Arch Pharm Res Vol 29, No 6, 514-519, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

HPLC Determination and Steady-State Bioavailability Study of Levodropropizine Sustained-release Tablets in Dogs

Lin Yan, Tongling Li[1], Rongqin Zhang[1], Xiaohong Xu[1], and Pengcheng Zheng[1]

Center For Drug Reevaluation, State Food and Drug Administration, Building 11, Fa-Hua-Nan-Li, Chongwen District, Beijing, China and [1]Division of Clinical Pharmacy, West China College of Pharmacy, Sichuan University, No. 17, Ren Min Nan Road 3 Section, Chengdu 610041, China

(Received July 27, 2005)

A simple HPLC method using UV detection was developed and validated for the determination of levodropropizine (LDP) in dog plasma. The sample was prepared for injection using a liquid-liquid extraction method with 1-phenypiperazine as the internal standard. The mobile phase was methanol - diethylamine solution (0.05 M) (20:80, v/v, pH adjusted to 3.0 with H_3PO_4) with a detection wavelength of 240 nm. The limit of quantitation (LOQ) of LDP in a biological matrix was determined to be 25.25 ng/mL. The calibration curve was linear across the concentration range of 25.25 to 2020 ng/mL. The intra-day and inter-day precision values (CV %) were within 7% and accuracy (R.E. %) was within 6% of the nominal values for medium (252.5 ng/mL) and high (2020 ng/mL) LDP concentrations. For the LDP concentration at the LOQ, the intra-day and inter-day precision and accuracy were within 20% and 10%, respectively. The average absolute recovery for LDP was 70.28%. This method was successfully used to analyze plasma samples in a steady-state bioavailability study of a newly developed sustained-release LDP tablets (SR) using immediate-release tablets (IR) as the reference. The relative bioavailability of the SR was determined to be 106.3 ± 12.8% (n=6). The C_{max} of the SR was significantly lower ($P<0.05$), and the t_{max} was significantly longer than that of the IR ($P<0.05$). The results of ANOVA and two one-sided tests indicated that the SR exhibited acceptable sustained release properties and was bioequivalent to the IR.

Key words: Levodropropizine, Bioavailability, Sustained-release

INTRODUCTION

Levodropropizine (LDP), (2*S*)-3-(4-phenylpiperazin-1-yl) propane-1, 2-diol (Fig. 1), is an anti-cough drug with a peripheral mode of action that has been developed by Dompe S.p.A (Italy). In several pharmacological and clinical studies, LDP has been shown to exhibit an antitussive activity comparable to dropropizine, with a reduced sedative effect on the central nervous system (Malandrino *et al.*, 1988; Melillo *et al.*, 1988). A sustained-release formulation would further improve the safety profile of LDP and allow for a more convenient dosing regimen as compared to immediate-release formulations.

After oral administration to the dog, LDP is absorbed from the intestine, undergoes first pass metabolism to reach peak plasma concentrations (Cmax, 270 ng/mL) at approximately 60 min (2 mg/kg, Hu *et al.*, 2000). In a study involving six healthy volunteers administered 60mg of LDP, Cmax was reached within 40 to 60 min, with individual Cmax values of 500, 920, 610, 960, 390, 470 ng/mL, (average 641 ng/mL, Zaratin *et al.*, 1988). The pharmacokinetics of LDP were linear over a wide range of doses in 12 healthy male volunteers (30 to 90 mg); dose-dependent increases of Cmax and area under the plasma concentration time curve (AUC) were observed without concomitant changes in the elimination half-life (Borsa *et al.*, 1991).

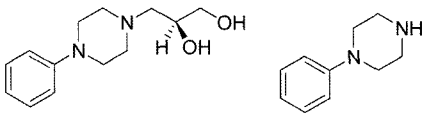
Compared with the immediate-release formulations, a sustained-release formulation would offer three potential benefits: 1. Sustained blood levels. The slower the rate of absorption, the less will the blood concentrations fluctuate within a dosing interval. For LDP, a sustained-release formulation may maintain therapeutic concentrations over prolonged periods of time. 2. Further improve the safety profile. With conventional dosage forms, high peak blood

Correspondence to: Lin Yan, Center For Drug Reevaluation, State Food and Drug Administration, Building 11, Fa-Hua-Nan-Li, Chongwen District, Beijing, China
Tel: 86-10-67102266; Fax: 86-10-67164985
E-mail: yanlin@cdr.gov.cn

concentrations may be reached soon after administration with possible adverse effects related to the transiently high concentration levels in the blood. 3. Improved patient compliance. LDP needs to be taken at frequent time intervals to maintain effective blood concentrations within the therapeutic range. A reduction in the number of daily doses offered by a sustained-release formulation has the potential to improve patient compliance.

