

Simultaneous Determination of Levocetirizine and Pseudoephedrine in Dog Plasma by Liquid Chromatography-Mass Spectrometry in the Presence of Dextrocetirizine

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ABSTRACT - Purpose. This study describes the development of a rapid and sensitive LC-ESI-MS assay for simultaneous enantioselective determination of levocetirizine and pseudoephedrine in dog plasma in the presence of dextrocetirizine. **Methods.** Separations were achieved on an Ultron ES-OVM chiral column using the mobile phase consisting of 10 mM aqueous NH₄OAc (pH 6.6) and acetonitrile (9:1 v/v). **Results.** The retention times of pseudoephedrine, dextrocetirizine, levocetirizine and diazepam (internal standard) were 5.2, 8.3, 9.6 and 11.6 min, respectively, and the total run time was less than 15 min. The assay was validated to demonstrate the linearity, accuracy and precision, recovery and stability. The calibration curves were linear over the concentration range from 1 – 200 ng/mL for levocetirizine and from 5 – 1000 ng/mL for pseudoephedrine. **Conclusions.** The developed assay was successfully applied to a pharmacokinetic study after oral administration of the racemic cetirizine (0.5 mg/kg, or 0.25 mg/kg as levocetirizine) and pseudoephedrine (12 mg/kg) in the dog.

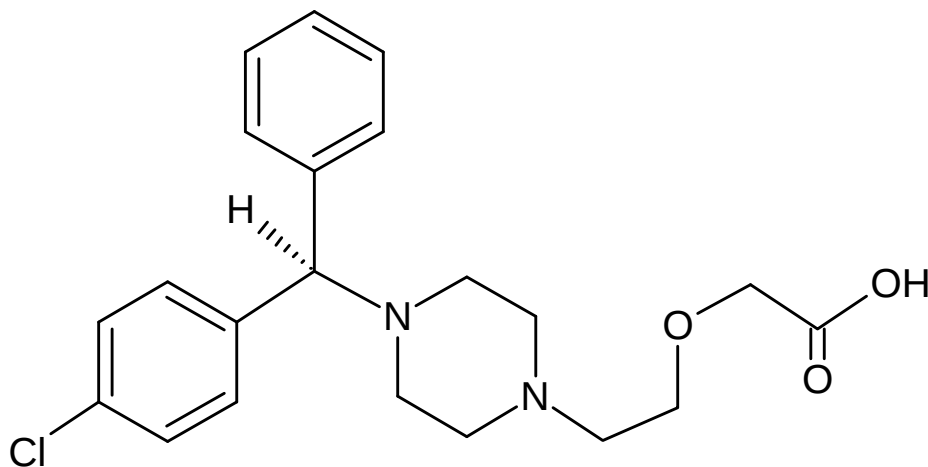
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INTRODUCTION

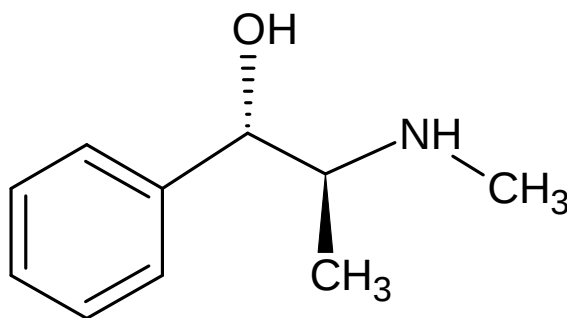
Cetirizine, (±) - [2 - (4 - [(4 - chlorophenyl) phenylmethyl]-1-piperazinyl) ethoxy] acetic acid, is an orally active histamine H₁-receptor antagonist used for the treatment of seasonal allergic rhinitis, perennial allergic rhinitis and chronic idiopathic urticaria. It is a racemate with the R enantiomer (levocetirizine) (Figure 1) being pharmacologically active, exhibiting approximately 30-fold higher affinity to human histamine H₁-receptors than its antipode, dextrocetirizine (1). Pseudoephedrine, [1S, 2S]-2-methylamino-1-phenylpropan-1-ol] (Figure 1), is an alpha-adrenergic agonist used as a nasal decongestant in patients with allergic rhinitis and in acute rhinitis in patients with upper-respiratory infections. It is a stereoisomer of ephedrine and is less potent than ephedrine in producing tachycardia and CNS stimulation. Combination therapy involving levocetirizine and pseudoephedrine may effectively reduce the nasal congestion due to allergic rhinitis. In developing and evaluating a sustained release oral dosage formulation containing levocetirizine and pseudoephedrine, it may be desirable to use a rapid and sensitive assay method that can simultaneously measure plasma concentrations of both drugs in the presence of dextrocetirizine.

