution of doxazosin mesylate was prepared by dissolving 12.5 mg in 25 ml methanol to yield a final concentration of 0.5 mg/ml and stored at 4°C until analysis. This stock solution was used to prepare a set of working standard by diluting in methanol . A 25 µl of each concentration of working standard was pipetted into 225 µl of drug-free human plasma to yield concentration of 1, 5, 10, 20, 40 and 50 ng/ml . In the same manner, plasma quality controls (QC) which stock solution of doxazosin mesylate QC sample was seperately prepared at concentration of 0.5 mg/ml. Three concentrations of QC sample (3, 15 and 30 ng/ml) were prepared in plasma and included in every analytical run. Internal standard solution (diazepam) was prepared in acetonitrile. A 25 µl of internal standard working solution (1000 µg/ml) was added to a 250 µl of drug-free human plasma to yield concentration of 100 µg/ml . Plasma standards were extracted by the process mentioned above before injecting into the HPLC system.

Analytical procedure A 0.25 ml aliquot of the spiked plasma or human plasma sample was pipetted into a microcentrifuge-tube and 25 µl of 1000 µg/ml diazepam (internal standard) was mixed. A 500 µl of acetonitrile was added into plasma sample. The samples were closed with a cap. After that the contents were vortex-mixed for 15 minutes and centrifuged at 10900 rpm for 15 minutes. A 750 µl of 10 mM ammonium acetate was added into plasma sample and centrifuged at 10900 rpm for 10 minutes. Then supernatant was collected into clean vial and 50 µl was injected onto the HPLC column.

Validation of the analysis method, including specificity/selectivity, extraction recovery, accuracy and precision, sensitivity and stability of the sample was performed accordingly to the Guidance for Industry: Bioanalytical Method Validation (Guidance of Industry, 2001).

Specificity and selectivity Peaks of drug and internal standard were separated from other interfering peaks in blank plasma by comparing the chromatograms of the following samples:

1. blank plasma of 6 normal volunteers
2. standard solution of doxazosin mesylate and the internal standard (diazepam)
3. blank plasma spiked with doxazosin mesylate and the internal standard

Recovery (extraction efficiency) Extraction recoveries from human plasma were determined by comparison of HPLC responses (peak area) from extracted sample (quality control sample) and internal standard, containing known amounts (3, 15 and 30 ng/ml respectively), to those from unextracted and directly-injected standard, spiked with the same amount.

Accuracy and Precision Accuracy and precision of the method were determined by five replicated analysis of known doxazosin mesylate concentration over the calibration curve. Inter-day (between-run) and intra-day (within-run) accuracy were expressed as percentage from spiked concentration, following equation:[(observed concentration-spiked concentration)/spiked concentration] x 100, where C_{obs} is the observed concentration for each standard, and C_{spike} is the spiked theoretical concentration. Intra-day (within-run precision) was studied by analyzing 5 sets of plasma spiked with doxazosin mesylate on the same day. Inter-day (between-run precision) was determined by quantifying the observed concentrations of quality control (QC) sample at three concentration levels, i.e., 3 ng/ml (low conc.), 15 ng/ml (medium conc.) and 30 ng/ml (high conc.) on 5 different days along with a daily-prepared standard curve. Inter-and intra-day precision of the method was expressed as the coefficient of variation (C.V.) of the mean peak ratio for each standard or QC sample.

Calibration curve Calibration curves were constructed in the range of 1.0 -50.0 ng/ml. Curves were obtained daily for 5 days by plotting between the peak area ratio and concentrations of standards.

