A LC–MS–MS Method for Determination of Low Doxazosin Concentrations in Plasma after Oral Administration to Dogs

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Abstract

A rapid and sensitive reversed phase liquid chromatography–tandem mass spectrometry (LC–MS–MS) method is developed for the determination of doxazosin in canine plasma. The samples are prepared by precipitation of proteins using a mixture of methanol and acetonitrile, followed by freezing and evaporation of the organic solvent. The remaining dry residue is redissolved in mobile phase and analyzed by LC–MS–MS with positive electrospray ionization using the selected reactions monitoring mode. An XTerra MS C_{18} column, a mobile phase composed of acetonitrile and 2mM ammonium acetate with gradient elution, and a flow rate of 400 µL/min are employed. The elution times for prazosin (internal standard) and doxazosin are ~ 8 and 10 min, respectively. Calibration curves are linear in the 1–20 ng/mL concentration range. Limits of detection and quantification are 0.4 ng/mL and 1.2 ng/mL, respectively. Recovery is higher than 94%. Intra- and inter-day relative standard deviations are below 7% and 8%, respectively. The method is applied for the determination of doxazosin plasma levels following a single administration of doxazosin base and doxazosin mesylate tablets (2 mg dose) to dogs in the fed state. The results indicate possible superiority of the mesylate salt on the plasma input rates of doxazosin.

Introduction

Doxazosin [(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-(1,4-benzodioxan-2-yl-carbonyl) piperazine] is a postsynaptic α_1-adrenoreceptor antagonist used either alone or in combination with diuretics or α_1-adrenergic-receptor-antagonist for the treatment of hypertension and benign prostatic hyperplasia (1). It is structurally related to prazosin (Figure 1), but doxazosin shows a more gradual onset of hypotensive effect and a longer half-life, making it a possible candidate for once-daily oral dosing (2,3).

For determination of doxazosin in human plasma, several analytical methods have been reported, mainly chromatographic methods coupled with fluorescence (4–10), UV (11), mass spectrometry (12) or MS–MS detection (13,14). Sample treatment involves primarily liquid–liquid extraction of doxazosin from plasma (4,5,8–10, 12–14), although offline and online solid-phase extraction (6,11) as well as protein precipitation (7) have also been applied.

Most of the developed methods have been applied to human pharmacokinetic studies after oral administrations of 4–10 mg doses of doxazosin tablets (6,9–12,14).

The objective of this study was to develop a method that would be suitable for the determination of low concentrations of doxazosin in canine plasma (i.e., after oral administration of a 2 mg dose in the fed state). Although literature data suggest that oral bioavailability and maximum plasma concentration (C_{max}) are not affected significantly by dosing conditions in humans (15), the lower absorption rates in the fed state (as a consequence of the slower gastric emptying rates) (16) frequently lead to lower plasma concentrations during absorption in the fed state. Furthermore, the number of interfering compounds in plasma samples is expected to be bigger in the fed state. Finally, because there are no published data on the superiority of mesylate salt over the free base, in the present study, we applied the developed method for a preliminary evaluation of the superiority of mesylate salt over the free base using the canine model.

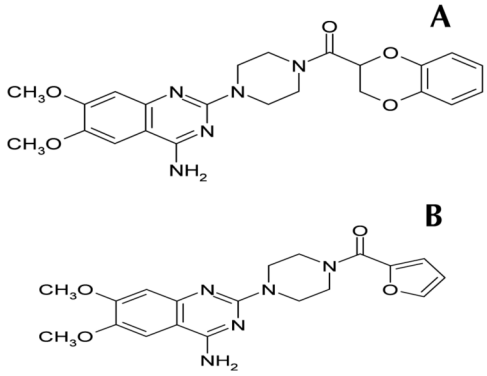

Figure 1. Structure of doxazosin (A) and prazosin (IS) (B).

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Experimental

Instrumentation

The liquid chromatograph (LC) (Agilent 1100; Agilent Technologies, Palo Alto, CA) was coupled to a mass spectrometer (MS) with a turbo electrospray ion source (Qtrap; Applied Biosystems, Foster City, CA) and an electrospray ionization (ESI) interface. An Analyst software 1.3.1 (Applied Biosystems) was used for LC–MS–MS control and signal acquisition. LC system was equipped with an Agilent 1100 gradient pump, Thermo autosampler, column oven, and diode array detector.