HPLC (2-3) and subcritical fluid chromatography (4) with UV detection methods are available for the determination of levocetirizine. These methods generally have poor assay sensitivity and require long analytical run times. Recently, a LC-MS/MS assay has been reported for the determination of plasma levels of levocetirizine (5). This assay, however, requires the time-consuming two-step sample extraction procedures and the normal phase liquid chromatographic separation using organic solvents that are toxic, expensive and difficult to remove eco-friendly. A number of assay methods have been reported for the analysis of pseudoephedrine, including HPLC (6-8), GC (9-10) and LC-MS/MS (11-13). The HPLC assays involve either time-consuming double liquid-liquid extraction (6, 8) or solid phase extraction adapted to an automated column switching method for online sample preparation (7). The sample preparation procedures of the GC assay needs laborious derivatization reactions (9-10). The LC-MS/MS assays are achiral methods (11-13) and cannot be used for the simultaneous enantioselective determination of levocetirizine and pseudoephedrine.

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Levocetirizine



Pseudoephedrine

Figure 1. Chemical structures of levocetirizine and pseudoephedrine.

As pseudoephedrine is a diastereomer of ephedrine, the m/z ratios of parent and daughter ions in the LC-MS/MS methods (11-13) are identical with each other and the physicochemical properties are very similar. The retentions of pseudoephedrine in the chromatographic column in those methods (11-13) might be too short to separate the drug from a diastereomer, ephedrine and other endogenous or exogenous substances with identical molecular formula and similar chemical structures to the drug. To our knowledge, no assay method is available for the simultaneous determination of levocetirizine and pseudoephedrine in biological fluids.

This study for the first time reports the development of a novel reversed-phase LC-MS assay for the simultaneous determination of

levocetirizine and pseudoephedrine in dog plasma in the presence of dextrocetirizine. The developed assay was successfully applied to a pharmacokinetic study after oral administration of racemic cetirizine (0.5 mg/kg, or 0.25 mg/kg as levocetirizine) and pseudoephedrine (12 mg/kg) in the dog. It was assumed that 50% of the racemic cetirizine was levocetirizine and the other 50% was dextrocetirizine.

EXPERIMENTAL

Chemicals

Levocetirizine dihydrochloride, cetirizine dihydrochloride and pseudoephedrine hydrochloride, were provided by Hanmi Pharmaceutical Co. (Seoul, Korea). Diazepam and

ammonium acetate (NH_4OAc) were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ethyl acetate was obtained from Merck (Darmstadt, Germany). Water used in this study was purified by the Milli-Q-Grade water system (Millipore, Bedford, MA, USA).

Instrumentation

The HPLC was performed with a Waters Alliance HT 2795 Chromatography System (Waters Corp., Milford, MA, USA). Chromatographic separations were achieved on an Ultron ES-OVM chiral analytical column (150 x 4.6 mm, 5 μm , Shinhwa Chemical, Kyoto, Japan) coupled with a guard column containing the identical packing material to that of the analytical column. The mobile phase was a mixture of 10 mM aqueous NH_4OAc (pH 6.6) and acetonitrile (9:1 v/v). The flow rate of the mobile phase was set at 1.0 mL/min (flow was split 20% to the source, and 80% to waste) and the column temperature was held at 30°C.

The HPLC system was coupled to a Waters ZQ 4000 mass spectrometer (Waters Corp., Milford, MA, USA) equipped with electrospray ionization (ESI) source. The ESI source was operated in a positive mode. The source temperature (150°C), de-solvation temperature (200°C), cone voltage (3.00 kV) and capillary voltage (16 V) were optimized. A single ion recording (SIR) was observed at m/z 389.1 for levocetirizine. Dextrocetirizine was also detected at m/z 389.1 but the retention time was different from that of levocetirizine. SIR was observed at m/z 166.1 and 285.0 for pseudoephedrine and diazepam, respectively. The system control and data acquisition was performed using MassLynx 3.5 (Waters Corp., Milford, MA, USA).