Stability To determine the influence of different time on the stability of drug, doxazosin mesylate was spiked to blank plasma at the concentration of 3, 15 and 30 ng/ml, each portion of spiked plasma was repeatedly analyzed five times for doxazosin mesylate concentration (n=5) under different storage conditions at-40°C. The first portion (initial concentration) was immediately extracted and analyzed as mentioned above, another portion was extracted and analyzed at time described under the following stability studies. The stability of doxazosin mesylate was expressed as observed concentration of doxazosin mesylate comparing with initial concentration (t=0). This study will be referred to in detail as follow:

Freeze-thaw stability and long-term stability The study of freeze-thaw stability was extracted and analyzed after 3 cycles of freeze-and-thaw study and long-term stability was extracted and analyzed after being stored for 14 days. After these, comparison with the concentrations determined on the initial concentration was made.

Bench top stability Preparation of sample was done as mentioned above which extracted and analyzed after sitting on a laboratory bench at room temperature for 5 hours. These were compared with the concentrations determined on the first time.

Autosampler stability Preparation of sample was done as mentioned above which extracted and analyzed for doxazosin mesylate concentrations immediately and were repeated after 24 hours. Auto sampler stability of doxazosin mesylate was expressed as observed concentration of doxazosin mesylate comparing with initial concentration (t = 0).

RESULTS AND DISCUSSION

The simple extraction method, utilizing protein precipitation with acetonitrile, was used for sample preparation.The chromatographic seperation was achieved by reversed–phase column, and mobile phase used was acetonitrile: 10 mM ammonium acetate (40:60) at flow rate of 1.0 ml/min. The optimum wavelength was detected with fluorescence detector at Ex = 246 nm, Em = 376 nm which had much better detector response.

No interfering peak was observed at the retention times of both doxazosin mesylate and the internal standard. The retention times of either doxazosin mesylate or the internal standard were approximately the same in all chromatograms (Figure 2).

The method was validated with regard to linearity, limit of detection and qualitation, recovery, precision, accuracy and specificity.

The lowest standard on the calibration curve should be accepted as the limit of quantification(LLOQ) when the response at LLOQ is at least 5 times greater than that of blank response and the analyte peak (response) should be identifiable, discrete and reproducible with high precision (% CV $\leq$ 20 and accuracy of 80 - 120%). The lowest concentration on the standard curve with acceptable accuracy, precision and variability was 1 ng/ml with the coefficient of variation (CV) of 5.94 % and the percent accuracy of observed plasma doxazosin mesylate concentration (n=5) from the spiked concentration was 101.0 %. Therefore, this concentration was accepted as the lower limit of quantification.

Peak area ratios of doxazosin mesylate of calibration standards were proportional to the plasma concentration of doxazosin mesylate in the range of 1.0-50 ng/ml.Each concentration was tested 5 times. The coefficient of correlation(r^2) , slope and intercept of linear regression line are reported in Table 1.

Table 1. Statistical data (n=5) for linearity of doxazosin mesylate.

Compound	r^2	slope (mean ± S.D)	intercept (mean ± S.D)
doxazosin mesylate	0.9998 ± 0.00007	0.0599 ± 0.00175	0.1963 ± 0.02396