Zarantin, *et al.* (1988) have reported on a gas chromatographic-mass spectrometric (GC-MS) method that enables the measurement of levodropropizine concentrations in the plasma of humans. This GC-MS method was found to be extremely sensitive (5 ng/mL) and specific, but unfortunately sophisticated instrumentation was required and the derivatization procedure with β-bis (trimethylsilyl) trifluroacetamide was found to be difficult and tedious. Tagliaro, *et al.* (1996) have more recently developed a high-performance liquid chromatographic method using fluorescence detection. This method was validated and is both simple and specific; however, a large volume of toxic organic solvent (5 mL of chloroform-2-propanol for each sample) must be used in sample preparation. For both of the preceding analytic methods *p*-methoxylevodropropizine was used as an internal standard (I.S.).

The present paper describes a simpler specific and accurate HPLC-UV method for measuring LDP in dog plasma with 1-phenypiperazine used as the I.S. (Fig. 1). This method has been validated according to the criteria established by the State Food and Drug Administration of China and was successfully applied to a multiple-dose steady state bioavailability study of newly developed LDP sustained-released tablets in dogs (SFDA: Guidance, 2004).



A. levodropropizine B. 1-phenylpiperazine

Fig. 1. Chemical structures of levodropropizine (A) and I.S. (B)

EXPERIMENTAL

Chemicals and reagents

LDP immediate-release tablets (IR, 30 mg/tablet, No. 030801) were used as the reference formulation and were purchased from the Shenzhen Haiwang Drug Company (Shenzhen, China); LDP sustained-release tablets (SR, 90 mg/tablet, No. 030627) were used as the test formulation and were developed by a pharmaceutical laboratory at the Sichuan University (Sichuan, China). The LDP (99.58% purity) and 1-phenypiperazine (I.S.) were supplied by the Yunnan Yikang Drug Company (Yunnan, China). HPLC grade methanol and other reagents of analytical grade were purchased from the Chengdu Reagent Company (Sichuan, China).

HPLC system

The chromatographic HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-10ATvp pump, and an SPD-10Avp UV detector set at 240 nm. The analytical column used for these studies was a Diamonsil C18 5 μm, 4.0 mm × 200 mm. The mobile phase was methanol and 0.05 M diethylamine solution (20:80, v/v, pH adjusted to 3.0 with H_3PO_4). The mobile phase flow-rate was maintained at 1 mL/min with a column temperature of 37°C.

Standard solutions

The LDP stock solution was prepared as a 0.202 mg/mL solution in redistilled water. Standards for the calibration curves and QC samples were prepared using serial dilutions of this stock solution in drug-free dog plasma. The standard solution of 1-phenypiperazine (I.S.) was 0.05 mg/mL in redistilled water with 20 μL of this solution added to plasma samples prior to extraction.

Sample preparation

One mL each of the plasma samples (with heparin as an anticoagulant) were spiked with 20 μL of 0.05 μg/mL I.S., 100 μL 0.1M NaOH and extracted with five mL of ethyl acetate. After vortex mixing for one min and centrifugation (at 4,000 rpm) for five min, four mL of the organic phase was collected and evaporated under a nitrogen stream. The residue was dissolved with 200 μL of the mobile phase and a sample of 50 μL was injected.

Method validation

The HPLC-UV method was validated according to previously established criteria (SFDA: Guidance, 2004; Bressolle *et al.*, 1996; Causon, 1997; Hartmann *et al.*, 1998). Calibration curves were developed using standards of LDP in plasma with six concentrations varying between 25.25 ng/mL and 2020 ng/mL. The calibration curves were generated by plotting the ratio of the peak area of the LDP and I.S. against the LDP concentration in plasma. Linear regression techniques were used to assess the calibration curves and the determination of the correlation coefficients.

The limit of quantitation (LOQ) was defined as the LDP concentration at which: (1) the response was 10 times the response for the blank plasma and, (2) a concentration at which there was reproducible precision (coefficient of variation of less than 20%) and accuracy (measured concentration within 20% variation of the nominal concentration).

