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Short communication

Development of an HPLC method for determination of diphenidol in plasma and its application in an oral multi-dose bioequivalence study in a healthy female mexican population

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Abstract

Diphenidol was determined by an HPLC method developed in our laboratory. It was validated and proved to be linear in the 40–400 ng/ml range. Accuracy for quality-control samples for intra and inter day assays ranged from 96.1–98.9% and 98.8–101.4%, respectively. This method was applied to a multi-dose bioequivalence study. No serious side effects were observed in the multi-dose design. Pharmacokinetic parameters (mean ± standard error [S.E.]) of Cavg (ng/ml) and AUC_{tau} (ng h/ml) for reference and test products were 139.54 ± 12.66 versus 148.60 ± 16.51 and 551.07 ± 53.53 versus 588.78 ± 69.02, respectively. Log-transformed values were compared by analysis of variance (ANOVA) followed by the classical 90% confidence interval (CI 90%) test and Schuirmann's test. Confidence limits ranged from 91.48–116.18% for C_{max} and from 91.24–117.65% for AUC_{tau}. These results suggest that the analytical method was linear, precise, and accurate for our purpose, and that both assayed formulations were bioequivalent.

Keywords: Diphenidol pharmacokinetics; Reversed-phase HPLC; Multi-dose clinical trial

1. Introduction

Diphenidol (DPN) [1,1-diphenyl-4-piperidino-1-butanol hydrochloride] (Fig. 1) [CAS No. 3254-89-5] is an non-phenothiazinic antiemetic agent employed for some time as a treatment for vomiting and vertigo, principally in patients with Meniere's disease and labyrinthopathies. DPN has been also used as a prophylactic against nausea and vomiting during surgery, cancer chemotherapy, and radiotherapy. The mechanism by which diphenidol exerts its antiemetic and antivertigo effects is not precisely known. It is thought to diminish vestibular stimulation and depress labyrinthine function. Action on modulating the chemoreceptive trigger zone may also be involved in the effect. DPN also possesses a weak peripheral antimuscarinic action [1,2]. It has been reported to cause serious adverse effects including hallucination and confusion (usually within the third day of therapy or at elevated doses) and occasionally drowsiness, dry mouth, depression, restlessness, headache, and transitory hypotension [1–3].

Following oral administration of DPN, peak concentrations – usually achieved between 1.5 and 3 h and with elimination half-life of approximately 4 h – have been reported [1]. However, no information with regard to its pharmacokinetic profile has been reported, in part due to the fact that determination of DPN in plasma by any method has always been hampered by the problem of stet selectivity and sensitivity due to poor detectability, in that its molar absorption coefficient in UV region is very low. Moreover, the structure does not present either fluorescence or electrochemical properties that can be used for detection by these conventional techniques.

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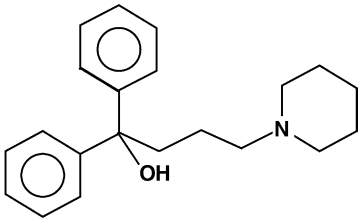

Fig. 1. Structure of diphenidol.

Thus, the aim of present work was to describe a reliable and simple high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection for quantitative determination of DPN in human plasma and its application in a multi-dose clinical trial for bioequivalence purposes.

2. Materials and methods

2.1. Drug and reagents

DPN hydrochloride was USP standard-grade. Triethylamine was reagent-grade and was purchased from Sigma (St. Louis, MO, USA). Acetonitrile, methanol, ethyl ether, and isobutyl alcohol were HPLC-grade and were purchased from J.T. Baker (Xalostoc, México), while sodium carbonate, hexane, and phosphoric acid were reagent-grade and were purchased from Tecsiquim (Mexico City). Water used during this study was HPLC grade (>18 MΩ cm) obtained for ultrafiltration from Milli-Q system prior to use (Millipore, México). DPN hydrochloride tablets 25 mg, test and reference, were all obtained from Mexican pharmaceutical companies. Human blank plasma comprised a pool obtained from our hospital's blood bank.