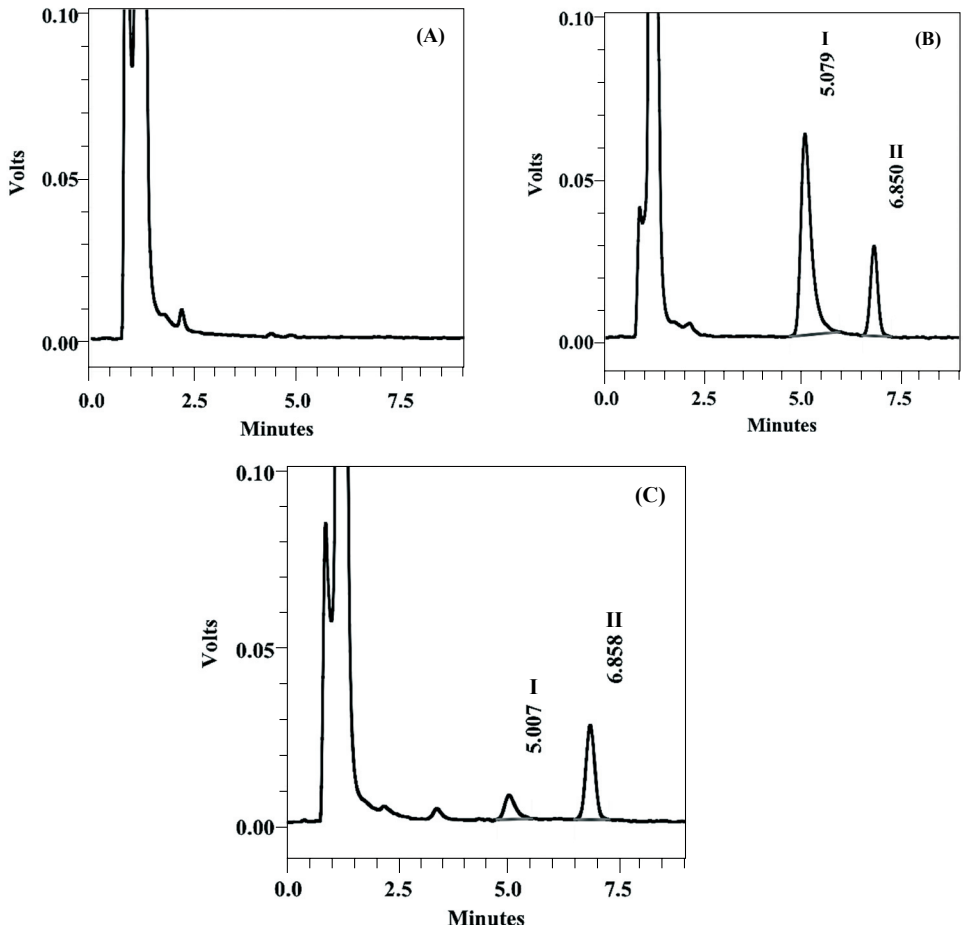

Figure 2. Representative chromatograms of blank plasma from a volunteer (A), Plasma standard containing 50 ng/ml doxazosin mesylate (peak I) and internal standard (peak II)(B), Plasma standard containing 5 ng/ml doxazosin mesylate (peak I) and internal standard (peak II) (C).

The absolute recovery was calculated by comparing the areas under the peak obtained from standard working solutions with the peak areas from standard sample. The recovery of doxazosin mesylate (3, 15 and 30 ng/ml) and diazepam were 101.93±2.66 %, 107.75±1.43 %, 101.90±1.31 % and 104.03±1.65 % , respectively. The results are shown in Table 2.

Table 2. Recovery of extraction for the analysis of doxazosin mesylate and diazepam in human (n = 5).

Compound	Added concentrations (ng/ml)	% Recovery ± (S.D.)	% CV
doxazosin mesylate	3	101.93±2.66	2.61
	15	107.75±1.43	1.33
	30	101.90±1.31	1.29
diazepam	100 (μg/ml)	104.03±1.65	1.59

Intra-day assay precision of the method is illustrated in Table 3. It was estimated by assaying the quality control samples five times in the same analytical runs. The precision was less than 4.0% and the accuracy ranged from 100.30-110.67% at all concentration levels.

Inter-day-assay precision and accuracy was evaluated by processing a set of calibration curve and quality control samples (three levels analyzed five times, results averaged for statistical evaluation) in the same analytical runs (Table 3). The precision was less than 5.9% and accuracy ranged from 97.47-103.33% at all concentration levels.

Table 3. Precision and accuracy of the HPLC method for the analysis of doxazosin mesylate in human plasma.

Conc. Added (ng/ml)	Intra-day studies (n=5)			Inter-day studies (n=5)		
	Conc. Found mean±S.D (ng/ml)	%CV	%Accuracy	Conc. Found mean±S.D (ng/ml)	%CV	%Accuracy
3	3.32±0.04	1.20	110.67	3.10±0.18	5.81	103.33
15	15.80±0.62	3.92	105.33	14.62±0.31	2.07	97.47
30	30.09±0.48	1.60	100.30	29.51±0.88	2.98	98.37

The stability of doxazosin mesylate in plasma was determined by periodic analysis of spiked samples. The results indicated less degradation which showed that doxazosin mesylate had a good stability in human plasma, either short-term or long-term stability test (Table 4).

