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Haemolysis Effect Estimation of Doxycycline Hyclate 150 mg Delay Release Tablet in Bio-analysis (Human Plasma) by Liquid Chromatography-Tandem Mass Spectrometry

Paresh Mistry[1,2]* and Shobhna Menon[1]

[1]School of Sciences, Chemistry Department, Gujarat University, Ahmedabad, Gujarat, India
[2]Accutest Research Laboratories (I) Pvt. Ltd, S.G. Highway, Ahmedabad-380054, India

Abstract

A fast, simple and particular method for estimation of Doxycycline in healthy human plasma was validated using Minocycline as IS. The analyte and IS were extracted from plasma using SPE. The compound was estranged on a RP column with an isocratic mobile phase consisting of 0.1% formic acid in water and acetonitrile (12:88, v/v), and detected by tandem mass spectrometry in positive ion mode. The ion transition recorded in several reaction monitoring mode were m/z 294.1→225.1 for Doxycycline and *m/z* 286.1→217.1 for IS. Linearity in plasma was observed over the concentration range 0.3-30 ng/mL for Doxycycline. The mean recovery for Doxycycline was 83.7%, with a lower limit of quantification of 0.3 ng/mL. The coefficient of variation of the assay was less than 6.8%, and accuracy of 96.1% to 102.2%. The validated method was applied to bioequivalence study of 150 mg Doxycycline Hyclate tablet in healthy human volunteers. The validated method was used to expose study samples of bioequivalence study of 150 mg Doxycycline Hyclate delay release tablet in 36 healthy human volunteers. Total 50 samples from individual volunteers identified as Haemolyzed, which were analyze initial and repeat again to cross check the method reproducibity for Haeamolysis effect and compared which found acceptable range. Bioequivalence was prove for test and reference using validated method to experimental samples (Figure 1).

Keywords: Doxycycline hyclate; Minocycline; LC-MS-MS; Human plasma

Introduction

In BA/BE studies, Haemolysis effect can be responsible for analysis of drug estimation in plasma. Validation of LC-MS/MS assays includes an assessment of hemolytic effects though the experiment. Recognition of this effect in attendance of drug can be supportive tool for estimation of assay in plasma. Validated method is used to evaluate Haemolysis effect can simply prove the impact on drug estimation. Validation of drug estimation can be challenged through the Haemolysis samples which are unknown though Incurred sample reanalysis to compare the method ruggedness, with respect to Haemolysis sample which are extracted directly from subjects (volunteers).

Tactic was applied to reduce Haemolysis effect through proper extraction techniques like SPE. We establish method with proper extraction techniques to reduce the Haemolysis effect, and then challenge our method in subject analysis, specifically for Haemolysis samples along with normal plasma samples [1].

Summarize the assay value for combination drugs for Haemolysis samples and through Incurred subject reanalysis cross check the techniques used for validation is properly applied as per regulation described in USFDA guidance [2].

Doxycycline Hyclate (Delayed-Release Tablet) contains particularly coated pellets of Doxycycline hyclate, a broad-spectrum antibiotic synthetically derived from oxytetracycline, in a delayed-release formulation for oral administration (Figure 1).

Molecular prescription of Doxycycline Hyclate is $(C_{22}H_{24}N_2O_8{\cdot}HCl)_2{\cdot}C_2H_6O{\cdot}H_2O$ and a molecular weight of 512.94. Doxycycline hyclate is a yellow crystalline soluble powder in water and in solutions of alkali hydroxides and carbonates. Doxycycline has a high degree of lipid solubility and a low affinity for calcium binding. It is highly stable in normal human serum.

It is used in malaria. It should not be used alone for initial cure of malaria. This delay is related to its mechanism of action, it is also used for the treatment of Rickettsial infections, sexually transmitted infections, Respiratory tract infections, Specific bacterial infections, ophthalmic infections, Anthrax, including inhalational anthrax (Post-Exposure) [3].

Quantitative Analysis of Quinine and Doxycycline in Formulations was reported through LC-MS method. It was carried out on a Sun Fire Waters C_{18} column and the mobile phase consisted of acetonitrile: 0.1% formic acid (75:25, v/v), run at a flow rate of 0.45 mL/min (split 1:3). The injection volume was 10 µL for both standard and samples.

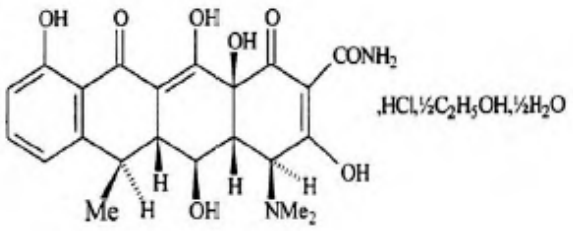

Figure 1: Structure of doxycycline hyclate.