2.2. Chromatographic conditions

The HPLC system consisted of pump (model 515), autosampler (model 717), a dual-wavelength UV detector (model 2487) (Waters Inc., Milford, MA, USA), and a degasser (model Degasit) (Metachem, Torrance, CA, USA). Separation was performed on a reversed-phase column (Xterra® RP8 4.6 mm × 150 mm, 3.5 μm) (Waters Inc.) maintained at 40 °C by a temperature control module (Metatherm™, Metachem), while the autosampler was kept at 10 °C. The Mobile phase consisted of an isocratic mixture of 0.3%, (v/v) triethylamine aqueous solution pH = 3 (adjusted with phosphoric acid) and acetonitrile (70:30, v/v), and was pumped at a constant flow rate (1.3 ml/min); eluent absorbance was monitored at 210 nm. Data were obtained and processed using Millennium® 32 software (Waters Inc.). Standard calibration curves were constructed by spiking drug-free human pool plasma with known amounts of DPN at concentrations of 40, 60, 100, 150, 250, 300, and 400 ng/ml.

2.3. Validation assay

Validation was carried out following the criteria established in the Mexican regulatory guidelines [4]. Intra-assay validation was performed with spiked plasma ($n = 5$) at low, medium, and high concentration levels on the calibration curve (75, 125, and 275 ng/ml). Inter- and intra-day precision and accuracy were determined. Selectivity, stability, limit of quantification, and limit of detection were also evaluated. Absolute extraction recovery of DPN was determined by comparing heights of the respective peaks from control points extracted above an aqueous calibration curve.

2.4. Determination of diphenidol in plasma

Briefly, 2 ml of plasma (calibration curve or unknown samples) were pipetted into a 12-ml glass centrifuge tube and alkalinized by addition of 1 ml of Na_2CO_3 (0.1 M). DPN was extracted using 4 ml of a cold mixture of hexane:ethyl ether:isobutyl alcohol (70:25:5, v/v) and was vortex-mixed for 1 min followed by centrifugation at 1370 × *g* for 10 min (4 °C); tubes were placed into an ultra-freezer for 10 min at −70 °C. The organic phase was transferred to another tube and evaporated to dryness at 40 °C under N_2 The residue was reconstituted with 125 μl of a mixture of acid water:acetonitrile (70:30, v/v), and 100 μl were applied onto the HPLC system.

2.4.1. Clinical design

2.4.1.1. Subjects. Twenty-two healthy female Mexican volunteers with mean age (±standard error [S.E.]) of 22.8 (±0.74) years were included for participation in our study according to ethical guidelines. Participants were excluded from the study if one of more of the following criteria were present at time of screening: allergic history to DPN; history or clinical data of renal of liver disease; positive test for presence of hepatitis B, HIV, or pregnancy; history of addiction (alcohol and/or drugs); use of any drug during the 14 days prior to day 1 of the study; participation in another clinical trial within 30 days of initiation of the previous study, or if the participant had donated blood within 72 days prior to the study.

Drug administration and sample collection. Participants were admitted to the Clinical Pharmacology Research Center at 8:00 p.m. At that time, blood samples were collected as predose samples ($t = 0$) and participants ate dinner at 9:00 p.m. Subjects received a charge dose of 50 mg and two more doses each of 25 mg of DPN 4 and 8 h after the initial dose, all doses was taken with 250 ml of water. All subjects received two oral formulations containing DPN in a two-period, two-sequence, double-blind, crossover, randomized trial and were separated for a 1-week washout period between each session. Blood samples (10 ml) were collected from volunteers at 8, 8.33, 8.66, 9, 9.5, 10, 10.33, 10.66, 11, and 12 h after the first dose. Blood was immediately centrifuged (1790 × *g*, 10 min) and plasma samples were stored at −70 °C until assayed.