Preparation of stock and standard solutions and QC samples

The stock solutions levocetirizine (100 $\mu\text{g/mL}$), pseudoephedrine (100 $\mu\text{g/mL}$) and diazepam (internal standard, 100 $\mu\text{g/mL}$) were prepared by separately dissolving 23.8 mg of cetirizine dihydrochloride, 12.2 mg of pseudoephedrine hydrochloride and 10.0 mg diazepam in 100 mL of water, respectively, and stored at -80°C. The racemic cetirizine rather than levocetirizine (R enantiomer) was used to ensure the separation of dextrocetirizine (S enantiomer) and levocetirizine. The standard working solutions were prepared by serial dilution with 60% acetonitrile, yielding concentrations of 10, 20, 50, 100, 200, 500, 1000

and 2000 ng/mL for levocetirizine and 50, 100, 200, 500, 1000, 2000, 5000 and 10000 ng/mL for pseudoephedrine. The stock solution of diazepam was diluted with 60% acetonitrile to provide the internal standard working solution (concentration, 2000 ng/mL). Quality control (QC) samples of levocetirizine were prepared by spiking the standard working solutions to blank dog plasma to provide low (3 ng/mL), medium (100 ng/mL) and high (160 ng/mL) concentrations and LLOQ (1 ng/mL). Similarly, QC samples of pseudoephedrine were prepared by spiking the standard working solutions to blank dog plasma to provide to provide low (15 ng/mL), medium (500 ng/mL) and high (800 ng/mL) concentrations and LLOQ (5 ng/mL). The prepared QC samples were stored at -20°C until analysis.

Sample preparation

Frozen dog plasma samples were thawed at room temperature. For sample extraction, 50 μL of the internal standard solution (2000 ng/mL) and 0.5 mL each of water and acetonitrile were added to 0.5 mL of dog plasma. After vortex-mixing for 1 min, 3 mL of ethyl acetate was added, and the mixture was vortex-mixed for 5 min and centrifuged for 3 min at 12,000 g . The supernatant (2.5 mL) was transferred to a clean glass tube and evaporated to dryness under nitrogen at 40°C. The residue was dissolved in 150 μL of the mobile phase and centrifuged for 3 min at 12,000 g . The supernatant were transferred to an auto-sampler vial, and a portion (50 μL) was injected into the chromatograph.

Calibration curves

Calibration curves were prepared by spiking blank dog plasma with levocetirizine standard working solutions at concentrations of 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL and with pseudoephedrine standard working solutions at concentrations of 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL. The calibration curves were constructed by the weighted regression method (1/x) of the peak area ratios of drug-to-internal standard vs. actual concentrations. The calibration curves were prepared on a daily basis. To determine the between-run variability of the slopes and intercepts, calibration curves were constructed on three different days.

Linearity

The weighted regression method (1/x) which takes into account the peak area ratios as a function of the theoretical concentrations was applied to each standard curve [$y = ax + b$, where

x = concentration (ng/mL), y = peak area ratio, a = slope and b = intercept]. The equation parameters (slope and intercept) of each standard curve were used to determine concentration values for unknown samples. Concentrations were back-calculated and compared with the nominal concentrations, and the relative concentration residuals (RCRs) were calculated (14).

Specificity, precision and accuracy

The assay specificity was investigated by screening six different batches of blank dog plasma to check whether endogenous components co-eluted with levocetirizine, pseudoephedrine and diazepam. The precision and accuracy were assessed from the results of the QC samples. Each QC sample with four concentration levels was analyzed consecutively six times within one day ($n = 6$) to determine the within-run precision and accuracy, and once a day for six successive days ($n = 6$) to determine the between-run precision and accuracy. The precision was expressed as the coefficient of variance of each concentration, and the accuracy was expressed as the percentage of mean calculated vs. theoretical concentrations.

Determination of quantification limits

The lower limit of quantification (LLOQ) was defined as the lowest plasma concentration that can be measured with acceptable accuracy and precision. The measured concentration of the proposed LLOQ should lie between 80 and 120 % of its theoretical concentration, and the relative standard deviation should be less than 20% (10). The LLOQ was derived from measurements in the low concentration range and determined based on the signal-to-noise (S/N) ratio. A concentration of a S/N ratio > 10 , with precision of $<20\%$ and accuracy between 80 and 120% was used as the LLOQ, which was the lowest concentration point in the calibration curve.