***Corresponding author:** Paresh Mistry, Accutest Research Laboratories (I) Pvt. Ltd, S.G. Highway, Ahmedabad-380054, Gujarat, India, Tel: 91-982-568-1583, E-mail: paresh.mistry@accutestindia.com

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The triple quadruple mass spectrometer equipped with an electro spray source in positive mode (ES+) was set up in Multiple Reactions Monitoring mode (MRM), monitoring the transitions of 325.0>307.0 and 445.0>428.1, for quinine and Doxycycline, respectively [4].

In another reported method, Quantitative Analysis of Doxycycline in Healthy Human Plasma was done through LCMS/MS. Method was developed and validated for the determination of Doxycycline in plasma for a range of 1.0 ng/mL to 200 ng/mL Demeclocycline was used as the IS. The analytes in plasma were extracted by acetonitrile protein precipitation. HPLC conditions included a Phenomenex Columbus C_{18} 2.0×50 mm column utilizing a gradient operation with water and acetonitrile [5].

LC/MS Method for the fortitude of Doxycycline in Human EDTA K3 Plasma was also reported. Doxycycline and its IS were extracted from EDTA K3 plasma by PPT. Analysis was done on an Agilent MSD GL 1100 Series single mass spectrometer using Electro spray interface. Positive ions were measured using SIM with m/z 445.20 for Doxycycline. The chromatography was achieved on an X-Terra C18 MS column, with a run time of 4.6 minutes per sample. This method used two mobile phases, which are a mixture of trifluoroacetic acid and acetonitrile. This assay was validated at a nominal range of 4 to 400 ng/mL [6].

RP-HPLC method also published with fluorescence detection was optimized and validated for determination of Doxycycline in human saliva and Gingival Crevicular Fluid (GCF) with tetracycline as IS. Single step extraction with acetonitrile for both types of samples was performed. The separation was achieved at Zorbax Extend-C18 analytical column at 30°C. Mobile phase was consisted of an aqueous phase containing magnesium acetate, ammonium acetate, Na_2 EDTA, triethyl-ammonium acetate buffered to pH 7.5 with ammonium hydroxide solution and acetonitrile. The volume ratio of the buffered water mixture of salts and acetonitrile was 86:14. Fluorescence detector was set at λ_{ex}=380 nm and λ_{em}=520 nm. This method was successfully applied for Doxycycline in saliva and GCF obtained from patients with chronic periodontal disease [7].

Method was established and validated to verify Doxycycline in human plasma using SPE technique, which give good analysis in terms of number of samples daily and give proper selectivity and recovery. For the method, IS was used as Minocycline.

Experimental Procedure

Total number of 26 samples of different time points has been collected with individual subjects in each period. Blood samples will be collected in Na-Heparin Vaccutainer. All the blood samples will be centrifuged under refrigeration with the machine set at, RPM, 10 minutes and 5°C. Plasma samples will be placed in deep freezer maintained at -20°C ± 5°C. Total number of 50 samples was identified as Haemolyzed samples. The Haemolyzed samples were initially analyzed and results were collected as initial concentration. The same samples were again analyzed to cross check method reproducibility in terms of Haemolysis effect in plasma as incurred subject reanalysis. Overall subjects concentration were reported and analyzed to demonstrate the Bioequivalence of test and reference product of Doxycycline 150 mg DR Tablet for the design of two way cross over, two periods for test and reference product.

Materials and chemicals

Reference standards of Doxycycline and Minocycline were obtained from Sun Pharmaceuticals Industries Ltd. (Baroda, India). These standards had purity ≥ 99%. HPLC grade methanol and acetonitrile were purchased from J.T. Baker INC (Phillipsburg, NJ, USA). Formic acid of AR grade was procured from Merck Ltd (Mumbai, India). (Phenomenex Strata-X (30 mg/1 mL) SPE cartridges were procured from Orochem India Pvt. Ltd., (Mumbai, India). Water used in the entire analysis was prepared through Milli-Q water purification system from Millipore (Bangalore, India). Na Heparin plasma was collected through volunteer's drug free blood.

Liquid chromatography and mass spectrometric conditions: The liquid chromatography system coupled with mass spectrometer (API, SCIX) consisted of Shimadzu/LC-20 AD Prominence, auto sampler and temperature controlled compartment for column. The analytical column, Chromolith RP18e (100 mm×4.6 mm) was used for separation of analyte and IS. Mobile phase of 0.5% (v/v) Formic Acid in Water was pumped isocratically at flow rate of 1 mL/min. Auto sampler temperature was set at 4°C and the injection volume was 5 µL. The column oven temperature was maintained at 40°C and the Retention time is 1.29 min and 1.27 for Doxycycline and Minocycline, respectively [8].

The Applied Biosystems/API 2000 LC-MS-MS apparatus was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ion *m/z* 445.0 to the product ion *m/z* 428.1 for Doxycycline, and the transition of the protonated molecular ion *m/z* 458.2 to the product ion *m/z* 441.1 for the IS, Minocycline (Table 1 and Figures 2 and 3). The instrument response was optimized for Doxycycline and Minocycline by infusing a constant flow of a solution of the drug dissolved in mobile phase. Electro Spray Ionization (ESI) was performed in the positive ion mode. The instrument was interfaced with a computer running Analyst Version 1.4.2.