QC samples were concentrations of 25.25, 252.50, and 2020 ng/mL of LDP in dog plasma representing the limit

of quantitation (LOQ) and the medium and high concentration quality controls, respectively. The intra- and inter-day accuracy and precision were determined for each of these concentrations. The intra-day accuracy and precision were determined using five replicates of QC samples on the same day; the inter-day accuracy and precision were determined by analyzing three calibration curves with five replicates of QC samples on three separate days.

The recovery of LDP was assessed by comparing the response of five replicates of extracted QC samples (at LOQ, mid and high ranges) to the response of the pure standard at the same concentration level. The recovery of I.S. was determined by comparing the mean response levels of 15 I.S. samples to the responses of the pure standard.

To assess the analyte stability, the accuracy and precision of the measured concentrations were compared to the nominal concentrations. The calibration standard and QC samples were prepared on the day of the analysis. Six stored replicates of the low (25.25 ng/mL) and high (2020 ng/mL) concentrations of LDP in dog plasma were analyzed for each storage condition. Short-term temperature stability was assessed at room temperature (21°C) for 8 h. Freeze-thaw stability was evaluated after three cycles of thawing at room temperature followed by re-freezing to -20°C. Long-term stability evaluations involved an analysis of QC samples that were stored at -20°C for 30 days. Post-preparation stability was also determined by using the extracted samples that were kept at room temperature for 12 h prior to injection and analysis by HPLC.

Pharmacokinetic study

The bioavailability study was designed as a multiple-dose, steady-state two-treatment, two-period, two-sequence crossover with a 10-day washout period between the two phases of the study. Six dogs (4 male, 2 female) weighing approximately 10 kg each were randomly assigned to one of the two dosing sequences and scheduled to receive either 90 mg sustained-release tablets (SR) twice daily for five days, or 60 mg immediate-release tablets (IR) thrice daily for five days and then crossed over to the alternative regimen following washout. The total daily dose of the two formulations was 180 mg. The sustained-release tablets were administered at 7 AM and 7 PM each day, and the immediate-release tablets were administered at 7 AM, 3 PM and 11 PM each day. The blood samples (1 mL) taken from dogs administered the oral sustained-release tablets were collected before administration and at 48, 72, 96, 96.5, 97, 97.5, 98, 98.5, 99, 100, 102, 105, and 108 hours after the initial oral dose. The blood samples (1 mL) taken from dogs administered the oral immediate-release tablets were collected before administration and at 48, 72, 96, 96.25, 96.5, 96.75, 97, 97.25, 98, 99, 100, 102, and 104 h after the initial oral dose. Three trough concentrations collected at 48, 72, and 96 h after the initial oral dose (on three consecutive days) were determined so as to ascertain that the subjects were at steady state. Samples were collected into heparinized tubes and centrifuged at 3,000 rpm for 5 min to separate the plasma, which was stored at - 20°C until analysis.

Statistical analyses

Peak drug concentration (C_{max}) and the time to peak drug concentration (t_{max}) were determined directly from the data. Area under the plasma concentration-time curve was from time zero to time τ over a dosing interval at steady state ($AUC_{0-\tau}$) which was calculated by the trapezoidal rule, where τ is the dosing interval. The average drug concentration at steady state (C_{av}) was calculated by $AUC_{0-\tau}$ divided by τ. Degrees of fluctuation (DF) at steady state were calculated as follows: DF = 100% × (C_{max} − C_{min}) / C_{av}, where C_{min} was the observed trough concentration over the last dosing. The relative bioavailability of SR (F) was calculated by

$$F = \frac{AUC_{0-\tau(SR)} \times D_{(IR)}}{AUC_{0-\tau(IR)} \times D_{(SR)}} \times 100\%$$

$D_{(IR)}$ was the dose of IR tablets (60 mg) and $D_{(SR)}$ was the dose of SR tablets (90 mg). A dosing adjustment was performed on the $AUC_{0-\tau}$ of the SR, which was calculated by $AUC_{0-\tau(SR)} \times 60/90$ when comparing the two formulations (SFDA: Guidance, 2004).