Extraction efficiency and matrix effects

The extraction efficiency was determined three times at the QC concentration levels for levocetirizine and pseudoephedrine and at the concentration level used during the assay for the internal standard (2000 ng/mL). The peak areas of analytes extracted from plasma samples were compared to those obtained from direct injections of the standard solutions prepared in the mobile phase at equivalent concentrations without extraction. Matrix effects were assessed qualitatively by post-column infusion experiments in order to identify chromatographic regions most likely to experience ion suppression or

enhancement of ESI mass spectrometry response in the presence of endogenous plasma interferences (5, 15).

Stability study

The stability of stock solutions was examined at -80°C over a period of 3 months, while that of working solutions containing both levocetirizine (50 ng/mL) and pseudoephedrine (200 ng/mL) was assessed immediately after preparation and a week after storage at room temperature and at 4°C . The short-term stability was tested with QC samples at concentrations of 3 and 160 ng/mL for levocetirizine and 15 and 800 ng/mL for pseudoephedrine in plasma over 24 h at room temperature and at 4°C . The stability of the drug in frozen samples (-80°C) was determined by periodic analysis over 3 months. The freeze-thaw stability of the drug at -80°C was confirmed after three freeze-thaw cycles on consecutive days. The drug was considered stable if the assay values were within the acceptable limits of accuracy and precision ($< 15\%$) (5).

Pharmacokinetic study

The validated assay was applied to a pharmacokinetic study in the dog after oral co-administration of the racemic cetirizine (0.5 mg/kg, or 0.25 mg/kg as levocetirizine) and pseudoephedrine (12 mg/kg). Male beagle dogs (body weight, 9–11 kg) were used in the study. The animals were housed individually with free access to Golden-pet dog diet (Agribrands, Seoul, Korea) and water. The animals were maintained at a temperature of $23 \pm 3^{\circ}\text{C}$ with a 12 h light-dark cycle and a relative humidity of $50 \pm 20\%$. The experiment was carried out after overnight fasting with free access to water. All procedures used in this study were performed in accordance with the Guide for the Care and Use of Laboratory animals, and the work was approved by the Ethics Committee of the Faculty. After drug administration, approximately 2 mL of blood was obtained from the foreleg vein immediately before and 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after administration. Blood samples were centrifuged immediately at 4°C and the harvested plasma samples were stored at -80°C until analysis. Plasma concentration-time data were analyzed by the non-compartmental method using the nonlinear least-squares regression program WinNonlin (Pharsight, Mountain View, CA, USA).

RESULTS

Assay specificity

Typical chromatograms of the extracted blank plasma spiked with levocetirizine, dextrocetirizine, pseudoephedrine and diazepam are shown in Figure 2. Chromatograms of the extracted plasma sample obtained at 90 min after drug administration are shown in Figure 3. The retention times of pseudoephedrine, dextrocetirizine, levocetirizine and diazepam were approximately 5.2, 8.3, 9.6 and 11.6 min, respectively. No endogenous or extraneous peaks were observed interfering with the assay.

Linearity

The calibration curve parameters demonstrate the linear relationship between the peak area ratios vs.

the internal standard over the concentration range from 1 – 200 ng/mL for levocetirizine and 5 – 1000 ng/mL for pseudoephedrine. Linearity was confirmed by the coefficient of determination (r^2) for both levocetirizine ($r^2 > 0.999$) and pseudoephedrine ($r^2 > 0.994$).

Assay accuracy and precision

The within- and between-run accuracy and precisions were assessed from the results of the QC samples (Table 1). The within-run precision showed R.S.D.s of 6.6 – 15.5% for levocetirizine and 6.5 – 16.0% for pseudoephedrine. The between-run R.S.D.s varied from 5.9 – 15.0% (LLOQ) for levocetirizine and 7.7 – 17.9% (LLOQ) for pseudoephedrine. The accuracy ranged from 97.2 to 117.8% for levocetirizine and 97.3 – 117.2% (LLOQ) for pseudoephedrine.