Preparation of standards and quality control samples: Two separate stock solutions of Doxycycline were prepared for bulk spiking of CC and QC samples for the MV test as well as the subject sample analysis. A 1 mg/mL stock solution for Doxycycline and Minocycline were organized by dissolving their exactly weighted compounds in methanol. This stock solution of Doxycycline thus prepared was

Drug	Scan type	Q1 mass (amu)	Q3 mass (amu)	Dwell time (msec)	Compound parameters (V)				
					DP	FP	CE	CXP	EP
Doxycycline	MRM	445.1	428.1	200	25.0	400.0	26.0	12.5	5.5
Minocycline	MRM	458.2	441.1	200	20.0	400.0	25.0	13.0	7.0

Source Parameters					
Ion Spray Voltage	CUR	GAS 1	GAS 2	Temperature	CAD GAS
3000 V	22	35	75	400°C	3

Table 1: Mass parameters and source parameter.

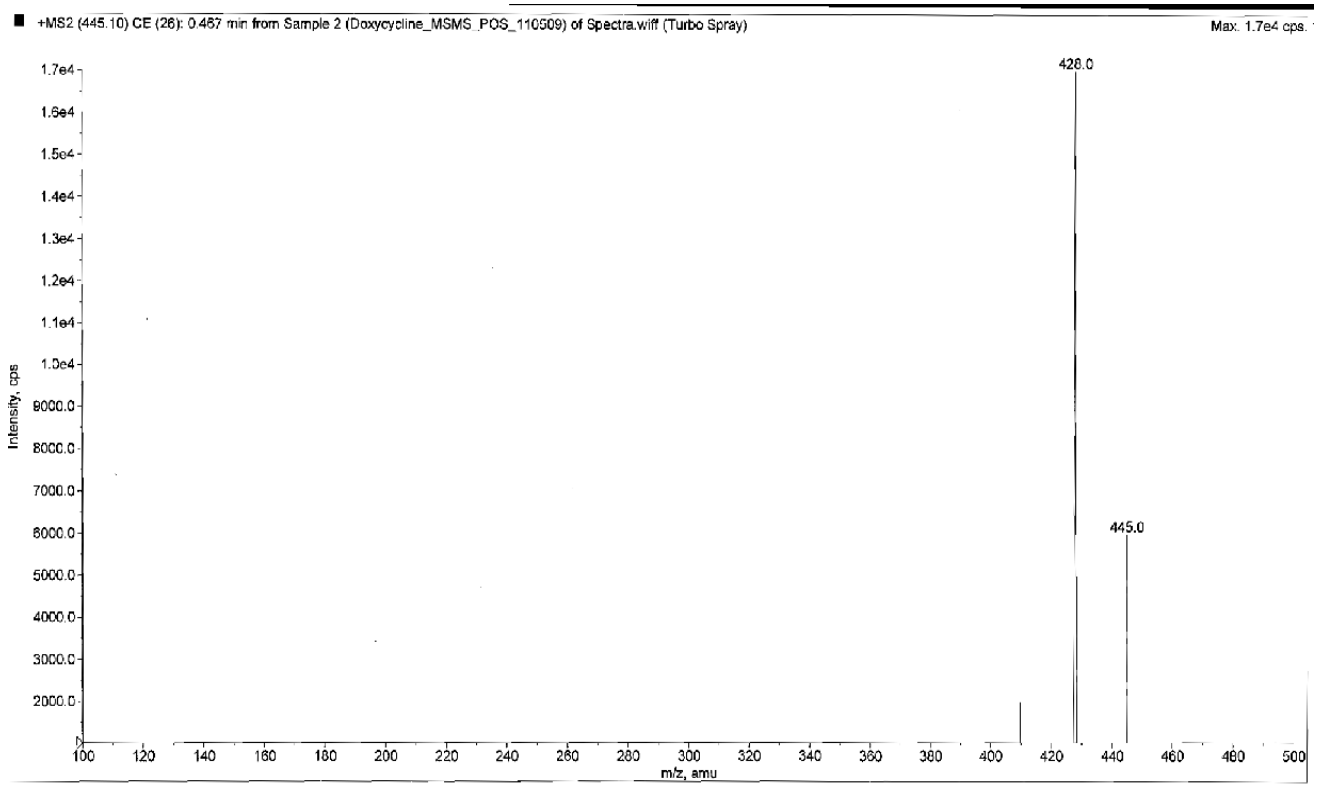


Figure 2: Product ion mass spectra of Doxycycline.

