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Bioanalytical method development and validation of Darifenacin in Human Plasma by LC-MS/MS and its application in Bioequivalence studies

Bhupinder Singh[1*], Naveen Dubey[1], Peeyush Jain[1], Sandeep Sharma[1], Ashish Dwivedi[1], Rama S. lokhandae[2]

[1]Jubilant Clinsys Limited, C-46, Sector-62 NOIDA (UP)-201307
[2]Jaipur National University, Jaipur, India

Abstract

An LC–MS method for the determination of Darifenacin (DRN) in human plasma was developed and validated. Sample preparation involved the extraction with Diethylether: Dichloromethane:: 80:20, v/v) and chromatographic separation was performed on a CC 150 x 4.6 NUCLEOSIL 100-5 NH2 column with the mobile phase consisting of acetonitrile: milli-Q water: formic acid (90:10:0.1 v/v/v). The interface used with the API 3000 LC-MS/MS was a turbo ion spray in which positive ions were measured in MRM mode. The method was validated over the concentration range of 0.025-10.384 ng/mL. The recovery was 90.94% -109.89% and the limit of quantitation (LOQ) was 0.025 ng/mL. The intra- and inter-day precision of the method at three concentrations was 0.84-2.69% and 2.01-7.47% while the intra- and inter-day accuracy was 94.79-108.00% and 102.61 -94.63%. Stability of compound was established in a series of stability studies. The method was successfully applied on bioequivalence study of extended release DRN tablet to obtain the pharmacokinetic parameters.

*Corresponding author, Mailing address:
Bhupinder Singh
Research Scientist, Bioanalytical Division
Jubiliant Clinsys Limited, Noida, New Delhi, India
Email: bs_khubber@yahoo.com

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INTRODUCTION

Overactive bladder (OAB) characterized increased frequency of micturition, urgency and urge (urinary incontinence) is a ubiquitous complaint particularly

with increasing age mediated chiefly by cholinergic activation of muscarinic M3 receptors present in the urinary bladder. Antimuscarinics are first-line drug for OAB treatments [1, 2]. Selective M3 receptor blockers have the potential to convene pronounced relief from the OAB by adjusting detrusor contractility while reducing the tolerability and safety problems related to non-selective muscarinic blockers [3]. Darifenacin hydrobromide chemically, (3S)-1-[2-(2,3-Dihydro-5-benzofuranyl)ethyl]-alpha,alpha-diphenyl-3-pyrrolidineacetamide, is a potent and competitive M3 selective receptor antagonist having low selectivity for the other muscarinic receptor sub types [3,4,5]. The clinical relevance and immense market potential of DRN requires a developed and validated bioanalytical method which could be applied for the bioequivalence study of various formulations. Some of the previous analytical researches performed on this molecule are presented in table 1. It is evident from the table that previous analytical studies were either used for in vitro quantification or related to degradation studies except one by Kaye et al, which applied the method for determination of plasma profile on single human volunteer after multiple oral dosing [7]. The present study is concerned with the development and validation of DRN in human plasma by HPLC–MS/MS and its application in bioequivalence study after single oral dose of in-house developed DRN (15mg) extended release tablet versus Enablex® (DRN) extended- release 15 mg reference tablet in 12 healthy adult, human subjects, under fed conditions.

Table 1: Some reports related to the methodology applied for the analysis of DRN

Instrumentation	Purpose	LLOQ	Recovery	Remarks	Year	Reference
HPTLC-densitometry	Stability – indicating analysis of DRN in bulk and in tablet dosage form	90.79 ng/spot	-	Not applied for bioequivalence study	2012	[6]
HPLC/APCI-MS/MS	Solid phase extraction technique for the high-throughput assay of DRN in human plasma	0.025ng/mL	~50%.	Low recovery, Not applied for bioequivalence study, Plasma profiling was done on 1 human volunteer	1996	[7]
RP-UPLC/MS	Determination of DRN, its related compounds and its degradation products	-	-	Not applied for bioequivalence study	2012	[8]
HPLC/LC-ESI/MS(n).	Structural elucidation of two process impurities and stress degradants in DRN	-	86.6 - 106.7%	Not applied for bioequivalence study	2012	[9]
HPLC-MS/MS	Method development and validation of DRN in human plasma	0.025 ng/mL	102.61-94.63%.	Applied for Bioequivalence study on 12 human volunteers	2012	Present study

