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Original Paper

Quantification of doxazosin in human plasma using hydrophilic interaction liquid chromatography with tandem mass spectrometry

Hydrophilic interaction LC with MS/MS (HILIC-MS/MS) was described as a rapid, sensitive, and selective method for the quantification of doxazosin in human plasma. Doxazosin and cisapride (internal standard) were extracted from human plasma with ethyl acetate at alkaline pH and analyzed on an Atlantis HILIC Silica column with the mobile phase of ACN/ammonium formate (100 mM, pH 4.5) (93:7 v/v). The analytes were detected using an ESI MS/MS in the selective-reaction-monitoring mode. The standard curve was linear ($r = 0.9994$) over the concentration range of 0.2 – 50 ng/mL. The LOQ for doxazosin was 0.2 ng/mL using 100 μL plasma sample. The CV and relative error for intra- and interassay at four QC levels were 3.7 – 8.7% and 0.0 – 9.8%, respectively. The matrix effect for doxazosin and cisapride were practically absent. The recoveries of doxazosin and cisapride were 67.4 and 61.7%, respectively. This method was successfully applied to the pharmacokinetic study of doxazosin in humans.

Keywords: Doxazosin / Human plasma / HILIC-MS/MS

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1 Introduction

Doxazosin is a selective α_1-adrenergic receptor antagonist used to treat hypertension and benign prostatic hyperplasia [1 – 5]. Doxazosin is extensively metabolized in the liver *via O*-demethylation and hydroxylation, which is <5% of the administered dose excreted unchanged in feces [1]. In order to minimize the risk of a first-dose hypotensive effect, the use of doxazosin in standard formulation requires a multistep titration regimen [1, 3, 6]. Doxazosin gastrointestinal therapeutic system has been developed to change the pharmacokinetic profile of doxazosin and minimize the possible adverse effects such as postural hypotension [4, 6, 7].

For the determination of doxazosin in human plasma, RP-HPLC with fluorescence [8 – 13], UV [14], MS [15], and MS/MS detection [16] were reported. The clean-up procedures for the extraction of doxazosin from plasma consist of protein precipitation [10], offline or online SPE [9, 14], and liquid – liquid extraction (LLE) [8, 11 – 13, 15, 16]. These methods use a large amount of plasma samples (300 – 500 μL plasma) in order to obtain high sensitivity [8 – 13, 15, 16].

Hydrophilic interaction LC (HILIC) technique using bare silica or polar bonded phase and low aqueous/high organic mobile phase has been proved to be a valuable tool for MS analysis of the polar compounds in biological samples [17 – 24]. HILIC can result in advantages compared to RP-HPLC as follows: MS sensitivity improvement due to higher organic content in the mobile phase, higher flow rates, and less matrix effect [17 – 20]. For the quantitative analysis of doxazosin in plasma, the rapid, robust, and sensitive HILIC-MS/MS method was described using a small volume of human plasma (100 μL) and the present method has been successfully applied to the pharmacokinetic study of doxazosin in humans.

2 Experimental

2.1 Materials

Doxazosin mesylate and cisapride (internal standard) were the gifts from Dong-A Pharm (Yongin, Korea). ACN

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Abbreviations: HILIC, hydrophilic interaction liquid chromatography; **QC,** quality control; **RE,** relative error; **SRM,** selective reaction monitoring

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and ethyl acetate (HPLC grade) were obtained from Burdick & Jackson (Muskegon, MI, USA) and the other chemicals were of HPLC grade or the highest quality available. Drug-free human plasma containing sodium heparin as the anticoagulant was obtained from healthy volunteers.

2.2 Preparation of calibration standards and quality control (QC) samples

Primary stock solutions of doxazosin and cisapride (1 mg/mL) were prepared in ACN. Working standard solutions of doxazosin were prepared by diluting the primary stock solution with ACN. The working solution for internal standard (25 ng/mL) was prepared by diluting an aliquot of stock solution with ACN. All doxazosin and cisapride solutions were stored at *ca*. 4°C in polypropylene bottles in the dark when not in use.