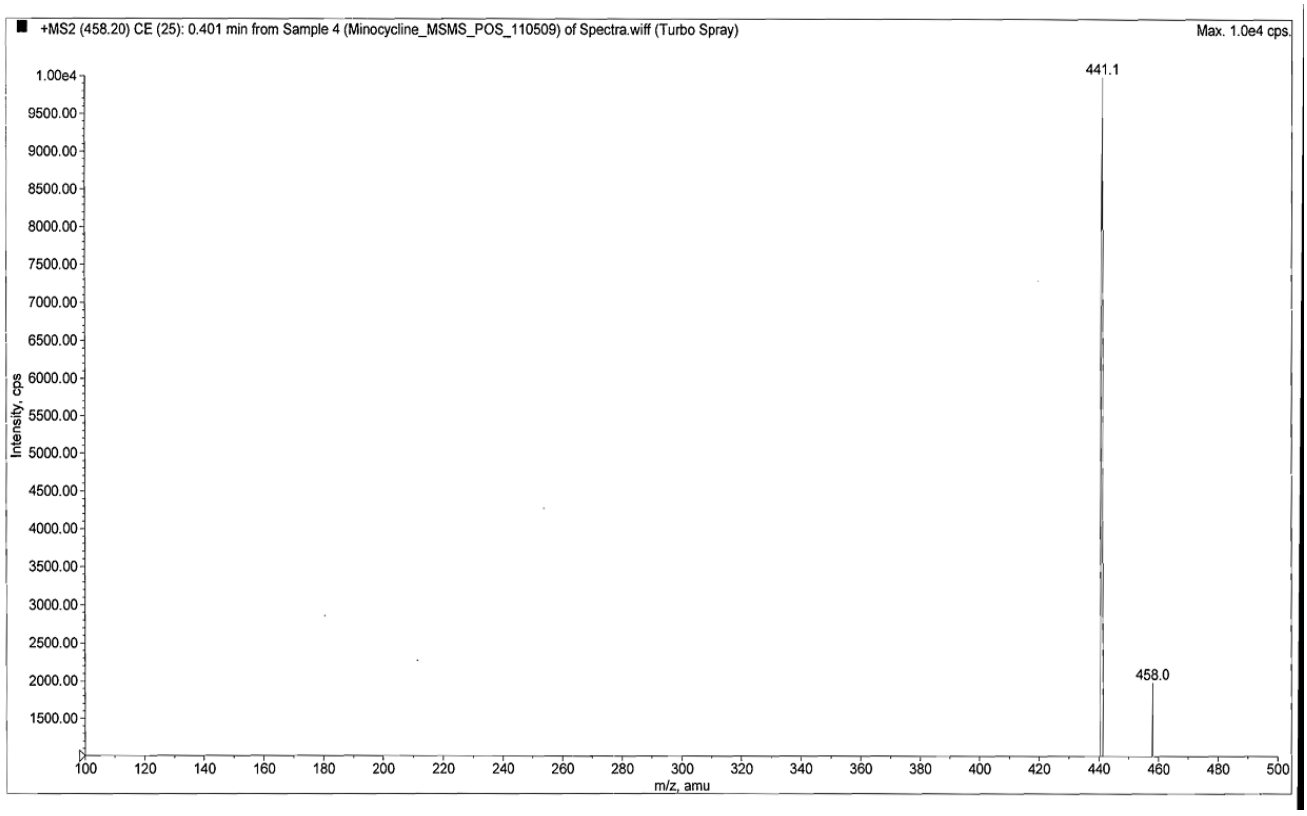


Figure 3: Product ion mass spectra of Minocycline.

serially diluted to prepare working solution in required concentration range with diluents methanol: water (70:30, v/v). The calibration standards and Quality Control (QC) samples were prepared by spiking (5% of the total plasma volume) with working solutions. Calibration standards were prepared at concentration of 50.000, 100.000, 250.000, 500.000, 1250.000, 2000.000, 3000.000, 4000.000, and 5000.000 ng/mL for Doxycycline. Similarly, Quality Control standards (QC's) were prepared at four different concentrations namely, 51.000 (LLOQ QC), 140.000 (LQC), 2250.000 (MQC) and 3750.000 (HQC) ng/mL. Enough calibration standards and quality control standards were prepared to validate the method, and to serve as standards and controls during the assay of all study samples. However, during the study, only three levels of controls were prepared as LQC (Lower Quality Control), MQC (Middle Quality Control) and HQC (Higher Quality Control). Aliquots of the standards and quality controls were stored together with the study samples at -20°C, until used for sample processing.

Extraction method

The plasma samples (200 µl) were transferred to 1.7 mL clear tubes (Tarsons, India) and added 25 µl of IS (working solution of 40.000 µg/mL of Minocycline). The samples were vortexed. 0.200 mL of 0.5% (v/v) Formic Acid in water was added in each tube, followed by vortexing for 30 second. Tubes were centrifuged at 14000 RPM at 10°C for 5 minutes. Conditioning and equilibration of SPE cartridge (Phenomenex Strata-X (30 mg/1 mL) was done by passing 1 mL Methanol, followed by 1 mL of Water. Than plasma samples were load on the cartridges. Cartridges were washing with 1 mL of 5% (v/v) Methanol in Water twice. Samples were eluted by passing 1 mL of Elution Solution. Samples were centrifuged at 14000 rpm at 10°C for 5 minutes. Samples were transferred into auto sample vials.