EXPERIMENTAL

Chemicals and materials

Darifenacin hydrobromide (mol.wt. 507.46; 99.24% w/w) and Darifenacin D4 hydrobromide (mol.wt. 511.48, 100.00 %) were obtained from Vivan life sciences. Control buffered (K_2EDTA) human plasma was procured from Laxmi sai clinical lab., India. All other reagents/chemicals were of AR grade.

LC–MS/MS instrumentation and settings

A high performance liquid chromatography system (Shimadzu Co., Kyoto, Japan) with CC 150/4.6 NUCLEOSIL 100-5 NH2 column was used in this study. The column oven and autosampler temperature were maintained at 35±2°C and 5±1°C respectively while the flow rate was set at 1.200 mL/min with split (1:2). Ionization and detection of analyte and IS was performed on a triple quadrupole mass spectrometer, API 3000 LC-MS/MS equipped with turbo ion spray®, from MDS SCIEX (Toronto, Canada) operated in the positive ion mode. Quantitation was done using MRM mode to monitor protonated precursor product ion transition of m/z

427.30 →147.10 amu and 431.40 →151.10 amu for DRN and DRN D4 respectively. All the parameters of HPLC and MS were controlled by analyst software version 1.4.2. The source dependent parameters maintained for analyte and IS were nebulizer gas (NUB): 14.00 psi, ion spray voltage (ISV): 2500.00 V, turbo heater temperature (TEM): 450.00 ºC, collision activation dissociation (CAD): 6.00 psi, curtain gas (CUR): 11.00 psi. The compound dependent parameters like declustering potential (DP) were optimized at 62.00 V (DRN) and 60.00 V (DRN D4), collision energy (CE) was 40.00 V and 50.00 V for DRN and DRN D4 respectively. Cell exit potential (CXP) was kept at 10.00 V and entrance potential (EP): 10.00 V.

Preparation of standard stock and plasma samples

The standard stock solution of DRN (1mg/mL) and DRN D4 (1mg/mL) were prepared by dissolving in requisite amount of methanol. Further dilutions from the stock solutions were prepared using diluent solution (methanol: milli-Q water :: 50:50, v/v) for spiking in plasma to obtain calibration curve (CC) standards and quality control (QC) samples. CC standards consisting of a set of eight non-zero DRN concentrations of 0.025 ng/mL, 0.050 ng/mL, 1.298 ng/mL, 2.597 ng/mL, 5.193 ng/mL, 6.750 ng/mL, 8.829 ng/mL and 10.386 ng/mL were prepared .

The quality control samples consisted of DRN concentrations of LLOQ QC 0.025 ng/mL, LQC 0.068 ng/mL, MQC 5.172 ng/mL and HQC 8.329 ng/mL were prepared. After bulk spiking, 500 µL of spiked plasma samples were pipetted out in pre-labeled polypropylene tubes. The calibration curve standards and quality control samples were logged in ultra low temperature deep freezer (Temp range: -55ºC to -75ºC) except 30 samples each of LQC and HQC which were transferred for storage in cell frost deep freezer (Temp range: -17ºC to -27ºC) for the generation of long term stability at – 22ºC±5ºC. These samples were used for performing the method validation.