Human plasma calibration standards of doxazosin, *i.e*., 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, and 50 ng/mL were prepared by spiking the appropriate amount of the working standard solutions into a pool of ten lots of drug-free human plasma. QC samples at 0.2, 0.8, 8, and 40 ng/mL were prepared in bulk by adding 250 μL of the appropriate working standard solutions (4, 16, 160, and 800 ng/mL) to drug-free human plasma (4750 μL). The QC samples were aliquoted (100 μL) into polypropylene tubes and stored at −20°C until analysis.

2.3 Sample preparation

Blank plasma (100 μL), calibration standards, and QC samples were mixed with 10 μL of internal standard working solution and 100 μL of 0.5 M NaOH to adjust pH of samples to more than 11. The samples were extracted with 1000 μL of ethyl acetate in 1.5 mL polypropylene tubes by vortex-mixing for 2 min at high speed and centrifuged at $3000 \times g$ for 5 min at 4°C. 900 μL of the organic layer was transferred and evaporated to dryness using a vacuum concentrator (Genovac, UK) at 35°C. The residues were dissolved in 40 μL of 93% ACN by sonication for 2 min, centrifuged at $3000 \times g$ for 3 min, and transferred to injection vials. The aliquot (5 μL) was injected into the LC-MS/MS.

2.4 HILIC-MS/MS analysis

The HPLC system consisted of a Nanospace SI-2 pump, an SI-2 autosampler, and an S-MC system controller (Shiseido, Tokyo, Japan). The analytes were separated on an Atlantis HILIC Silica column (5 μm, 3 mm id × 50 mm, Waters, Milford, MA, USA) using a mixture of ACN/ammonium formate (100 mM, pH 4.5) (93:7 v/v) at a flow rate of 0.5 mL/min. The column and autosampler tray temperature were 50 and 4°C, respectively. The analytical run time was 3.0 min. The eluent was introduced directly into the positive ionization electrospray source of a tandem quadrupole mass spectrometer (API 2000, Applied Bosystems/MDS SCIEX, Foster City, CA, USA) through the turbo ionspray source with typical settings as follows: curtain gas, 35 psi; nebulizer gas, 50 psi; turbo gas, 65 psi; ionspray voltage, 5500 V; temperature, 380°C; collision energies for doxazosin and cisapride, 44 and 28 V, respectively; collision gas, nitrogen at a pressure setting of 7 on the instrument. Selective-reaction-monitoring (SRM) mode was employed for the quantification: m/z 452.2 → 344.0 for doxazosin and m/z 466.1 → 183.8 for cisapride. Peak areas for all components were automatically integrated using Analyst software version 1.4 (Applied Biosystems/MDS SCIEX).

2.5 Method validation

Batches, consisting of triplicate calibration standards at each concentration, were analyzed on three different days to complete the method validation. In each batch, QC samples at 0.2, 0.8, 8, and 40 ng/mL were assayed in sets of six replicates to evaluate the intra- and interday precision and accuracy. The percentage deviation of the mean from true values, expressed as relative error (RE), and the CV serve as the measure of accuracy and precision, respectively.

The absolute matrix effect and recoveries of doxazosin and cisapride were assessed by analyzing three sets of four standard concentrations. The matrix effect for doxazosin was assessed by comparing mean peak areas of doxazosin at four standard concentrations (0.2, 0.8, 8, and 40 ng/mL) spiked after extraction into blank plasma extracts with mean peak areas for neat solutions of the analytes in ACN. Recovery of doxazosin was determined by comparing mean peak areas of the extract of analyte-spiked plasma with those of the analyte spiked postextraction into blank plasma at four standard concentrations.