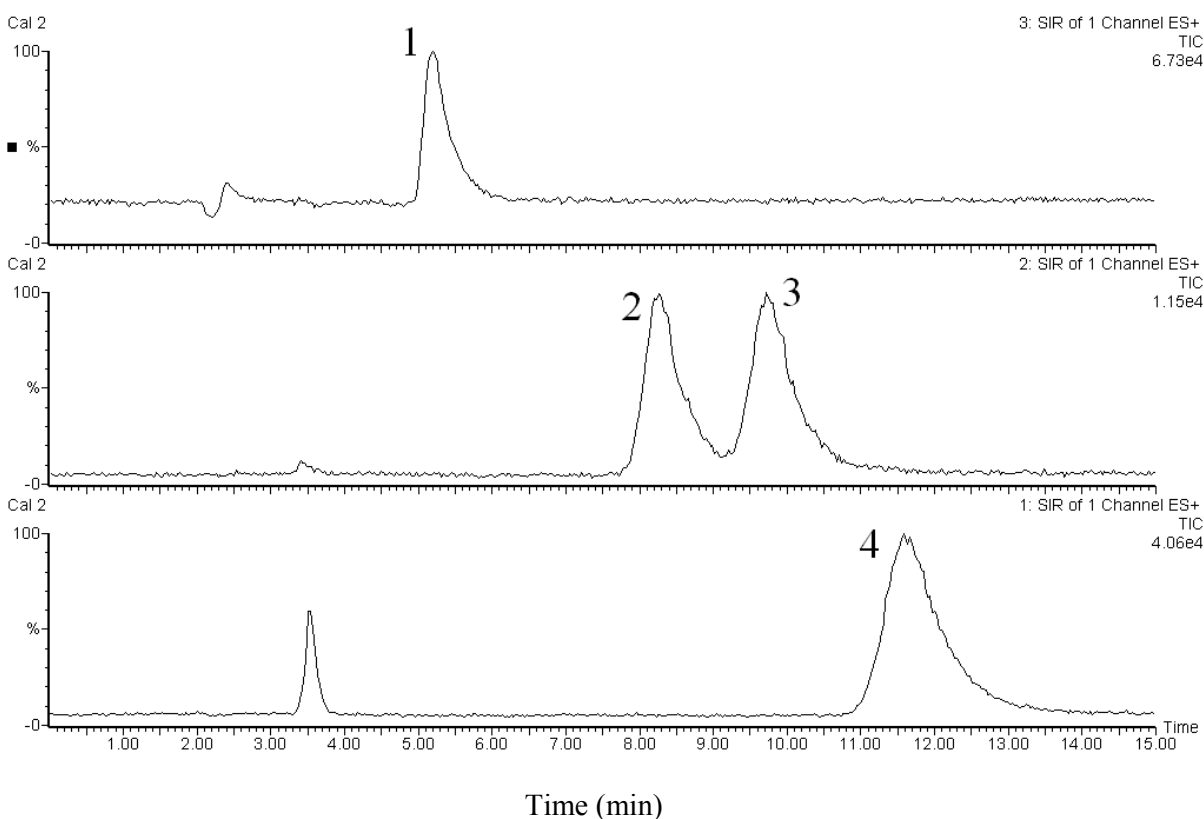


Figure 2. Chromatograms of blank plasma spiked with 200 ng/mL of cetirizine (or 100 ng/mL of dextrocetirizine and 100 ng/mL of levocetirizine) and 500 ng/mL of pseudoephedrine. Peaks 1, 2, 3 and 4 are pseudoephedrine, dextrocetirizine, levocetirizine and diazepam (internal standard), respectively. Their retention times were approximately 5.2, 8.3, 9.6 and 11.6 min, respectively.