Analysis of variance (ANOVA) was performed on $AUC_{0-\tau}$, C_{max}, C_{min}, C_{av} (*log* transformed) and t_{max} using SPSS General Linear Model procedures, and $P' < 0.05$ was considered statistically different. Two one-sided hypotheses at α=0.05 level of significance were tested for $\ln AUC_{0-\tau}$ and $\ln C_{max}$ by constructing the 90% confidence interval for the ratio between the test and reference (SFDA: Guidance, 2004; FDA: Guidance, 1997). Linear regression analysis of the trough concentrations (48, 72, and 96 h) versus time was performed to test the steady state. The means of the individual slopes for each preparation were tested by Student's t test versus a value of zero.

RESULTS AND DISCUSSION

Specificity

The plasma from six dogs was tested for interference and showed no endogenous interference at the retention times of LDP and the internal standard. Representative chromatograms of a blank plasma sample, plasma containing LOQ and medium concentration of LDP and a plasma sample at 0.5 h after the last dose (60 mg) of IR of LDP at a steady-state are shown in Fig. 2. Retention times of LDP and I.S. were 7.6 and 9.1 min, respectively.

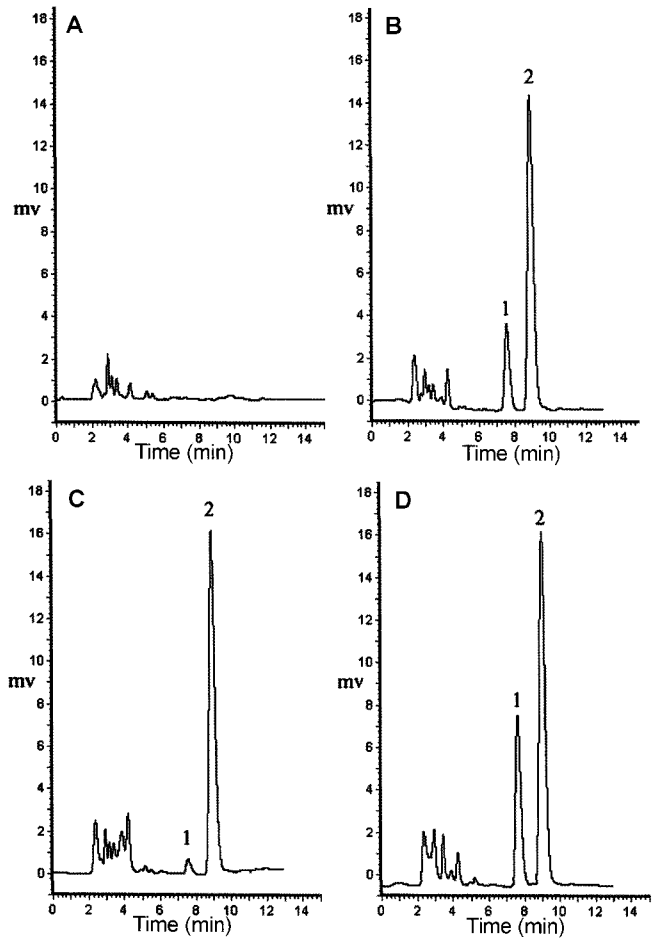


Fig. 2. chromatograms for the measurement of LDP in dog plasma: (A) blank plasma; (B) plasma sample containing 252.5 ng/mL of LDP; (C) plasma sample containing 25.25 ng/mL of LDP; (D) plasma sample at 0.5 h after administration of IR of LDP at a dose of 60 mg. Peaks: (1) LDP; (2) I.S.

LOQ, linearity and range

The LOQ for LDP was identified to be at 25.25 ng/mL. A linear response was observed for the peak area ratio versus LDP concentration over a concentration range of 25.25-2020 ng/mL. The data for five measurements of the calibration curve are presented in Table I, and indicates linearity within the examined concentration range.

Precision, accuracy and recovery

The intra-day and inter-day precision and accuracies were well within the limits for acceptance with coefficients of variation (CV%) or relative error (R.E. %) of less than 10% for the medium and high QC samples and 20% for the LOQ Sample (Table II). The recovery of LDP was consistently greater than 65% at all QC concentrations tested with a mean recovery of 70.28%. The mean recovery for I.S. was 61.70%. Results are shown in Table III.