To evaluate the three freeze/thaw cycle stability and room temperature matrix stability, six replicates of QC samples at each of the low and high concentrations (0.8 and 40 ng/mL, respectively) were subjected to three freeze/thaw cycles or were stored at room temperature for 4 h before processing, respectively. Six replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h and were assayed to assess postpreparative stability.

2.6 Application

The developed HILIC-MS/MS method was applied to a pharmacokinetic study after an oral administration of doxazosin to male volunteers. The protocol was approved by an institutional review board at the Research Institute

for Pharmaceutical Sciences, Sungkyunkwan University (Suwon, Korea) and the informed consent was obtained from the subjects after explaining the nature and purpose details of the study. Eight healthy volunteers, fasted for 24 h, received a single oral dose of doxazosin (4 mg tablet) with 200 mL of water. Blood samples (2 mL) were withdrawn from the forearm vein at 0, 2, 4, 6, 8, 10, 12, 14, 16, 24, 48, 72, and 96 h postdosing, transferred to Vacutainer™ plasma glass tubes (sodium heparin, BD, NJ, USA), and centrifuged at 3000 × *g* for 10 min. The plasma samples were transferred to polypropylene tubes and stored at −20°C prior to analysis.

The maximum concentration (C_{max}) and the time to maximum concentration (T_{max}) were determined by visual inspection from each volunteer's plasma concentration–time curve for doxazosin. Area under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal method from 0 to 96 h.

3 Results and discussion

3.1 HILIC-MS/MS

The ESI of doxazosin and cisapride (internal standard) produced the abundant protonated molecular ions (MH^+) at *m/z* 452.2 and 466.1, respectively under positive ionization conditions, without any evidence of fragmentation and adduct formation. MH^+ ions from doxazosin and cisapride were selected as the precursor ions and subsequently fragmented in MS/MS mode to obtain the product ion spectra yielding useful structural information (Fig. 1). The prominent product ion for doxazosin was *m/z* 344.0 [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-ethylcarbonyl-piperazine] and the prominent product ion for cisapride was *m/z* 183.8 (5-chloro-4-amino-2-methoxy-phenyl-ketone). The SRM data acquisition was used due to the high selectivity and sensitivity: *m/z* 452.2 → 344.0 for doxazosin and *m/z* 466.1 → 183.8 for cisapride.

The drastic change in the selectivity of HILIC separations can be obtained by the judicious selection of column and the nature of buffer and mobile phase pH [18–20]. Using Atlantis silica column with ACN/100 mM ammonium formate buffers (93:7 v/v), the effect of ammonium formate pH at pH 3.0, 4.5, and 6.0 on the retention of doxazosin was investigated. Doxazosin showed good retention and peak shape using ammonium formate at pH 4.5. MS sensitivity using HILIC technique is enhanced due to the high organic content in the mobile phase and the high efficiency of spraying and desolvation techniques [18–20]. The use of 93% ACN as mobile phase in HILIC-MS/MS analysis of doxazosin resulted in the sensitivity enhancement of doxazosin in MS/MS detection, and therefore, the plasma sample volume (100 μL) used in this study was smaller than that