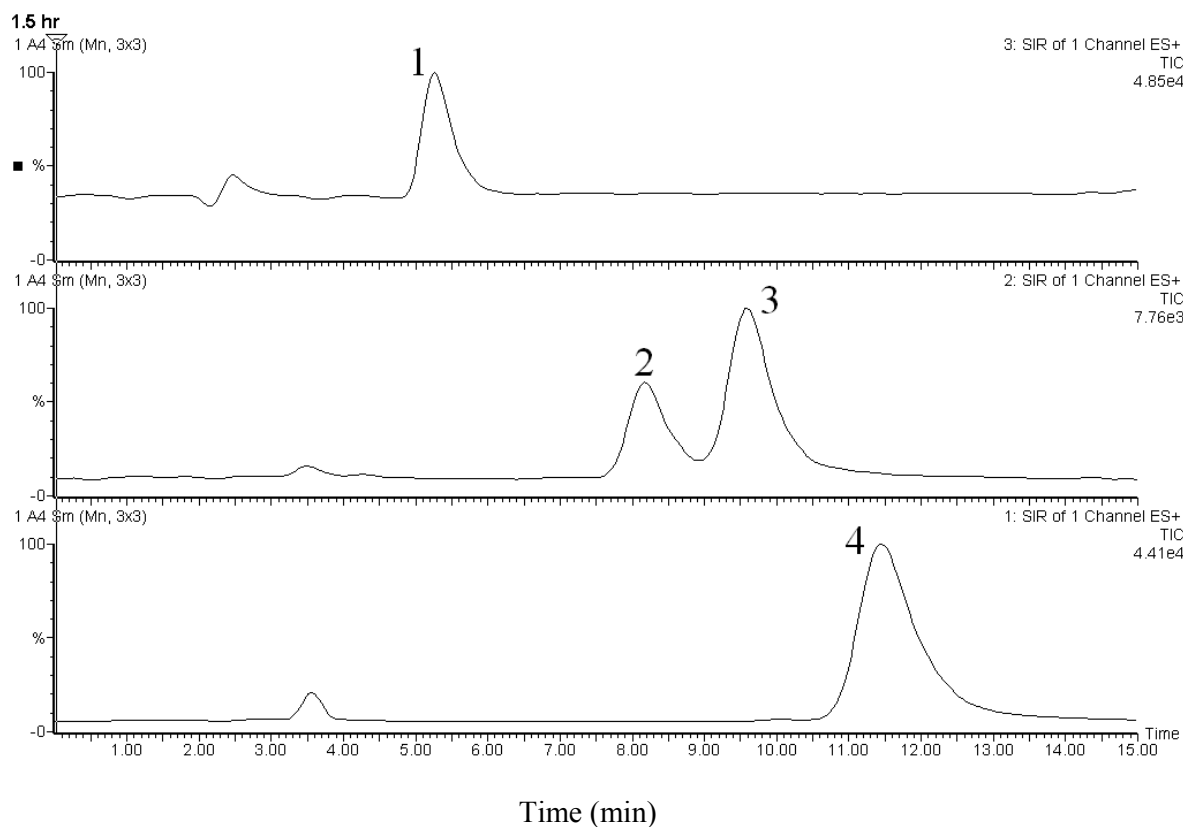


Figure 3. Chromatograms of extracted plasma sample obtained at 90 min after oral administration of the racemic cetirizine (0.5 mg/kg, or equivalent to 0.25 mg/kg of levocetirizine) and pseudoephedrine (12 mg/kg) in the dog. Peaks 1, 2, 3 and 4 are pseudoephedrine, dextrocetirizine, levocetirizine and diazepam, respectively. Their retention times were identical to those of Figure 2.

Table 1. Accuracy and precision data for levocetirizine and pseudoephedrine.

Nominal concentration (ng/mL)	Concentration found (ng/mL)	R.S.D. (%)	Recovery (%)
<i>Levocetirizine</i>			
Within-run (n = 6)			
1	1.1	15.5	111.9
3	3.2	7.3	107.4
100	98.5	9.6	98.5
160	155.6	6.6	97.2
Between-run (n = 6)			
1	1.2	15.0	117.8
3	3.3	11.6	111.2
100	98.9	10.0	98.9
160	163.9	5.9	102.4
<i>Pseudoephedrine</i>			
Within-run (n = 6)			
5	5.7	16.0	117.2
15	15.2	9.6	101.4
500	518.3	8.5	103.7
800	792.7	6.5	99.1
Between-run (n = 6)			
5	5.9	17.9	117.1
15	15.9	6.6	106.0
500	486.3	9.4	97.3
800	795.1	7.7	99.4

Extraction efficiency and matrix effects

The mean extraction efficiency averaged $80.5 \pm 6.7\%$ for pseudoephedrine, $87.0 \pm 5.8\%$ for levocetirizine and $91.3 \pm 5.5\%$ for diazepam (Table 2). The extraction efficiency was within the acceptable range. The post-column infusion system was used to assess matrix effects. There was no significant ion suppression or enhancement at the region for retention time of levocetirizine, pseudoephedrine and diazepam. It is noted that no endogenous substances significantly interfered with the ionization of the analytes.

Lower limit of quantification

The LLOQ for levocetirizine was 1 ng/mL, and the precision ($n = 6$) at this concentration was 15.5%. The LLOQ for pseudoephedrine in dog plasma was 5 ng/mL, and the precision ($n = 6$) at this concentration was 17.9%.