2.5. Pharmacokinetic and statistical analysis

Profiles of individual plasma concentrations versus time for steady-state were constructed. Pharmacokinetic parameters were calculated considering a multi-dose design in a non-compartmental model using a WINNONLIN™ Version 3.1 computer software [8] with a 4 h dose interval (tau). Maximum and minimum observed concentration (C_{max} and C_{min}) and the time to reach C_{max} (T_{max}) were experimentally obtained by observation; area under curve to last measurable concentration (AUC_{tau}), clearance in steady-state (CL_{ss}/F), minimum concentration (C_{min}), average concentration (C_{avg}), half-life ($t_{1/2}$), and % fluctuation were software outputs. ANOVA for a standard 2 × 2 crossover design was used to evaluate fixed effects such as period, sequence, formulation, and carryover. For bioequivalence analysis, C_{max}, C_{avg}, and AUC_{tau} were considered for constructing the classical 90% confidence interval (CI 90%) with significance level of 0.05 and assuming normal data distribution. Moreover, interval hypothesis based on Schuirmann's procedure was tested, with a significance level of 0.05. Both data analyses were conducted according to FDA and Mexican recommendations for establishing bioequivalence [4,9]. All statistical procedures were performed with SAS/STAT® Version 8.2 software [10].

3. Results and discussion

To measure DPN in plasma, we developed a novel sensitive, precise, and accurate HPLC method that proved suitable for pharmacokinetic studies. We tested different solvent mixtures for extraction of DPN in plasma, such as methyl-*t*-butyl ether, hexane-ethyl ether-isobutyl alcohol, ethyl ether, *n*-hexane-isobutyl alcohol, heptane-isopropylic alcohol, and chloroform. All were evaluated for recovery and sample clean up. The Best extraction was obtained using chloroform followed by hexane-ethyl ether-isobutyl alcohol; however, the solvent mixture was chosen to avoid use of chlorinated aliphatic hydrocarbon solvents. Fig. 2 shows typical chromatograms obtained using this extraction. During validation, the selectivity of the assay was established. DPN sharp peak was obtained with a retention time of 3.9 min. Peak height versus concentration of DPN was linear in a first-order model [height = 343.14 (concentration)–437.69] that ranged from 40–400 ng/ml (r^2 = 0.9996). We tested different analytes as internal standard candidates, some having nearly similar chemical structure; nonetheless, retention times (RT) were either to close to DPN RT or to long. Therefore, the analytical method was validated to maintain FDA and Mexican guidelines without an internal standard.

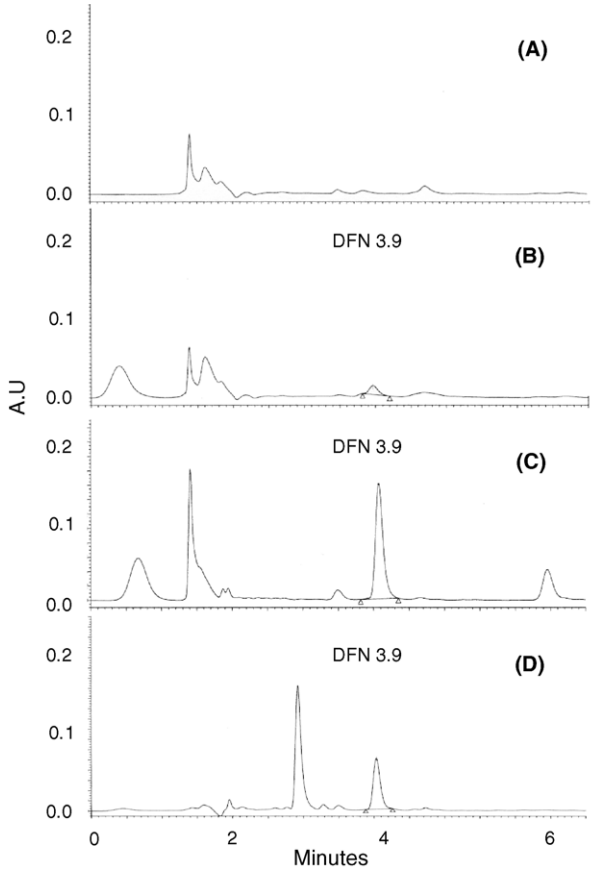

Fig. 2. Representative chromatograms of: (A) blank human plasma; (B) and (C) human plasma spiked with DFN at lower (40 ng/ml) and upper (400 ng/ml) limits of quantitation; (D) plasma obtained from volunteer 9 h after receiving the initial drug administration.