A Hettich centrifuge Universal 32R (Tuttlingen, Germany) was utilized to centrifuge plasma samples. The vacuum evaporator used in sample extraction procedure was a Concentrator Eppendorf 5301 (Eppendorf, Hamburg, Germany).

Chemicals and reagents

Methanol and acetonitrile were of high-performance LC grade. All other chemicals were of analytical-grade. Ammonium acetate was purchased from Merck (Darmstadt, Germany). Doxazosin mesylate (purity 99.8%) was synthesized and standardized in house (PLIVA, Zagreb, Croatia). Doxazosin base substance (purity 97.2%) was also prepared in-house. Prazosin hydrochloride (purity > 99%) was used as internal standard (IS), and it was purchased from Sigma (Sigma Aldrich Chemie, GmbH, Steinheim am Albuch Germany). Water purified with Q-system (Millipore, Milford, MA) was used in all procedures.

Canine blank plasma was obtained from dogs hosted in an animal facility that operates at the Faculty of Pharmacy, National and Kapodistrian University of Athens (Greece), which operates in accordance with European Union regulations for the maintenance and experimentation on animals and which has been approved by the Veterinary Directorate of the Municipality of Athens. Aliquots of doxazosin-free pooled canine plasma were used for preparation of spiked plasma standards.

Chromatographic conditions and MS settings

Separation was achieved on an XTerra MS C_{18} column (150 mm × 2.1 mm, 3.5 µm particle size) equipped with an XTerra MS C_{18} guard column (20 mm × 2.1 mm, 3.5 µm particle size), both from Waters (Milford, MA). The gradient mobile phase was composed of acetonitrile–2 mM ammonium acetate (10:90, v/v) as mobile phase A and acetonitrile–2 mM ammonium acetate (90:10 v/v) as mobile phase B. Mobile phase A at times 0, 1, 8, 10, and 15 min was 90%, 90%, 30%, 90%, and 90%, respectively. The flow rate was 400 µL/min, and the injection volume was 100 µL. The column temperature was maintained at 35°C, and the autosampler cooler was maintained at 10°C. The elution time for prazosin and doxazosin was ~ 8 and 10 min, respectively. The HPLC effluent was sprayed directly into the MS at 400 µL/min flow rate. The Q-TRAP MS was operated with turbo ion-spray interface in positive ion mode at unit resolution. Doxazosin and prazosin (IS) were detected by selected reaction monitoring (SRM) in the multi reaction monitoring mode using the following settings: transitions *m/z* 452.3 → 344.4 and 384.3 → 247.2 with a dwell time 200 msec. Ion source and other instrument parameters were optimized for the transition and the following settings were used: 30 psi, curtain gas; 300°C, temperature; 30 psi, nebuliser gas; 60 psi, heater gas; 4 eV, nebulizer current; 60 V, declustering potential; 12 V, entrance potential; 40 eV, collision energy; and collision cell exit, 4 eV.

Optimization of sample treatment

For the isolation of doxazosin from plasma samples, protein precipitation was applied and optimized. Because liquid–liquid extraction is still the most widely used technique for extraction of doxazosin from spiked plasma samples, in our study it was tested for comparative purposes only.

Various solvents (methanol, ethanol, and a combination of methanol and acetonitrile) and conditions (4°C and –20°C) were evaluated for protein precipitation.

Liquid–liquid extraction was performed with 500 µL of plasma by alkalinization with 1M NaOH, followed by extraction with 30% dichloromethane in hexane. The upper organic layer was evaporated to dryness, and the remaining dry residue was dissolved in mobile phase and injected into the LC–MS–MS system. Recovery was calculated by comparing the peak areas obtained from plasma samples with those obtained by direct injection of the working standard solutions of doxazosin. Extraction was performed so that the entire volume of supernatant was obtained and subsequently evaporated to dryness. In both cases, IS was contained in the solution.

Method validation

Validation procedures were based on relevant guidelines (17,18).

Calibration curves

Stock solutions of doxazosin base, doxazosin mesylate, and prazosin hydrochloride (100 µg/mL) were prepared by dissolving 10 mg of each compound in 100 mL of methanol. All solutions were prepared freshly every day. Concentration of the working solution of IS was 60 ng/mL. All dilutions to volume were performed with water.