Method Validation

Selectivity

Selectivity was performed using 10 different sources of blank plasma comprising of 6 normal, two Haemolyzed and two lipemic. They were processed as per the extraction method, and their response was assessed at the retention time of analytes, and the IS with six LLOQ samples for Doxycycline were prepared from the screened blank plasma samples which had the least interference [9].

Carry over

Carryover effect was evaluated to ensure that the rinsing solution used to clean the injection needle and port is able to avoid any carry forward of injected sample in subsequent runs. The design of the experiment comprised blank plasma, LLOQ, Upper Limit of Quantitation (ULOQ), followed by blank plasma to check for any possible interference due to carryover.

Linearity and lower limit of quantification

The linearity of the method was determined by analysis of five standard plots associated with a nine-point standard calibration curve. The ratio of area response for analyte to IS was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression. The calculation was based on the peak area ratio of analyte versus the area of IS. The concentration of the analyte were calculated from calibration curve (y=mx+c; where y is the peak area ratio), using linear regression analysis with reciprocate of the drug concentration as a weighing factor ($1/x^2$). Several regression types were tested and the linear regression (weighted with 1/concentration^2) was found to be the simplest regression, giving the best results ($r^2 \geq 0.9985$). The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. The deviation of standards other than LLOQ from the nominal concentration should not be more than ± 15.0% for LLOQ, it should not be more than ± 20.0%.

Accuracy and precision

The intra-batch and inter-batch accuracy and precision were determined by replicate analysis of the four quality control levels on three different days. In each of the precision and accuracy batches, six replicates at each quality control level were analyzed. Mean and Standard Deviation (SD) were obtained for calculated drug concentration over these batches. Accuracy and precision were calculated in terms of Relative Error (%RE) and Coefficient of Variation (% CV), respectively.

Matrix effect

The assessment of matrix effect (co-eluting, undetected endogenous matrix compounds that may influence the analyte ionization) was performed by processing six lots of different normal controlled plasma samples in replicate (*n*=4). LQC and HQC working solutions were spiked post extraction in duplicate for each lot. The results found were well within the acceptable limit set, i.e. the RSD of area ratio to be within ± 15% at each level tested. Also, the ion suppression/enhancement of analyte signal due to endogenous matrix interferences does not affect the quantification of analyte and IS peak which was confirmed by post-column infusion experiment. A standard solution containing Doxycycline (at MQC level) and IS was infused post column *via* a 'T' connector into the mobile phase at 10 µL/min employing in-built infusion pump. Aliquots of 5 µL of extracted control plasma were then injected into the column by the auto sampler and MRM LC-MS/MS chromatogram was acquired for analytes. Any dip in the baseline upon injection of double blank plasma (without IS) would indicate ion suppression, while a peak at the retention time of analyte would indicate ion enhancement.

Recovery

Absolute recoveries of the analyte were determined at the three different quality control levels *viz.* LQC, MQC and HQC, by comparing the peak areas of the extracted plasma samples with those of the unextracted standard mixtures (prepared in the elution solution at the same concentrations as the extracted samples), representing 100% recovery.

Dilution integrity

The dilution integrity experiment was intended to validate the dilution test to be carried out on higher analyte concentrations (above ULOQ), which may be encountered during real subject samples analysis. It was performed at 1.6 times the ULOQ concentration. Six replicates samples of ½ and ¼th concentration were prepared and their concentrations were calculated by applying the dilution factor of 2 and 4, respectively, against the freshly prepared calibration curve.

Stability

All stability results were evaluated by measuring the area response (analyte/IS) of stability samples against comparison samples of identical concentration. Stock solutions of Doxycycline and IS were

checked for short term stability at room temperature and long term stability at 2-8°C. The solutions were considered stable if the deviation from nominal value was within ± 10.0%. Bench top stability, auto sampler stability (process stability), freeze thaw stability and long-term stability in plasma were performed at LQC and HQC level using six replicates at each level. Freeze-thaw stability was evaluated by successive cycles of freezing (at -20°C) and thawing (without warming) at room temperature. To meet the acceptance criteria, the difference between the stability and fresh samples should be within ± 15%.

The same method was applied for pharmacokinetic study as A Randomized, Open Label, Balanced, Two-Treatment, Four-Period, Two-Sequence, Replicate, Crossover, Single Dose, Bioequivalence Study of Doxycycline 150 mg delay release tablet in Normal, Healthy, Adult, Human Subjects Under Fasting Condition to evaluated bioequivalence.