Preparation of mobile phase and liquid-liquid extraction method

Mobile phase was prepared as the mixture of acetonitrile: milli-Q/HPLC grade water: formic acid in the ratio 90:10:0.1,v/v/v. For bioanalysis a set of calibration curve standards and/or quality control samples were withdrawn from the deep freezer and allowed to thaw at room temperature. 50µl of DRN D4 as an internal standard (2500.000 ng/mL) was added into ria vials and 400µl of plasma was aliquoted from the pre-labeled polypropylene tubes into ria vials followed by vortexing the samples. Liquid-liquid extraction was performed using diethylether:dichloromethane::80:20, v/v as an extraction solution. Briefly, 2.0 ml of extraction solution was added and vortex for 10 minutes. Samples were centrifuged at 4500rpm for 5 min at 4ºC temperature and flash freezed for approx. 0.2-2 min. The supernatant was decanted off and evaporated to dryness at 40°C (at constant pressure) in nitrogen evaporator. Residue was reconstituted in 300µl of mobile phase and analysed.

Method validation

The method was validated for selectivity, sensitivity, linearity, matrix effect, calibration curve standards and quality control samples, precision and accuracy batches. The results of various stabilities i.e. (stock dilution stability at refrigerator temperature and room temperature, standard stock solution stability in refrigerator temperature and room temperature and photo degradation test in dark, auto sampler stability, re-injection reproducibility, freeze thaw stability, long term stability at -65ºC±10°C and at -22ºC±5ºC, reagent stability, bench top stability, dry ice stability, dry extract stability, extended bench top stability, wet extract stability in refrigerator, lipemic and haemolysed plasma stability), blood stability, effect of potentially interfering drugs, dilution integrity, recovery, ion suppression through infusion, ruggedness, robustness and extended batch verification meeting the acceptance criteria as per the USFDA guidelines (FDA, 2001). Selectivity was

performed in eight lots of normal, four lots of lipemic and four lots of haemolysed plasma containing K_2EDTA (Potassium salt of ethylene diamine tetra acetic acid) as an anticoagulant. Sensitivity of the method was determined on six LLOQ samples. For matrix effect, 12 blank samples were processed from 6 normal plasma lots (two aliquots prepared from each plasma lot) and six blank samples were processed from 3 lipemic plasma lots and six blank samples were processed from 3 haemolysed plasma lots respectively. After drying these processed blank samples from each plasma lot were reconstituted with aqueous LQC and aqueous HQC dilution respectively. For comparison of matrix effect same prepared aqueous LQC and HQC dilution were used and six replicates were injected from each prepared aqueous LQC and HQC. Matrix effect was calculated as per the following formula:

Matrix factor = (peak response in the presence of matrix ions)/(peak response in the absence of matrix ions)

%Matrix effect = (1-mean of matrix factor) X 100

The precision of the assay was calculated as percent coefficient of variation over the concentration range of LLOQ QC, LQC, MQC and HQC samples, respectively. The accuracy of the assay was calculated as the ratio of the calculated mean values of the LLOQ QC, LQC, MQC and HQC samples to their respective nominal values. The data of three precision & accuracy batches were subjected for goodness of fit analysis. The back-calculated concentrations of calibration curve standards using 1/x and $1/x^2$ weighing were considered for finding the best fit for regression. Linearity was calculated using a regression equation with a weighting factor of $1/x^2$ for drug to IS (DRN D4) concentration to produce the best fit for the concentration-detector response relationship for DRN. Stock solution and stock dilution stability in refrigerator for DRN and DRN D4 (IS) was carried out for 8 days while stock solution and stock dilution stability at room temperature was carried out for 21 hours. Photo degradation test of DRN and DRN D4 was performed for 22 hours in dark. For all the aqueous related stability studies two aqueous mixtures were prepared, one from the stability standard stock solution and the other from fresh standard stock solution (comparison stock). Six replicates of aqueous mixture from each stability stock and comparison stock were injected. The response of stability sample was corrected using a correction factor.

Correction factor= (conc. of fresh standard sol.)/(conc. of stability standard sol.)

Corrected response=stability stock response X correction factor

% Change=[(mean response of comparison samples - mean corrected response of stability samples)]/(mean response of comparison samples) X 100

Aqueous recovery comparison samples (LQC, MQC and HQC) were prepared by adding 8µL each of aqueous dilution of DRN from respective quality control samples, 50µL of internal standard dilution (2500.000 ng/mL) and 242µL of mobile phase (representing 100% extraction). The aqueous samples (LQC, MQC and HQC) of DRN were compared against 6 sets of extracted LQC, MQC and HQC samples . Recovery of internal standard was compared at LQC, MQC and HQC level.