Stability

Stock solutions of pseudoephedrine, levocetirizine and the internal standard were stable at -80°C for 3 months. The stability of the working solution was good at both 4 and 20°C for at least a week. QC samples were stable at -80°C for 3 months. No significant decreases in the concentrations of pseudoephedrine and levocetirizine in plasma were observed during the three freeze-thaw cycles (Table 3). In plasma samples stored at 4°C and 20°C during 24 h, no significant losses were detected for levocetirizine and pseudoephedrine. The percentages of deviation of calculated versus theoretical concentrations at 4°C were less than 4.1% for levocetirizine determined at 3 and 160 ng/mL and less than 4.7% for pseudoephedrine determined at 15 and 800 ng/mL. The deviation of calculated versus theoretical concentrations at 20°C was less than 5.5% for levocetirizine and less than 5.1% for pseudoephedrine.

Pharmacokinetic study

The mean plasma concentration-time profiles of levocetirizine and pseudoephedrine found in dogs after oral administration of the racemic cetirizine (0.5 mg/kg, or 0.25 mg/kg as levocetirizine) and pseudoephedrine (12 mg/kg) are shown in Figure 4. The pharmacokinetic parameter values of the maximum concentration (C_{max}), time to the maximum concentration (T_{max}) and area under the curve (AUC) are shown in Table 4.

DISCUSSION

This study reports the development of a LC-MS assay method for the simultaneous determination of levocetirizine and pseudoephedrine in dog plasma in the presence of dextrocetirizine. The total run time of this assay (<15 min) is shorter than those of the previously reported assays (approximately 40 min) (3,16). Dextrocetirizine could not be obtained in this study, but the peak eluting at approximately 8.3 min (Figs. 2 – 3) was not detected either after oral administration of levocetirizine (0.25 mg/kg) in dogs, or in the blank plasma spiked with levocetirizine (200 ng/mL), pseudoephedrine (1000 ng/mL) and diazepam (data not shown). Therefore, the corresponding peak was regarded as the dextrocetirizine peak.

The LLOQ of this assay (1 ng/mL) is significantly improved compared with those of previously reported HPLC methods, e.g., 2.5 $\mu\text{g/mL}$ for 0.1 mL of rat plasma (2), 400 ng/mL for 5 mL of human urine (16), and 20 ng/mL for 2 mL of human plasma (3). The previously reported LC-MS/MS method allows a sensitive measurement of levocetirizine (LLOQ 0.5 ng/mL for 0.3 mL of human plasma) (5), but this assay has disadvantages including time-consuming two-step sample extraction procedures and normal phase chromatographic separation using organic solvents.

The oral pharmacokinetic studies of levocetirizine, racemic cetirizine or pseudoephedrine in dogs have not been found in the literature to be compared with this study. In this study, however, the peak areas of levocetirizine were larger than those of dextrocetirizine after oral administration of the racemic cetirizine (Figure 3), while the peak intensities of levocetirizine and dextrocetirizine were almost the same in blank plasma spiked with cetirizine (Figure 2). Although plasma dextrocetirizine concentrations were not determined due to the unavailability of the standard material, these concentrations appear to be lower than levocetirizine concentrations after oral administration of the racemic cetirizine in this study. This is consistent with the previous finding that the clearance of levocetirizine is lower than that of dextrocetirizine in human (1).

Table 2. Extraction recovery (%) for levocetirizine, pseudoephedrine and diazepam (mean $\pm$ S.D.) ($n = 3$).

Recovery	Levocetirizine (ng/mL)			Pseudoephedrine (ng/mL)			Diazepam (ng/mL)
(%)	3	100	160	15	500	800	2000
Mean $\pm$	86.8	88.5	85.7	79.0	77.7	84.7	91.3
S.D.	6.0	6.5	7.1	5.9	6.1	8.1	5.5