Results and Discussion

The mean absolute recoveries of Doxycycline determined at 140.000, 2250.000 and 3750.000 ng/mL were 78.05 (RSD 5.00%), 75.42 (RSD 0.51%) and 77.21% (RSD 2.01%), respectively. The mean absolute recovery of Minocycline was 97.44 %(RSD 1.64%). Minimal matrix effect for Doxycycline was observed from the six different plasma lots tested. The RSD of the area ratios of post spiked recovery samples at LQC and HQC levels were less than 2.62% for Doxycycline. For the IS, the RSD of the area ratios over both LQC and HQC levels was less than 3.57%. This indicated that the extracts were "clean", with no co-eluting

A: Intra day precision and accuracy of doxycycline

S. No.	Run ID	Back calculated concentration (ng/mL)			
		LLOQ QC	LQC	MQC	HQC
Nominal		**51.000**	**140.000**	**2250.000**	**3750.000**
1	1_PA01_DI01	52.054	135.890	2138.233	3894.285
		54.541	147.006	2143.167	3655.231
		52.213	132.561	2277.448	3602.057
		50.892	144.378	2163.433	3753.031
		55.299	135.245	2234.456	3671.566
		51.913	145.184	2201.595	3703.162
Mean		52.819	140.044	2193.055	3713.222
SD		1.709	6.164	55.283	101.907
% CV		3.24	4.40	2.52	2.74
% Accuracy		103.57	100.03	97.47	99.02
N		6	6	6	6

B: Inter day precision and accuracy of doxycycline

Sr. No.	Run ID	Back calculated concentration (ng/mL)			
		LLOQ QC	LQC	MQC	HQC
Nominal		**51.000**	**140.000**	**2250.000**	**3750.000**
1	2_PA01_DI01	52.054	135.890	2138.233	3894.285
		54.541	147.006	2143.167	3655.231
		52.213	132.561	2277.448	3602.057
		50.892	144.378	2163.433	3753.031
		55.299	135.245	2234.456	3671.566
		51.913	145.184	2201.595	3703.162
2	3_PA02_ABSOLUTE RECOVERY	55.405	137.067	2247.117	3606.683
		54.149	143.092	2316.983	3601.448
		53.430	140.503	2172.055	3740.801
		50.001	142.142	2188.159	3601.126
		51.115	137.198	2062.007	3651.585
		51.173	137.447	2155.949	3515.588
3	4_PA03	45.613	131.408	2076.568	3638.903
		51.750	131.422	2125.969	4044.558
		47.921	131.279	2320.304	3616.170
		47.615	137.615	2097.308	3607.019
		49.777	134.943	2113.591	3958.929
		48.176	127.819	2141.915	3785.726
Mean		51.280	137.344	2176.459	3702.659
SD		2.758	5.442	76.688	139.659
% CV		5.38	3.96	3.52	3.77
% Accuracy		100.55	98.10	96.73	98.74
N		18	18	18	18

Table 2: Intra day and inter day precision and accuracy of doxycycline.

compounds influencing the ionization of the analyte and the IS.

The high selectivity of MS-MS detection allowed the development of a very specific and rapid method for the determination of Doxycycline in plasma. No significant interfering peak of endogenous compounds was observed at the retention time of analyte in blank human plasma containing Na Heparin as the anti-coagulant in ten different plasma lots which was compared versus six replicates of extracted samples at the LLOQ level.

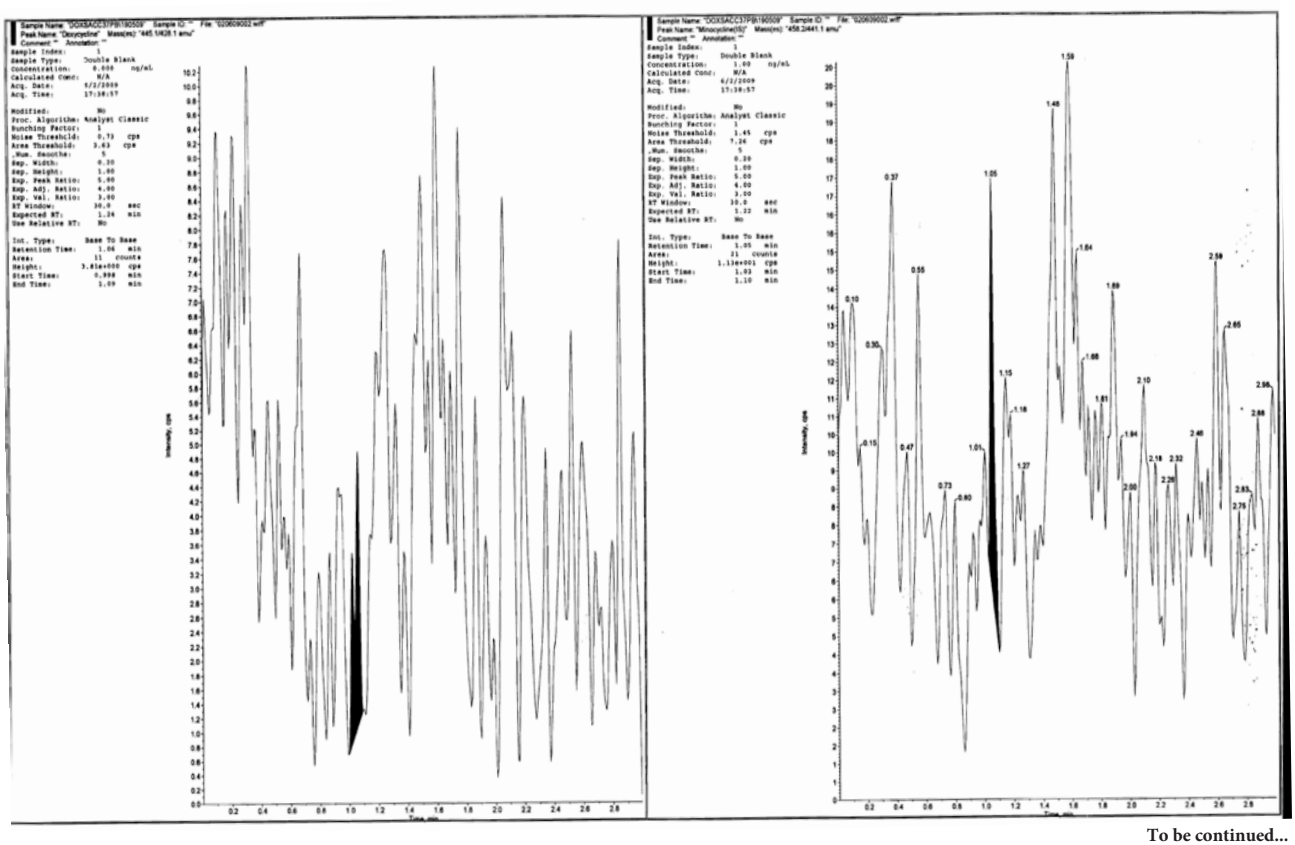
The LLOQ, defined as that concentration of Doxycycline, which can still be determined with acceptable precision (%RSD ≤ 20) and accuracy (bias within ± 20%) was found to be 50.000 ng/mL. Results of the intra-batch and inter-batch validation assays are presented in Table 2, respectively. The inter-batch and intra-batch precision were ≤ 5.38% and ≤ 4.40%, whereas the inter-batch and intra-batch accuracy in terms of % bias were within the range of -3.27 to 0.55 and -2.53 to 3.57 for Doxycycline, respectively.