% Recovery = (mean peak area response of extracted sample) / (corrected mean peak area response of unextracted sample) X 100

The effect of potentially interfering drugs (PIDs) i.e. ibuprofen, caffeine, acetaminophen and acetyl salicylic acid on DRN analysis was performed by spiking PID's at their approximately C_{max} concentration in the LLOQ sample in triplicate.

Bench top stability was determined for 13 hours using six sets each of LQC and HQC samples while extended bench top stability was determined in spiked samples to assess the stability of DRN at each step of extraction. The freeze and thaw stability was determined for five freeze thaw cycles. Six sets of

LQC and HOC samples were analyzed after five freeze-thaw cycles. Long term stability (at -65°C±10°C and -22°C±5°C) was carried out in plasma for 22 days by using six sets of LQC and HQC. Dry extract stability was carried out by processing six sets of LQC and HQC, stored at -22°C±5°C without reconstitution while wet extract stability was carried out by processing the six sets of LQC and HQC, stored at 2-8°C after reconstitution. The samples of wet extract and dry extract stabilities were analyzed after 51 hrs storage. All stability QC's were analyzed against the freshly spiked calibration curve standards and six sets of freshly spiked LQC and HQC (prepared from the fresh stock solution) to calculate the %change between the stability QC's and Comparison QC's.

Robustness experiment was performed at different column temperatures (38°C and 42°C), flow rates (1.17 mL/min and 1.23 mL/min) and at different mobile phase compositions (acetonitrile, milli-Q/HPLC Grade water: formic acid : : 92:8:0.1 and 88:12:0.1, v/v/v). To evaluate ruggedness, precision and accuracy batch was processed against calibration curve standards and analyzed by a different analyst using different column and different sets of solutions.

Bioequivalence study design

To compare the bioavailability and characterize the pharmacokinetic profile for assessment of bioequivalence of the in house developed test formulation of DRN 15 mg extended release tablet with the reference 15 mg DRN formulation [Enablex® extended- release tablet] in healthy adult, human subjects, under fed conditions. The protocol was approved by the relevant institutional ethics committee (IEC). Plasma samples of all evaluable subjects completing all the periods of the clinical phase of the study were to be analyzed. All participants gave written consent and were informed of the aims and risks of the study. Inclusion criteria comprise age (18–45years), body mass index (18.5–30.0kg/height^2) and absence of abnormalities on physical examination along with normal electrocardiogram and laboratory tests. Exclusion criteria comprise allergy to DRN, alcoholism, psychosis, smoking, diabetes or any disease which could compromise the haemopoietic, gastrointestinal, renal, hepatic, cardiovascular, and respiratory or central nervous systems. Moreover, all the procedures were based on the international conference on harmonization, E6 good clinical practice (ICH, E6GCP) guidelines. As per the study protocol 576 clinical blood samples were collected from 12 subjects during two period of the study. The clinical blood samples were collected in K_2EDTA vacationer from the subjects and intermediately stored in deep freezer at -22°C on the days of plasma sample collection. Plasma was obtained by centrifugation at 4500 rpm at 4°C for 10 min and was stored at -65°C until assayed. All pharmacokinetic values were gained by the non-compartmental model and expressed as mean±SD.

RESULTS AND DISCUSSION

LC–MS/MS settings

Internal standards DRN D4 was expected show nearly similar chromatographic behavior as of analyte because they are differing only in terms of possessing different isotopic atoms. Retention time (RT) of DRN and DRN D4 was found to be 0.74±0.3 min and 0.75±0.3 min respectively. Furthermore, as expected DRN D4 produces similar recovery as of DRN in the positive ion mode. Electro spray ionization (ESI) provided high ionization efficiencies for both analyte and IS in positive ion mode which resulted in admirable sensitivity of the method. The parent ion (Q1) mass spectra of the $[M-H]^+$ ions of DRN and IS are shown in Figure 1.