Usual doxazosin calibration curves in plasma were constructed in the concentration range of 1–20 ng/mL as follows: 100 µL of blank canine plasma were transferred in a centrifuge tube containing 50 µL of doxazosin working solutions (2.5–48 ng/mL in water). After vortexing, 20 µL of working solution of IS (60 ng/mL in water) was added. Further preparation procedure was according to the same manner as described in the "Analysis of plasma samples" section.

Regression equations were obtained through unweighted least square linear regression analysis with a regression equation $y = ax + b$, where y was the peak area ratio of doxazosin to IS, and x was doxazosin concentration in ng/mL.

Precision, recovery, and accuracy

Quality control (QC) standards for the determination of accuracy and precision of the method were independently prepared at 2, 5, and 10 ng/mL concentrations in the same manner as the calibration standards. QC standards represent the matrix of the samples with known amounts of the analyte, used for validation purposes and to validate the test run. For recovery, accuracy and intra-day precision QC standards were prepared and analyzed in triplicate. Inter-day precision was also tested using QC standards in triplicate.

Stability

To evaluate doxazosin stability in canine plasma, drug-free plasma samples were spiked with analytes at 5 and 10 ng/mL. To test the short- and long-term stability of doxazosin, quality control standards of 5 and 10 ng/mL were prepared in duplicate and stored at ambient temperature (25 ± 2°C) and at –20°C for 24 h and 60 days, respectively.

LOD and LOQ and carryover effects

For the determination of LOD and LOQ, calibration curves were prepared in the range of 0.5–12 ng/mL. The LOD and LOQ values were defined as follows (19):

$$\text{LOD} = \frac{3.3S_{y/x}}{b}$$

$$\text{LOQ} = \frac{10S_{y/x}}{b} \qquad \text{Eq. 1}$$

where b is the slope, and sy/x is the residual standard deviation of the regression line, calculated using working standards. QC standard for determination of carry over was prepared at the concentration of 20 ng/mL.

Analysis of plasma samples

150 µL of plasma samples were transferred in a centrifuge tube and 20 µL of working solution of IS (60 ng/mL) were added. After vortexing for 30 s, 400 µL of precipitation solvent (methanol–acetonitrile 50:50, v/v) were added. The new mixture was vortexed for 30 s and stored for at least 12 h in a freezer at –20°C. Then, the samples were centrifuged for 15 min at 12,000 rpm. The supernatant was filtered using 0.2 µm Gelman acrodisc CR PTFE syringe filters (Gelman Science, Ann Arbor, MI) and evaporated to dryness using vacuum evaporator at 40°C. Dry residue was then redissolved in 120 µL of mobile phase and the solution was vortexed for 1 min. Finally, 100 µL were injected into the LC–MS–MS system.

Pharmacokinetic application

A single dose oral administration of doxazosin was performed in four female, 4-year-old mongrel dogs weighing ~ 25 kg each. Before administration, each dog was made to fast for 16 h from food but not water. Each dog was administered one doxazosin base tablet or doxazosin mesylate tablet (both containing 2 mg doxazosin) with 500 mL of cow's milk (3.5% fat) via an orogastric tube. Blood samples were drawn by means of an indwelling catheter positioned in a suitable foreleg vein. Eight hours after drug administration, each dog was offered a standard meal (150 g pellets and 250 mL tap water). Twelve hours after dosing, the catheter was removed, and the dog was returned to her cage, where she was allowed to eat and drink ad libitum. Samples after 12 h were collected by individual venipuncture. Blood samples were centrifuged and plasma was stored in aluminum foil covered normal brown glass vials at stored at –20°C until assayed.

Pharmacokinetic parameters including C_{max} (peak plasma concentration), T_{max} (time to C_{max}), and $AUC_{p,base}$ [partial area under the plasma concentration *vs*. time curve from t = 0 up to the first peak of the profile after administration of the base (20)] were calculated using Prism Software (GraphPad Prism 3.02. Software, Inc., San Diego, CA).

Apparent terminal half life was calculated by log-linear regression of the terminal segment of the plasma concentration-time curve (0.693/λz), where λz is the apparent elimination rate constant.

Results and Discussion

Optimization of sample treatment

Liquid–liquid extraction was not adopted, primarily because it resulted in low recovery of analyte from plasma samples (Table I). In addition, it required significantly larger volumes of plasma and increased time of analysis.

A previously described precipitation method (7), which involved the use of methanol was found not to be appropriate for removal of proteins from our samples. Supernatant of samples collected in the fed state were not clear, and recoveries of doxazosin and prazosin were low (Table I).