Bench top and processed (auto sampler) stability for Doxycycline were performed at LQC and HQC levels. The results revealed that Doxycycline was stable in plasma for at least 15 h at room temperature and 49 h in auto sampler at 10°C. It was confirmed that repeated freeze and thawing (five cycles) of spiked plasma samples at LQC and HQC level did not affect the stability of Doxycycline. Doxycycline was found stable for minimum five freeze and thaw cycles. The long term stability results also indicated that Doxycycline was stable in human plasma for up to 96 days at a storage temperature of -20°C & -70°C. This period of long term stability was sufficient enough to cover the entire storage period from first day of storage of the plasma samples to the last day of analysis.

During method development, different options were evaluated to optimize sample extraction, detection parameters and chromatography. Electro spray ionization (ESI) was evaluated to get better response of analytes, as compared to Atmospheric Pressure Chemical Ionization (APCI) mode. In the nonionic forms, the strong binding of analytes to the copolymer of SPE cartridge enables sufficient clean up. Best signal for the analyte was achieved with the ESI positive ion mode. The effect of pH of buffer also checked on sensitivity and peak shape. But, the best signal and peak shape for Doxycycline was achieved using a mobile phase of 0.05% (*v/v*) formic acid in deionised water. Use of a short Chromolith RP18e (100 mm×4.6 mm) column resulted in reduced flow rate 1 mL/min with Splitter, 60% flow to waste and reduced run time. The retention times for Doxycycline and Minocycline were ~ 1.29 minutes and ~ 1.27 minutes, respectively.

Minocycline, used as IS, belonged to the same therapeutic category as Doxycycline. Ionization, retention and extraction characteristics were found to be similar to that of Doxycycline, and hence, it was selected as the IS of choice. The validated method was employed to analyze plasma samples containing Doxycycline obtained from a single oral dose of 150 mg Doxycycline per treatment in 36 healthy volunteers under fasting conditions. Blank sample, spike sample (CS1) and subject sample chromatogram with IS are presented (Figure 4a and 4b). The study design was a randomized, open label, Two-treatment, two-sequence, two-period, crossover, single dose, bioequivalence study. The study conducted was carried out after approval from an independent ethics committee and obtaining written consent from the volunteers.

Test vs. reference product of Doxycycline 150 mg Delayed release



To be continued...