Sample preparation

Liquid-liquid extraction method was used for sample preparation because of relatively low cost, good extraction efficiency as well as simple procedure. Various extraction solvents were tried but mixture of diethylether : dichloromethane :: 80:20, v/v was

found to be most effective for extraction of both DRN and DRN D4 with minimal interference.

Method validation

Selectivity and matrix effect

Figure 2 shows typical MRM chromatograms of a blank plasma sample, a plasma sample spiked with DRN at the LLOQ (0.025 ng/mL) and a plasma sample from a healthy volunteer 1.5 hr after the oral administration of the tablet. No significant interference was observed from endogenous substances at the retention times of the analyte and internal standard in normal, haemolysed or lipemic plasma. The variability of matrix factor (reported as %CV of matrix factor) was 1.54% (HQC) and 2.84% (LQC) for DRN and 0.96% (HQC) and 1.24% (LQC) for DRN D4 and the variability of IS-normalized matrix factor on normal plasma (reported as %CV of matrix factor) was 1.08% (HQC) and 2.20% (LQC). The % matrix effect ranged from -0.56% (LQC) to 0.83% (HQC). The results were within the acceptance criteria and indicate that ion suppression or enhancement due to the plasma matrix was consistent and would not interfere with the quantitation of analytes.

Linearity and sensitivity

The correlation coefficients (r^2) were greater than 0.99 over the range of 0.025 ng/mL and 10.386 ng/mL. Typical equations of calibration curves are as follows:

$$y = 0.154\ x + 0.00238,\ r^2 = 0.9990$$

Where y represents the analyte\I.S. peak area ratio and x represents the plasma concentration of the analyte. The precision and accuracy for DRN at LLOQ was 7.75% and 106.67%, respectively revealing a prodigious sensitivity of the method.

Precision and accuracy

Table 2 summarizes back calculated concentrations of calibration curve standards for DRN whereas Table 3 represents the intraday and inter days precision and accuracy data. The intra- and inter-day precision of the method at three concentrations was 0.84%-2.69% and 2.01-7.47% while the intra- and inter-day accuracy was 94.79%-108.00% and 102.61% -94.63%. The results showed that method is fairly precise and accurate within the acceptable limits.

Table 2 Back calculated concentration of calibration curve standards for DRN (n = 3).

	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7	STD 8	Slope	Intercept	r^2
DRN (ng/mL)	0.025	0.050	1.298	2.597	5.193	6.750	8.829	10.386			
MEAN	0.0247	0.0510	1.3183	2.6160	5.2593	6.4813	8.7920	10.3603	0.155	0.00227	0.9997
S.D	0.00058	0.00173	0.00379	0.02900	0.04842	0.13057	0.02951	0.11380			
%C.V	2.34	3.40	0.29	1.11	0.92	2.01	0.34	1.10			
% Nominal	98.67	102.00	101.57	100.73	101.28	96.02	99.58	99.75			

Table 3: Inter day and intraday precision and accuracy of the method for DRN

Levels	Concentration added (ng/mL)	Inter-day (n=6)			Intra days (n=18)		
		Mean concentration found (ng/mL)	% nominal	% CV	Mean concentration found (ng/mL)	% nominal	% CV
LLOQQC	0.025	0.0270	108.00	2.34	0.0256	102.44	7.47
LQC	0.068	0.0693	101.96	2.69	0.0698	102.61	6.77
MQC	5.172	4.9250	95.22	0.84	4.9382	95.48	5.55
HQC	8.329	7.8950	94.79	0.98	7.8814	94.63	2.01

Recovery

The mean % recovery of DRN was 98.067% with a precision of 10.52 % while DRN D4 showed mean % recovery of 93.321% with a precision of 1.88%. The data show that the simple LLE procedure efficiently extracts analyte as well as IS from human plasma.