The absolute recovery for DPN was 84 ± 2.65%, in agreement with previous reports [5]. Accuracy and precision (intra- and inter-assay) are presented in Table 1. Relative error was <10% for the three control points. The Intra-assay coefficient of variation calculated for the three control points was 8.2%, while assay accuracy was 100.1 ± 7.5%.

The detection limit for DPN was 15 ng/ml for a signal-to-noise ratio (S/N) = 3.4. The limit of quantification (LOQ) was 40 ng/ml, with a coefficient of variation of 5.2%.

We conducted stability tests throughout the study; DPN proved to be stable in biological samples for at least two

Table 1
Results of intra- and inter-day variability during validation of the proposed HPLC method

Concentration (ng/ml)	Within-day, n = 9		Between-day, n = 5	
	Accuracy (%)	Precision (C.V.)	Accuracy (%)	Precision (C.V.)
75	98.77	9.91	101.56	7.90
125	99.80	7.74	101.47	9.91
275	101.45	5.32	104.44	6.86

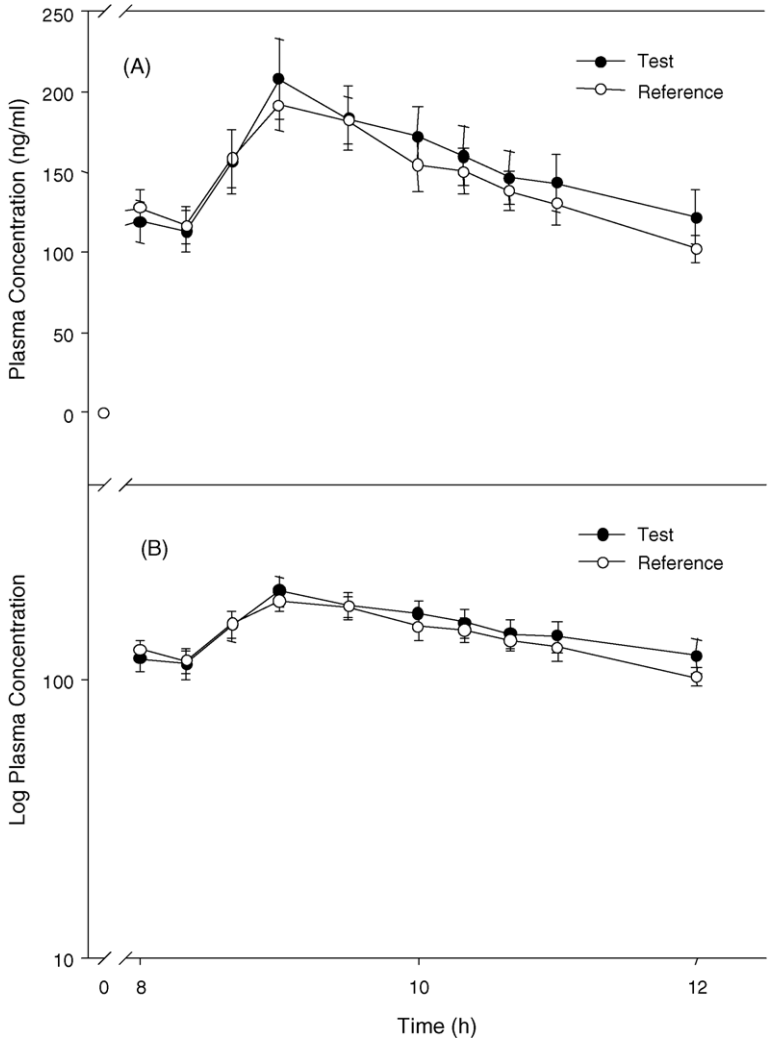

Fig. 3. Mean plasma concentration vs. time profiles in 22 healthy female volunteers after multi-dose administration of diphenidol hydrochloride tablets: (◯) reference and (●) test products. (A) linear and (B) log-transform scale.

freeze-and-thaw cycles (final mean recovery of 110.24% and C.V. of 4.3%). Samples were stable for at least 42 days at − 70 °C (99.01% and C.V. 3.4%) for at least 5 h on the worktable at room temperature (107.81% and C.V. 4.28%), and at least 18 h in the autosampler (97.74% and C.V. 2.45%).