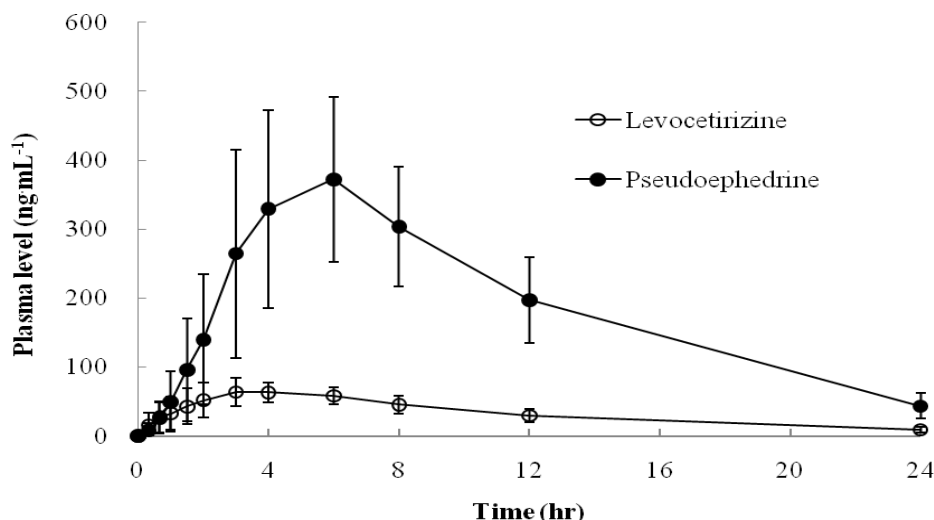
Table 3. Long-term and freeze-thaw stability data for levocetirizine and pseudoephedrine.

Concentration (ng/mL)	Long-term stability Storage period (month)	% Remaining after storage ^a	Freeze-thaw stability Freeze-thaw cycle	% Remaining after freeze- thaw cycles ^a
<i>Levocetirizine</i>				
3	1	98.9 $\pm$ 4.3	1	102.0 $\pm$ 4.7
	2	97.5 $\pm$ 3.1	2	98.5 $\pm$ 5.1
	3	96.7 $\pm$ 3.2	3	93.2 $\pm$ 6.3
160	1	102.5 $\pm$ 4.1	1	99.7 $\pm$ 4.1
	2	98.1 $\pm$ 3.3	2	95.3 $\pm$ 3.2
	3	95.7 $\pm$ 4.9	3	94.1 $\pm$ 5.1
<i>Pseudoephedrine</i>				
15	1	103.1 $\pm$ 5.1	1	99.7 $\pm$ 5.8
	2	99.1 $\pm$ 3.8	2	97.1 $\pm$ 3.6
	3	98.9 $\pm$ 3.2	3	95.0 $\pm$ 3.9
800	1	98.9 $\pm$ 3.4	1	101.0 $\pm$ 2.8
	2	97.1 $\pm$ 4.2	2	98.1 $\pm$ 4.1
	3	99.3 $\pm$ 3.7	3	95.5 $\pm$ 4.3

^a Calculated as the percentage of the initial concentration and expressed as mean $\pm$ S.D. ($n = 3$).

Table 4. Pharmacokinetic parameter values found in dogs ($n = 6$) after oral administration of cetirizine (0.5 mg/kg, or equivalent to 0.25 mg/kg) and pseudoephedrine (12 mg/kg).

Parameters	Levocetirizine (0.25 mg/kg)	Pseudoephedrine (12 mg/kg)
$C_{\max}$ (ng/mL)	74.4 $\pm$ 11.7	396.5 $\pm$ 106.2
$T_{\max}$ (hr)	3.6 $\pm$ 1.6	5.3 $\pm$ 1.4
AUC (ng·hr/mL)	791.6 $\pm$ 188.0	4437.2 $\pm$ 1278.2
$t_{1/2}$ (hr)	6.8 $\pm$ 1.1	5.6 $\pm$ 0.7

**Figure 4.** Plasma concentration-time profiles of levocetirizine and pseudoephedrine after oral co-administration of the racemic cetirizine (0.5 mg/kg, or equivalent to 0.25 mg/kg of levocetirizine) and pseudoephedrine (12 mg/kg) to beagle dogs ($n = 6$).

CONCLUSIONS

In summary, a rapid and sensitive LC-MS method capable of enantioselective separation of cetirizine was developed for the simultaneous quantitation of levocetirizine and pseudoephedrine concentrations in dog plasma. This method is simple, reproducible and specific and allows for the separation of pseudoephedrine, levocetirizine and diazepam from other components in plasma samples, including dextrocetirizine. To date, it is the first achiral and chiral method allowing for the simultaneous determination of pseudoephedrine and levocetirizine concentrations in dog plasma in the presence of dextrocetirizine.

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