Stability and Other parameters

Table 4 represents stability data and shows there were no stability-related issues that might cause problems in application of the assay to PK studies. The outcomes of other parameters like ruggedness, reinjection reproducibility, effect of potentially interfering drugs (PID), dilution integrity, extended batch verification and robustness were found to be within the acceptance criteria as per USFDA guidelines [10].

Table 4: Stability data of DRN in processed QC samples for different stability activities at different conditions (n = 6).

Stability	Storage condition	Levels	DRN				
			Mean comparison concentration (ng/ml, n=6)	%CV	Mean stability concentration (ng/mL, n=6).	%CV	%Stability (% mean change)
Bench top	Room temperature for 13 hrs	LQC	0.0723	6.16	0.0735	4.19	-1.61
		HQC	7.9783	1.21	7.9838	0.99	-0.18
Auto sampler	Auto sampler at 5°C for 74 hrs	LQC	0.0752	7.26	0.0740	4.68	1.55
		HQC	7.5697	1.65	7.7042	1.67	-1.81
Freeze and thaw	After 5th cycle at -70°C	LQC	0.0752	7.26	0.0737	7.85	2.00
		HQC	7.5697	1.65	7.7503	1.28	-2.42
Long term	22 days at -70°C	LQC	0.0723	6.16	0.0717	3.71	0.92
		HQC	7.9783	1.21	7.8835	1.11	1.08
	22 days at -22°C	LQC	0.0723	6.16	0.0738	2.61	-2.03
		HQC	7.9783	1.21	7.6458	1.14	4.06
Dry Extract	51 hrs at -22°C	LQC	0.0733	5.70	0.0710	5.04	3.18
		HQC	7.7252	1.65	7.7860	1.67	-0.90
Wet extract	51 hrs at 2-8°C	LQC	0.0733	5.70	0.0752	3.08	-2.50
		HQC	7.7252	1.65	7.7858	1.92	-0.89
Haemolysed Impact	2 days at -70°C	LQC	0.0723	6.16	0.0723	2.86	0.00
		HQC	7.9783	1.21	7.9262	1.05	0.55
Lipemic Impact	2 days at -70°C	LQC	0.0723	6.16	0.0732	2.01	-1.15
		HQC	7.9783	1.21	7.9062	0.76	0.80

Bioequivalence study

The method was applied for the analysis of plasma samples obtained from the pharmacokinetic study. Blood samples were collected using K_2EDTA vaccutainers at the following time points: Pre-dose and at 0.50, 1.00, 1.50, 1.75, 2.00, 2.33, 2.67, 3.00, 3.25, 3.50, 3.75, 4.00, 4.33, 4.67, 5.00, 5.50, 6.00, 8.00, 10.00, 12.00, 16.00, 24.00, 36.00, 48.00 and 72.00 hours post dose. Pharmacokinetic parameters were calculated from the subjects who had successfully completed periods I and II of the study. Some of the main pharmacokinetic parameters are given in Table 5. The mean plasma concentration versus time profile is shown in Figure 3.

Table 5 Pharmacokinetic parameters after oral administration of DRN extended release tablets in human subjects (n = 12)

Drug	Statistic	Cmax (ng/mL)	AUC o-t (ng*hr/mL)	AUC o-∞ (ng*hr/mL)
Test	Mean	6.3363	133.4701	144.3621
	RSD %	4.06121	92.34416	98.00689
Reference	Mean	6.4778	135.6333	156.3431
	RSD %	4.41563	131.93782	136.78608
90% Confidence interval for the ratio of the mean T/R*		78.913 - 115.022	90.675 - 138.424	82.360 - 125.310

T/R= test/reference

CONCLUSION

For the very first time a highly sensitive and selective method for the quantitative determination of DRN in human plasma was developed using HPLC–MS/MS

with turbo-ion spray in positive ion mode. The method was validated as per USFDA guidelines and found to be well suited for the PK study. This method allows for a much higher sample throughput due to the shorter chromatographic run time (2.0 min) in comparison to all published literature till now. The method has been successfully used in a PK study of orally administered DRN extended release tablets.