Table I. The linearity of calibration curves of LDP concentration in dog plasma

	run	Slope$\times10^{-2}$	intercept$\times10^{-2}$	Correlation coefficient
calibration curves	1	0.10	0.81	0.9999
	2	0.10	0.50	0.9999
	3	0.12	0.47	0.9998
	4	0.11	0.39	0.9997
	5	0.10	0.24	0.9994

Table II. Results of intra-day and inter-day validation in dog plasma

Concentration added (ng/mL)	Concentration founded (ng/mL)	CV (%)	R.E. (%)	n
Intra-day				
25.25	24.02± 1.64	6.81	-4.87	5
252.5	252.53± 10.47	4.15	+0.01	5
2020	1904.48± 69.64	3.66	-5.72	5
Inter-day				
25.25	24.62± 3.78	15.37	-2.50	15
252.5	255.56± 15.85	6.20	+1.21	15
2020	2037.01±101.56	4.99	+0.84	15

R.E. %, relative error

Table III. Extraction recovery of LDP and internal standard

Concentration (ng/mL)	Extraction Recovery (%)	CV (%)	n
Low(25.25)	66.64	3.25	5
Mid(252.5)	74.44	2.45	5
High(2020)	69.77	3.55	5
I.S.(1000)	61.70	5.03	15

Stability

Acceptable analyte stability was demonstrated for all phases of storage and processing. The accuracy of LOQ (25.25 ng/mL) and high (2020 ng/mL) concentrations of LDP in dog plasma for 8 h room temperature stability (93 and 95%), three cycle freeze-thaw stability (94 and 97%), 30 days at -20°C stability (89 and 92%) and 12 h post-preparative stability (108 and 102%) were all acceptable. The CV (%) for each of the stability experiments varied between 3 and 12%.

Pharmacokinetic study

The three trough concentrations collected at 48, 72, and 96 h after the initial oral dose were 65.45, 59.79, 68.08 ng/mL (SR tablets), and 33.07, 36.31, 47.90 ng/mL (IR tablets); data consistent with a steady state. The mean plasma concentration-time curves of the two formulations from 96 to 108 hours are shown in Fig. 3; the mean values of pharmacokinetic parameters at steady state are

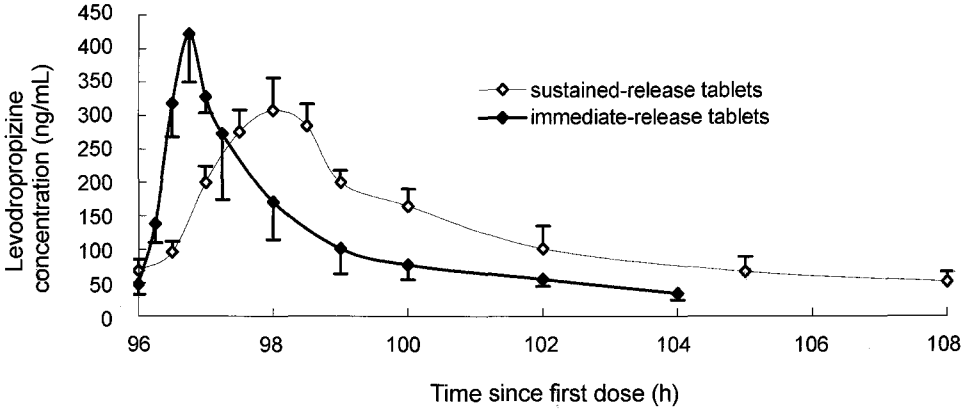


Fig. 3. Steady-state mean plasma concentrations of LDP from 96 to 108 h following administration of LDP SR tablets twice daily (dose: 90 mg) and IR tablets thrice daily (dose: 60 mg) in dogs.

summarized in Table IV.

The t test of the mean slopes of the trough regression lines versus zero (test for steady state) indicated that both SR and IR had slopes not significantly different from zero ($p>0.05$). The mean slope for SR and IR were 0.055 and 0.031, respectively.

ANOVA and two-one sided tests (Table V) indicated no statistical differences between the two preparations for *ln* $AUC_{0-\tau}$ but were statistically different for *ln*C_{max} and t_{max} ($p<0.05$). The C_{max} of SR was significantly lower than that of IR and the t_{max} was significantly prolonged, which suggested that the new SR formulation possessed acceptable sustained release properties. The relative bioavailability of the SR was 106.3 ± 12.8% (n=6), and the 90% CI of ln $AUC_{0-\tau}$ was 95.2~117.1% of the reference formulation, which lie within the acceptable range of 80~125%.