Our method is the first technique reported for measurement of DPN in plasma by HPLC that was developed in a regulatory context [4,9], which allowed us to obtain pharmacokinetic data with a high degree of confidence with respect to other techniques previously employed. A multi-dose design was used as analytical strategy because it was very problematic to carry out measurement of its physicochemical properties and no pharmacokinetic profile has been reported.

DPN kinetics exhibited a similar pattern after multi-dose administration of both formulations. Fig. 3 shows the mean DPN plasma concentrations observed in the 22 healthy volunteers studied with the two oral formulations, reference and test products; (A) linear and (B) logarithmic data. With this experimental scheme, we were able to obtain steady-state for plasma concentration because the concentration at the first sample time (8:00 a.m.) was nearly equal to last sample time (12:00 a.m.), as reported for multiple-dose kinetics by Gimaldi and Perrier [11]. Inter-individual variability in DPN plasma concentrations was low (Fig. 3), although the C_{max} obtained in this study was similar to that reported in the drug information for prescription [1]. The half-life obtained in this Mexican population is 3 h, which is slightly lower than that reported in references (4.0 h) [1] and suggesting that Mexican population may eliminate DPN faster than other populations, such as is suggested for other drugs and populations [12–15].

Pharmacokinetic parameters for the bioequivalence study are summarized in Table 2. These values were compared according to Mexican and FDA guidelines [4,9]. Comparison included analysis of variance test (ANOVA) for logarithmic transformed values and Schuirmann's test; neither was probed to find significant differences between the two formulations. Finally, CI 90% ranged from 91.48–116.18% for C_{max}, from 94.96–115.22% for C_{avg}, and

Table 2
Pharmacokinetic parameters of DPN after multi-dose administration of two different oral formulations in 22 healthy female Mexican volunteers

Parameter	Reference	Test	90% CI[a](80–125%)	Schuirmann's test[a]($p < 0.05$)	
				$P < 80\%$	$P > 125\%$
C_{max} (ng/ml)	201.69 ± 10.17	216.19 ± 12.27	91.48–116.18 %	0.0008	0.0059
T_{max} (h)	9.20 ± 0.45	9.18 ± 0.52			
AUC_{tau} (ng/ml h)	551.07 ± 53.53	583.68 ± 69.02	91.24–117.65 %	0.0011	0.0097
CLss/F (ml/h kg)	0.053 ± 0.005	0.052 ± 0.005			
C_{min} (ng/ml)	86.72 ± 7.66	84.01 ± 9.67			
C_{avg} (ng/ml)	139.54 ± 12.66	148.60 ± 16.51	94.96–115.22 %	0.0001	0.0024
$T_{1/2}$ (h)	2.96 ± 0.37	3.20 ± 0.37			
Fluctuation (%)	82.29 ± 5.46	85.16 ± 6.46			

Values are given as mean ± standard error.
[a] Statistics were applied on decimal logarithm-transformed data, $n = 22$.

from 91.24–117.65% for AUC_{tau}; limits of acceptance were fixed at 80–125% [4,6,7,9]. A concise decision of bioequivalence was taken based on confidence intervals because these were contained entirely within acceptability limits, with a statistical power >80%.

4. Conclusion

DPN is still extensively used; nevertheless, due to the risk of potential side effects an adequate drug monitoring method is necessary. Our rather simple analytical HPLC-UV method for determination of DPN in human plasma was demonstrated to be precise and accurate it can be successfully used in pharmacokinetic or bioequivalence studies and for continuous drug monitoring under a multi-dose administration regime. This pharmacokinetic data of DPN in Mexicans has not been previously reported, suggesting differences in pharmacokinetic profiles linked with ethnic genotype composition [12–15]. Finally, both oral formulations tested containing 25 mg of DPN and manufactured in Mexico are bioequivalent.

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