In contrast, when the plasma sample was diluted with a mixture of methanol–acetonitrile (50:50, v/v), vortexed for 30 s, stored for 12 h in a freezer at –20°C, and subsequently treated as described in the "Analysis of plasma samples" section, proteins were efficiently precipitated (i.e., supernatant was clear and suitable for further chromatographic analysis whereas recovery of spiked doxazosin and prazosin was higher than 90%) (Table I).

It should be noted that protein precipitation has recently been reported to be inefficient for accurately measuring doxazosin in pharmacokinetic studies (8). However, in the relevant study, although storing conditions of the plasma samples drawn from the PK study are mentioned, storing conditions of blank plasma sample are not specified.

Method validation

Selectivity

Typical chromatograms of blank canine plasma and canine plasma samples from the pharmacokinetic study are shown in Figure 2. Prazosin and doxazosin were eluted at ~ 8 and 10 min, respectively, with a total run time of 15 min, which is within range of other published methods (4–14). Product ion mass spectra of doxazosin and prazosin have already been published (14,21).

Table I. % Recovery of Doxazosin (20 ng/mL) and of Prazosin (IS, 30 ng/mL) from Canine Plasma using Various Sample Treatment Procedures

	Doxazosin	IS
Liquid–liquid extraction (with 30% dichloromethane in hexane after alkalinization with 1M NaOH)	43	37
Double precipitation with methanol and storage at 4°C for 12 h	12	18
Double precipitation with ethanol and storage at 4°C for 12h	16	16
Double precipitation with methanol–acetonitrile (50:50, v/v) and storage at 4°C for 12 h	22	38
Precipitation with methanol–acetonitrile (50:50, v/v) and storage –20°C for 12 h	91	98

A good separation of doxazosin and prazosin were obtained whereas no interfering peaks were found at the retention time of doxazosin and/or prazosin (Figure 2).

Table II. RSD of Measurements, % Recovery and % Accuracy of Three Doxazosin Concentrations Spiked in Canine Plasma*

Nominal Conc. (ng/mL)	RSD (%)	Mean relative recovery ± SD (%)	Accuracy (%)
Intra-day			
2	6.8	97.2 ± 6.6	–2.8
5	6.1	101.0 ± 6.1	1.0
10	5.2	99.7 ± 5.2	–0.3
Inter-day			
2	6.8	94.1 ± 6.4	–5.9
5	4.9	103.6 ± 5.1	3.6
10	7.9	101.3 ± 8.0	1.3

* Each standard was prepared and measured three times on three different days.