Table IV. The mean values of main pharmacokinetic parameters after multiple oral doses of LDP IR and SR tablets

Parameter	SR	IR	Difference (%)
$AUC_{(0-\tau)}$ (ng·h/mL)	1009.7* ±379.3	947.6 ±347.9	6.55
C_{max} (ng/mL)	349.06±150.31	452.03±270.20	-22.78
C_{min} (ng/mL)	51.89± 14.83	34.81± 10.04	49.07
C_{av} (ng/mL)	126.21± 47.41	118.45± 43.49	6.55
t_{max} (h)	2.17± 0.41	0.67± 0.13	223.88
$MRT_{(0-\delta)}$(h)	4.49± 0.35	2.58± 0.30	74.03
DF (%)	232 ± 47	330 ± 98	-29.69

Difference (%) = 100 % × (SR-IR)/IR; * Dose Adjusted Value

Table V. ANOVA and two-one sided test results of the parameters after multiple-dosing with LDP SR and IR tablets

Parameters	ANOVA tests (F value)			Two-one sided tests		
	preparation	period	individual	t_1	t_2	90%CI
$lnAUC_{(0-\delta)}$	1.24	1.98	35.19*	5.69*	3.47*	95.2-117.1
lnC_{max}	10.0*	1.05	37.64*	0.32	6.64*	71.2- 93.6
lnC_{min}	23.81*	0.0004	5.93			
lnC_{av}	1.24	1.98	35.19*			
t_{max}	72.0*	0.00	1.16			
DF	12.25*	0.52	4.16			

$F_{0.05\,(5,\,4)}$ =6.26; $F_{0.05\,(1,\,4)}$ =7.71; $t_{(1-0.05)4}$=2.132
*P<0.05

CONCLUSION

A simple HPLC-UV method was developed and validated for the determination of LDP in dog plasma. This method was successfluly applied to the analysis of plasma samples in a steady state bioavailability study of a newly developed LDP SR tablet. Based on the results of the bioavailability study, it can be concluded that the LDP SR tablets exhibited acceptable sustained release properties and was bioequivalent to the reference formulation.

REFERENCES

Borsa, M., Glavenna, G., Ferrari, M. P., Canali, S., Giachetti, C., and Zanolo, G., Linearity of levodropropizine, a new antitussive drug, in the healthy volunteer. *Eur. J. Drug. Metab. Pharmacokinet.*, 3,178-184 (1991).

Bressolle, F., Bromet-Petit, M., and Audran, M., Validation of liquid chromatographic and gas chromatographic methods applications to pharmacokinetics. *J. Chromatogr. B Biomed. Appl.*, 686, 3-10 (1996).

Causon, R., Validation of chromatographic methods in biomedical analysis. Viewpoint and discussion. *J. Chromatogr. B Biomed. Sci. Appl.*, 689, 175-180 (1997).

FDA, Guidance for Industry: Statistical Approaches to Establishing Bioequivalence. Center for Drug Evaluation and Research (CDER), United States Food and Drug Administration, (1997).

Hartmann, C., Smeyers-Verbeke, J., Massart, D. L., and McDowall, R. D., Validation of bioanalytical chromatographic methods. *J. Pharm. Biomed. Anal.*, 17,193-218 (1998).

Hu, X. Z., Yu, J. X., and Lin, Y. F., Determination of levodropropizine in serum by tandem gas chromatography-mass spectrometry. *Analysis And Testing Technology Andinstruments*, 6, 228-231 (2000).

Malandrino, S., Melillo, G., Bestetti, A., Borsa, M., Giuliani, P., and Tonon, G. C., Antitussive properties of levodropropizine. *Arzneimittelforschung.*, 38, 1141-1143 (1988).

Melillo, G., Malandrino, S., Rossoni, G., Caselli, G., Bestetti, A., Borsa, M., Tonon, G. C., and Berti, F., General pharmacology of the new antitussive levodropropizine. *Arzneimittelforschung.*, 38, 1144-1150 (1988).

SFDA, Guidance: Bioavailability and Bioequivalence studies for drug products in human being. The State Food and Drug Administration of China, (2004).

Tagliaro, F., Moffa, M., De Battisti, Z., Smith, F. P., and Gentile, M., High-performance liquid chromatographic determination of levodropropizine in human plasma with fluorometric detection. *J. Chromatogr. B Biomed. Appl.*, 685, 165-170 (1996).

Zaratin, P., De Angelis, L., and Cattabeni, F., Gas chromatographic-mass spectrometric determination of levodropropizine plasma levels in healthy volunteers. *Arzneimittelforschung.*, 38, 1156-1158 (1988).