Figure 1: The parent ion (Q1) mass spectra of the $[M-H]^+$ ions of (A) DRN and (B) DRN D4

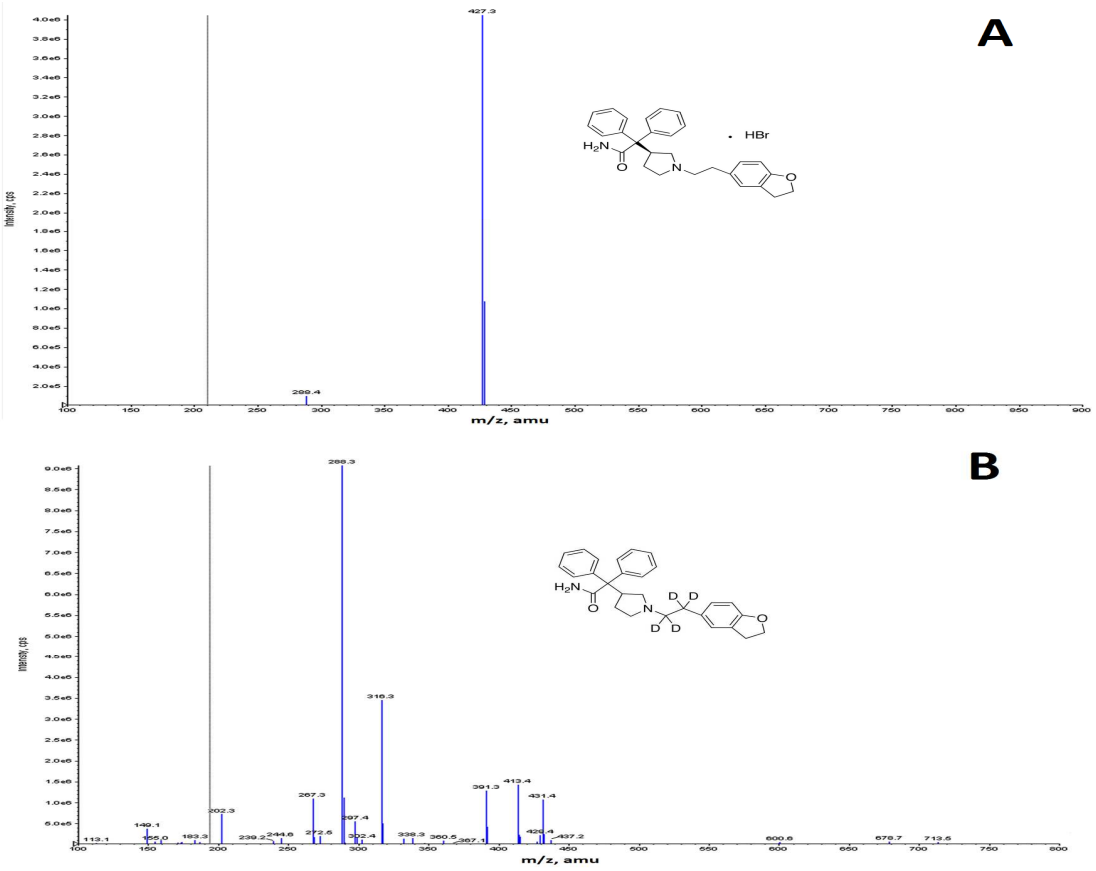


Figure 2 Representative MRM chromatograms of (A) blank plasma sample, (B) a plasma sample spiked with DRN at the LLOQ (0.025ng/mL for both analytes) and (C) a plasma sample from a healthy volunteer at 1.5 hr