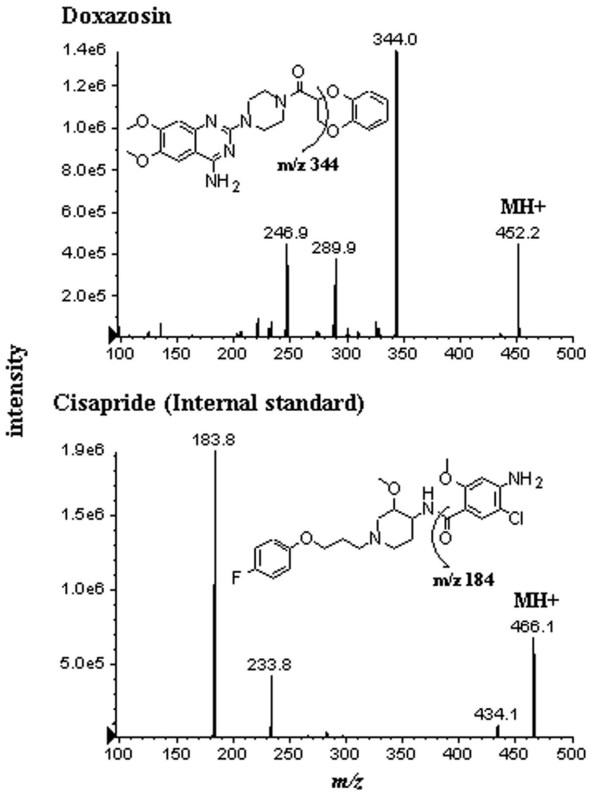

Figure 1. Product ion mass spectra of (a) doxazosin and (b) cisapride (internal standard).

(500 μL) of RP ultra-performance LC-MS/MS [16] to obtain the same LOQ (0.2 ng/mL).

No interference at the retention times of doxazosin (0.8 min) and cisapride (1.3 min) was observed in any of the 13 different lots screened as shown in representative chromatograms of the extracted blank plasma sample, confirming the specificity of the present method (Fig. 2). The retention times of doxazosin and cisapride were reproducible throughout the experiment and no column deterioration was observed after the analysis of 400 human plasma samples.

3.2 Method validation

Calibration curves were obtained over the concentration range of 0.2–50 ng/mL of doxazosin in human plasma. Linear regression analysis with a weighting of 1/concentration^2 gave the optimum accuracy of the corresponding calculated concentrations at each level (Table 1). The low CV value (5.5%) for the slope indicated the repeatability of the method (Table 1).

Table 2 shows a summary of intra- and interbatch precision and accuracy data for QC samples containing doxazosin. Both intra- and interassay CV values ranged from 3.7 to 8.7% at four QC levels. The intra- and interassay RE values for doxazosin were 0.0–9.8% at four QC levels.

Table 1. Calculated concentrations of doxazosin in calibration standards prepared in human plasma ($n = 9$)

	Theoretical concentration (ng/mL)								Slope	Intercept	r
	0.20	0.50	1.00	2.00	5.00	10.0	20.0	50.0			
Mean	0.20	0.53	1.00	2.01	5.07	9.66	20.3	49.9	0.1998	0.0144	0.9994
CV (%)	9.2	6.1	11.2	8.6	7.1	5.8	7.6	6.7	5.5		
RE (%)	0.0	6.0	0.0	0.5	1.4	−3.4	1.5	−0.2			