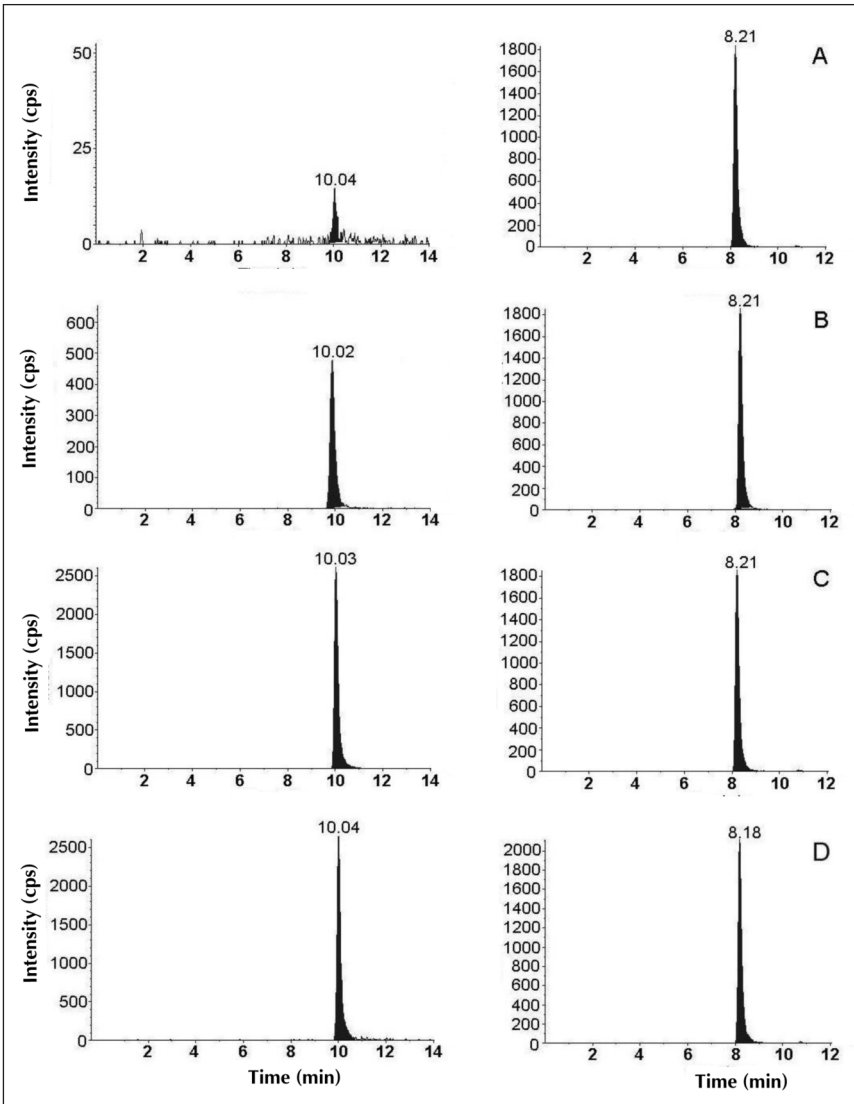

Figure 2. Chromatograms of (A) canine plasma spiked with 10 ng/mL prazosin, (B) canine plasma spiked with 2 ng/mL doxazosin and 10 ng/mL of IS (concentration of doxazosin is the lowest above LOQ), (C) canine plasma spiked with 10 ng/mL doxazosin and 10 ng/mL of IS and (D) plasma sample collected 5h after administration of one doxazosin mesylate tablet (2 mg doxazosin in tablet).

Calibration curves

Linear calibration curves for doxazosin were obtained throughout the concentration range studied (1–20 ng/mL) over three consecutive days. The number of points in each calibration curve was six. Linearity criteria imposed a correlation coefficient $r \geq 0.99$. Regression analysis was performed for the ratios of peak area of doxazosin to that of the IS (y) versus doxazosin concentration (x). The calibration curve [mean (SD), $n = 3$] could be described by the equation 2.

$$y = 0.134\ (\pm\ 0.017)x + 0.006\ (\pm\ 0.068) \qquad \text{Eq. 2}$$

In all three replications, intercept was not significant.

Precision, accuracy, and recovery

In the last few years, various authors have made efforts to improve the existing methods for determination of doxazosin in human plasma. Most of these methods were applied to pharmacokinetic studies of 4–10 mg doses of doxazosin tablets (6,9–12,14).

To our best knowledge, Sripalakit et al. (8) were the only who administered single 2 mg dose of doxazosin tablets to humans in the fasted state. In that study, 500 µL of sample was used for extraction vs. 150 µL used in the present study for achieving comparable accuracy, precision, and recovery.

The precision of the proposed LC–MS–MS method was examined in spiked canine plasma samples. After preparing and measuring QC samples of three different concentrations of doxazosin, each in triplicate, values of intra- and inter-day relative standard deviation (RSD) were calculated. Results showed that intra-day RSD was less than 7% while the corresponding inter-day value was less than 8%. Even at concentration level close to the LOQ, RSD values were in accordance with the relevant guidelines (17,18), where RSD for LOQ did not exceed 20%.

Accuracy of the developed method was examined on QC standards at three concentration levels by comparing the measured value with the nominal values. These standards were quantified using calibration curves prepared in plasma matrix. The results are summarized in Table II and are in agreement with the relevant guidelines (17,18).

Recovery was calculated by comparing ratios of integrated peak area of doxazosin to IS from the quality control samples to those from the standard solutions having the same concentrations of doxazosin and IS (direct injection of the corresponding unextracted standard solutions). The mean recovery of doxazosin from canine plasma at the concentrations of 2, 5, and 10 ng/mL was over 94.1% (Table II).