Table 4. Stability of doxazosin mesylate in human plasma (n = 5).

Type of stability	Conc. (ng/ml) at t = 0, (mean±S.D)	Conc. (ng/ml) at t = t, (mean±S.D)	% Deviation
Freeze - thaw (3 cycles)	3.32±0.04 15.80±0.62 30.09±0.48	3.31±0.07 15.17±0.18 29.91±0.30	-0.30 -3.99 -0.60
Long-term (14 days)	3.32±0.04 15.80±0.62 30.09±0.48	3.40±0.14 14.97±0.46 29.21±0.13	2.41 -5.44 -2.92
Short-term (5 hours)	3.32±0.04 15.80±0.62 30.09±0.48	3.36±0.06 15.12±0.62 30.85±0.66	1.20 -4.30 2.53
Autosampler (24 hours)	3.32±0.04 15.80±0.62 30.09±0.48	3.20±0.12 15.89±0.60 30.46±0.47	-3.61 0.57 1.23

The developed method was applied to determine plasma doxazosin mesylate in pharmacokinetic and bioequivalence study in 16 healthy male volunteers. Plasma samples were periodically collected up to 36 hours after oral administration of 4 mg doxazosin mesylate tablet (Cardura®). Figure 3 illustrates the mean ±SD plasma concentration time profile of doxazosin mesylate, following an oral dose of 4 mg doxazosin mesylate. The plasma level of doxazosin mesylate reached the maximum in 2.0 hours after administration.

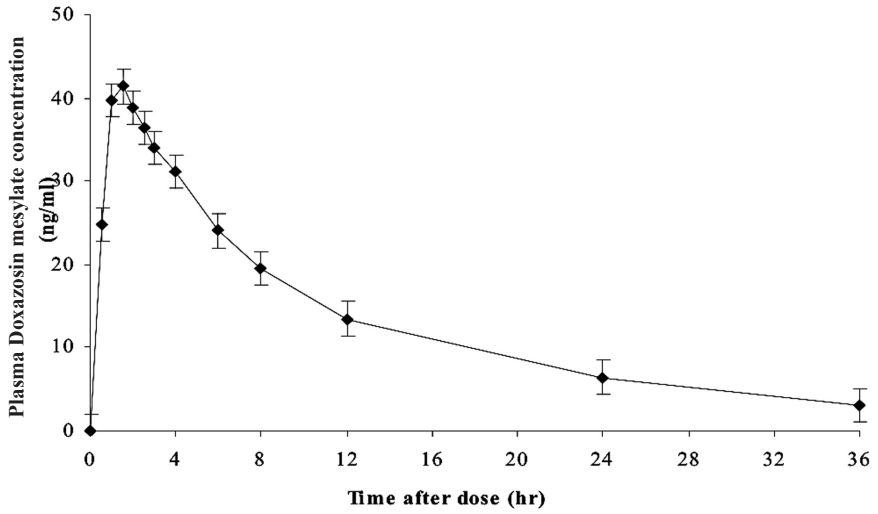

Figure 3. Mean plasma concentration time profile of doxazosin mesylate after oral administration of 4 mg doxazosin mesylate tablet in 16 healthy male volunteers.

CONCLUSION

This developed HPLC method for the determination of plasma doxazosin mesylate concentration was found to be a highly effective method since it used one-step precipitated extraction with acetonitrile which is simple, low cost of extraction and high extraction recovery. Moreover, this analysis method had high specificity and selectivity, sensitivity, accuracy and precision and also had a good stability, either short-term or long-term. The analytical method was successfully used in pharmacokinetic and bioequivalence study of doxazosin mesylate in healthy volunteers.

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