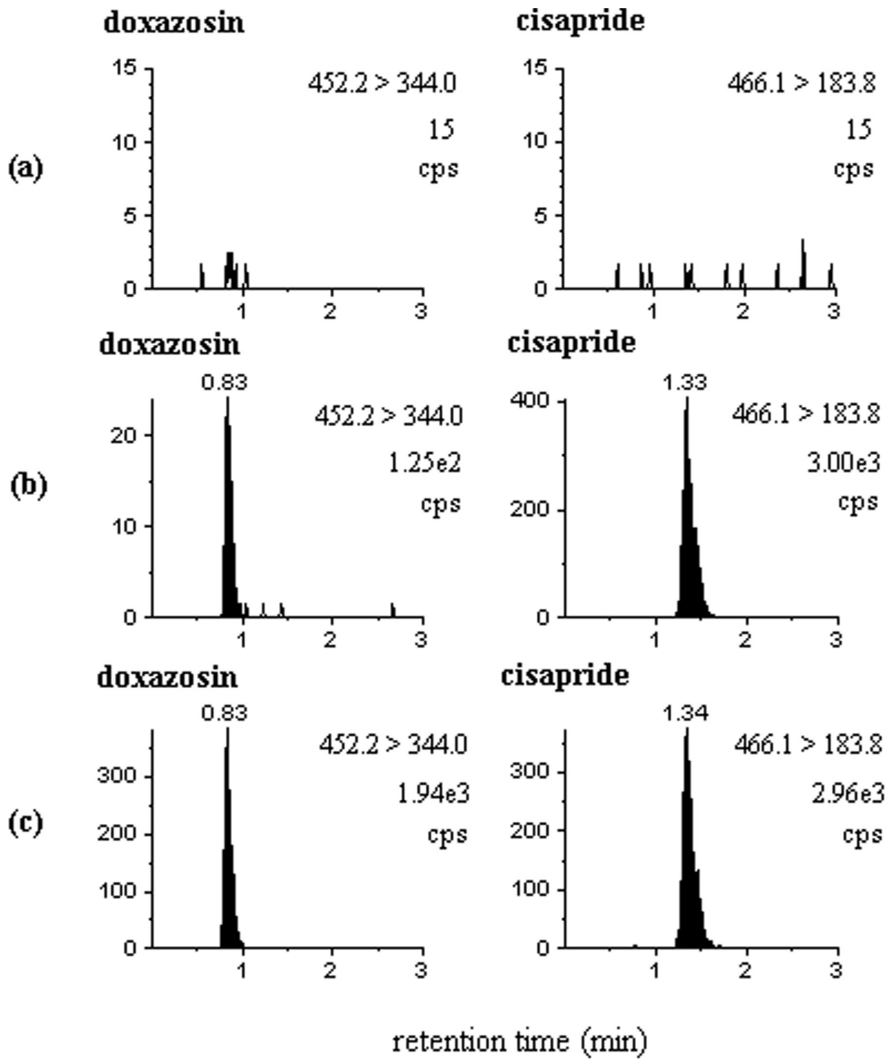

Figure 2. SRM HILIC-MS/MS chromatograms of (a) a blank human plasma, (b) human plasma sample spiked with 0.2 ng/mL of doxazosin, and (c) a plasma sample obtained 10 h after oral administration of doxazosin at a dose of 4 mg to a male volunteer.

These results indicated that the present method has an acceptable accuracy and precision [25]. LOQ was 0.2 ng/mL with the S/N of about 20, and a representative chromatogram of an LOQ for doxazosin is shown in Fig. 2b.

The matrix effect, the ratio of the mean peak area of an analyte spiked postextraction to the mean peak area of same analyte standards multiplied by 100, was 98.9 and 101.2% for doxazosin and cisapride, respectively (Table 3). A value of 100% indicates that the response in the solvent and in the plasma extracts was the same and no absolute matrix effect was observed. A value of <100% indicates an ionization suppression and a value of >100% indicates an ionization enhancement. There was little matrix effect for doxazosin and cisapride.

As shown in Table 3, the overall extraction recovery of doxazosin was 67.4%, which was consistent over the concentration range of 0.2 – 40 ng/mL. The recovery of cisapride was 61.7%. In the sample preparation procedure of doxazosin from plasma, diethylether, methyl *tert*-butylether, *n*-hexylane, and ethyl acetate have been used as

Table 2. Precision and accuracy of doxazosin in QC samples

	Intrabatch (n = 6)				Interbatch (n = 3)			
QC (ng/mL)	0.20	0.80	8.00	40.0	0.20	0.80	8.00	40.0
Mean (ng/mL)	0.20	0.82	8.69	43.9	0.21	0.82	8.45	42.4
CV (%)	8.7	7.5	3.7	4.1	7.3	7.5	5.7	5.6
RE (%)	0.0	2.5	8.6	9.8	5.0	2.5	5.6	6.0

Table 3. Matrix effect and recovery data for doxazosin and cisapride (I.S.) in human plasma (n = 5)

Nominal concentration (ng/mL)	Matrix effect[a] (%)		Recovery[b] (%)	
	Doxazosin	I.S.	Doxazosin	I.S.
0.2	99.4	103.1	68.9	60.3
0.8	98.2	101.9	67.8	61.3
8.0	98.9	100.7	68.7	61.6
40.0	99.0	99.1	64.3	63.6
Mean	98.9	101.2	67.4	61.7

[a] Matrix effect expressed as the ratio of the mean peak area of an analyte spiked postextraction to the mean peak area of same analyte standards multiplied by 100.