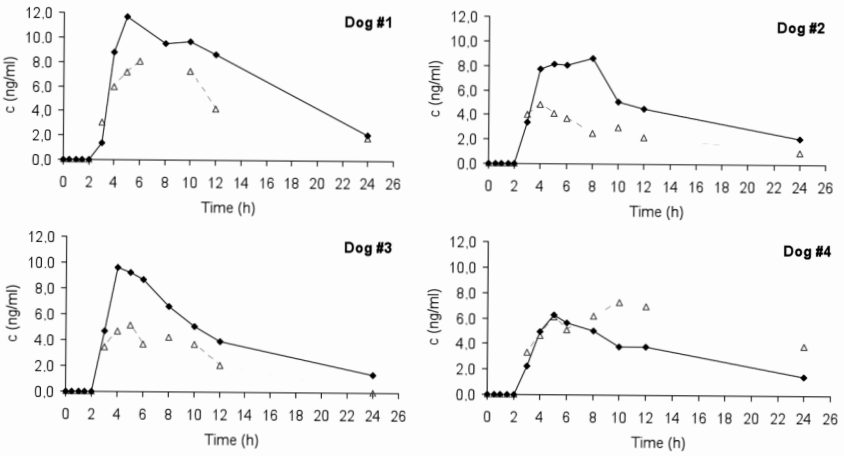

Figure 3. Doxazosin plasma profiles after single administrations of one doxazosin base tablet (Δ) and one doxazosin mesylate tablet (♦) to four dogs in the fed state. Tablets contained 2 mg doxazosin.

Table III. Individual AUCp,base values (ng/mL/h) After the Single Dose (2 mg) Administration of Doxazosin Base and Mesylate Salt of Doxazosin to Four Dogs in the Fed State

	Base	Mesylate salt
Dog #1	20.2	27.3
Dog #2	6.6	7.3
Dog #3	10.7	18.9
Dog #4	11.0	10.4

Stability

Doxazosin was found to be stable at room temperature for at least 24 h. Recoveries were 91.0% and 92.2% for 5 and 10 ng/mL samples, respectively. Similarly, doxazosin was stable at –20°C for at least 60 days. Recoveries at 5 and 10 ng/mL were 93.2% and 91.3%, respectively.

LOD and LOQ and carryover effects

Based on the Equation 1, LOD and LOQ values for doxazosin were found equal to 0.4 and 1.2 ng/mL of plasma sample, respectively.

Most of the reported methods have LOD and LOQ values close to the values obtained in our study. Only in one recently developed method that utilizes UPLC–MS–MS (13) were the LOD and LOQ values lower (0.02 and 0.07 ng/mL, respectively) than those of the method developed in the present study.

Carry over was tested at the concentration of 30 ng/mL, and it was determined to be equal or less than 0.3%.

Canine in vivo data

Individual doxazosin plasma concentration profiles after administration of 2 mg doxazosin base tablets and doxazosin mesylate tablets to four dogs are shown in Figure 3. In most cases, more than one peak is observed in agreement with literature data indicating that doxazosin is enterohepatically circulated (Cardura®, summary of product characteristics).

In men, the peak plasma level of ~ 9 ng/mL is achieved within 2 to 3 h after a single oral dose in the fasted state (2), but there are no published relevant data in the fed state. In our study the maximum doxazosin plasma concentration of doxazosin base (C_{max}) ranged from 4.9 to 8.1 ng/mL and was achieved 4 to 10 h post-dose (T_{max}). Doxazosin mesylate achieved maximum concentrations of 6.3 to 11.6 ng/mL 4 to 8 h after administration. Because gastric emptying of dogs in the fed state is similar with that of humans (22) and also terminal elimination half life in our dogs ranged from 4.0 to 15.1 h, [i.e., it was similar to that in humans (9–22 h) (1)], it can be claimed that the slightly lower plasma profiles in dogs and delayed T_{max} are both due to the fed state conditions.

It is interesting that there are no published human data on the advantage of using mesylate salt of doxazosin. Based on AUCp, base values the use of mesylate seems to lead to slightly faster rise of plasma levels in dogs (Table III). However, the superiority of the mesylate salt cannot be evaluated on a statistical basis due to the small number of dogs available. It should be noted that although there are published data that salts of weak acids might be absorbed with faster rates than free acids in vivo (23,24), similar data for weak bases are very limited (25,26).

Conclusion

A simple, rapid, and selective LC–MS–MS method for determination of doxazosin plasma concentrations after oral administration was developed and validated. Specific advantages over previously published methods include the low sample volume (150 µL), the short retention times (of both doxazosin and IS) and high sensitivity. Pharmacokinetic profiles in dogs provide for the first time some evidence that mesylate salt might be advantageous *vs.* the free base in regard to the absorption rates of doxazosin.

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