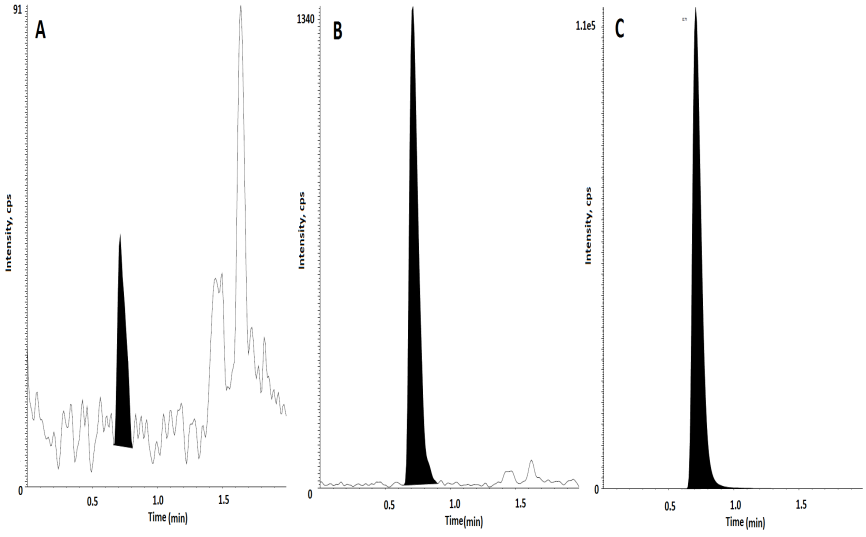
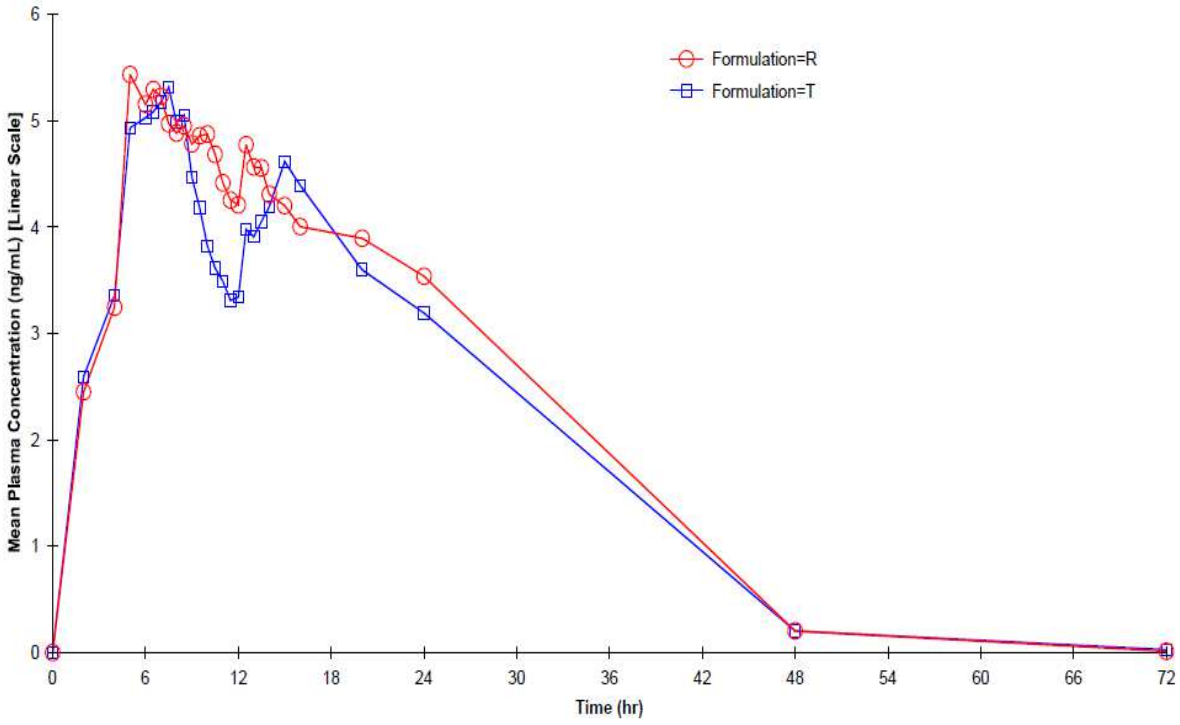


Figure 3: Mean plasma concentration-time curve for DRN as compared with standard extended release tablet



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