[b] Recovery calculated as the ratio of the mean peak area of an analyte spiked before extraction to the mean peak area of an analyte spiked postextraction multiplied by 100.

Table 4. Stability of samples (n = 6)

Statistical variable	Theoretical concentration (ng/mL)	
	0.80	40.0
Three freeze and thaw cycles		
Mean	0.84	41.6
CV (%)	6.1	3.5
RE (%)	5.0	4.0
4 h at room temperature		
Mean	0.87	43.9
CV (%)	1.3	5.2
RE (%)	8.7	9.8
Postpreparative stability (24 h at room temperature)		
Mean	0.84	41.6
CV (%)	6.5	5.2
RE (%)	5.0	4.0

extraction solvents [8, 11 – 13, 15, 16]. In this study, ethyl acetate was selected as an extraction solvent in order to obtain the selectivity as well as good recovery.

Stabilities of processing (freeze – thaw and short-term) and chromatography (reinjection) were tested and shown to be of insignificant effect (Table 4). QC samples that went through three freeze – thaw cycles showed the acceptable accuracy (RE: 4.0 – 5.0%) and precision (CVs: ≤6.1%). QCs showed the acceptable accuracy (RE: 8.7 – 9.8%) and precision (CVs: ≤5.2%) when exposed to room temperature for 4 h. The reanalysis of the reconstituted extracts stored for 24 h at 4°C showed the acceptable accuracy (RE: 4.0 – 5.0%) and precision (CVs: ≤6.5%) for QC samples.

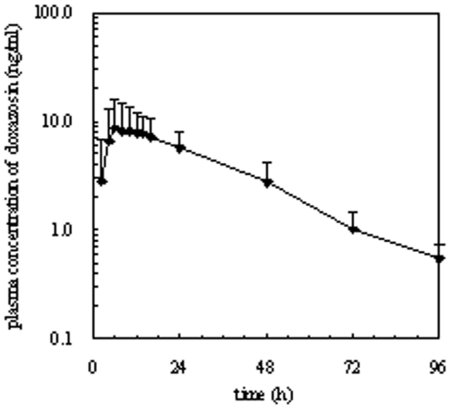

Figure 3. Mean plasma concentration – time plot of doxazosin after a single oral dose of doxazosin (4 mg tablet) to eight male volunteers. Each point represents the mean ± SD.

3.3 Application study

This method has been successfully applied to the analysis of 104 plasma samples in a pharmacokinetic study of doxazosin. Representative chromatograms of the extract of a plasma sample obtained 10 h after oral administration of doxazosin at a dose of 4 mg to a human are shown in Fig. 2c. Figure 3 shows mean plasma concentration profiles of doxazosin obtained after a single oral dosing of doxazosin (4 mg) to eight healthy male volunteers. C_{max}, T_{max}, and AUC of doxazosin were 10.3 ± 6.5 ng/mL, 10.5 ± 4.1 h and 324.0 ± 127.1 ng · h/mL, respectively.

4 Conclusion

A rapid, sensitive, and reliable HILIC-MS/MS method for the determination of doxazosin in human plasma has been successfully developed and validated using one step LLE as a sample preparation procedure. This assay method demonstrated acceptable sensitivity (LLOQ: 0.2 ng/mL), precision, accuracy, selectivity, recovery and stability, and less absolute matrix effect. The validated method was successfully applied to assay human plasma samples from the pharmacokinetic study of doxazosin.

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