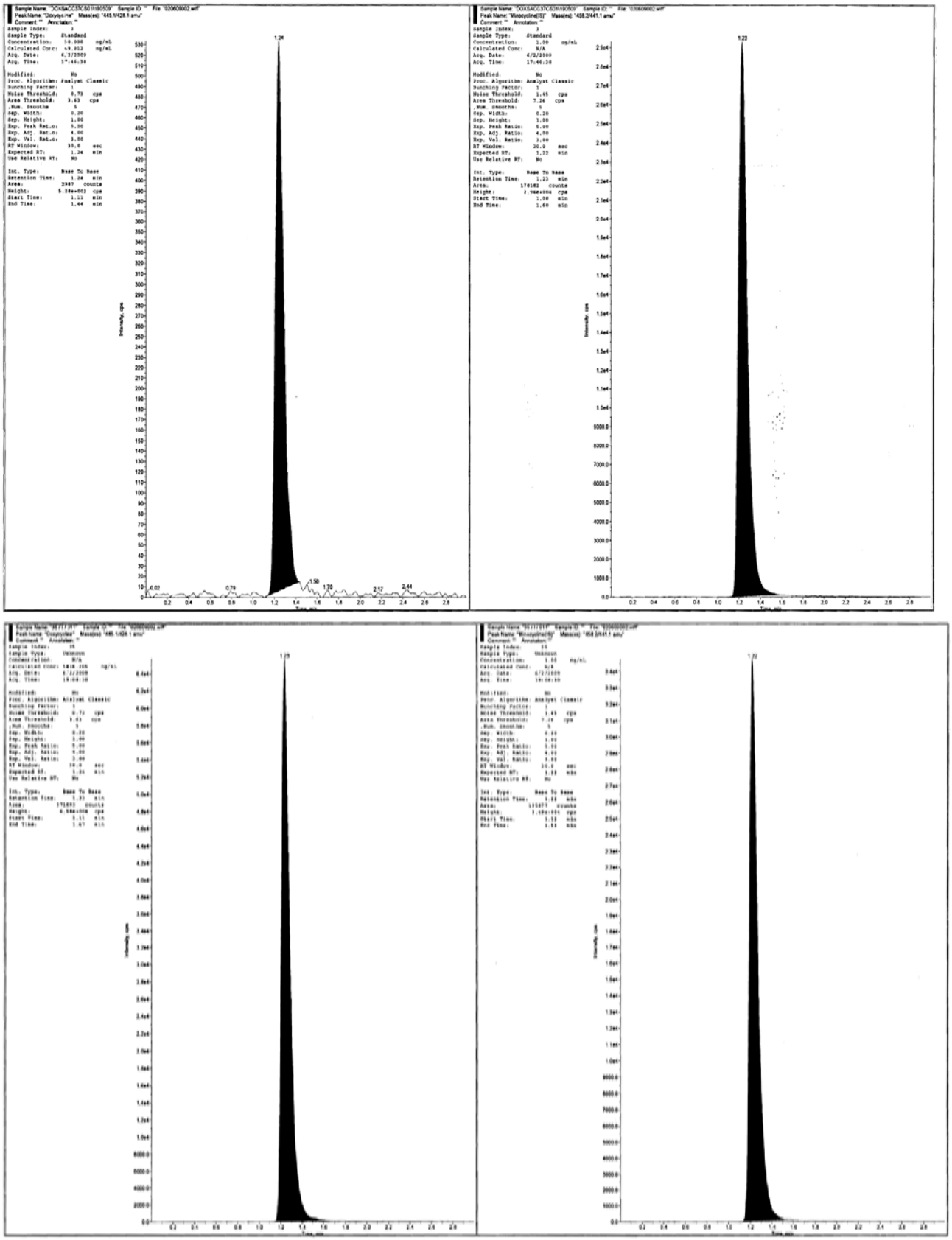


Figure 4: a. Chromatogram of blank check. b. Chromatogram of spike sample.

tablet result, as shown in Table 3, detailed that the both the product meets bioequivalence.

Conclusion

A simple, selective and rapid method for the estimation of Doxycycline in human plasma was developed and validated, using LCMS/MS API 2000. The validated method was used in a bioequivalence study in which 36 healthy volunteers were enrolled; each subject dosed 150 mg Doxycycline tablets (reference and test) as a single oral dose. The method allows higher sample throughput due to the short chromatography and simple sample preparation. Robust LC-MS-MS performance was observed, with acceptable variation in instrument response within batches. This method is an excellent analytical option for rapid quantification of Doxycycline in human

Sr. No.	Sample I.D	Initial Conc. (ng/mL) (A)	Repeat Conc.(ng/mL) (B)	Mean C:(A+B)/2	% Diff
1	01 / I / 006	3450.686	3349.363	3400.025	-2.98
2	01 / I / 009	3494.588	3412.553	3453.571	-2.38
3	01 / I / 020	740.183	728.439	734.311	-1.60
4	01 / I / 021	316.661	346.565	331.613	9.02
5	01 / II / 009	3132.694	3334.969	3233.832	6.25
6	01 / II / 010	3018.738	3389.569	3204.154	11.57
7	01 / II / 021	314.951	353.057	334.004	11.41
8	01 / II / 022	153.357	147.811	150.584	-3.68
9	02 / I / 006	2302.093	2695.926	2499.010	15.76
10	02 / I / 007	2325.019	2542.780	2433.900	8.95
11	02 / I / 019	654.602	651.265	652.934	-0.51
12	02 / I / 020	268.152	296.138	282.145	9.92
13	02 / II / 005	2570.680	2708.345	2639.513	5.22
14	02 / II / 006	2408.683	2537.141	2472.912	5.19
15	02 / II / 019	687.463	715.645	701.554	4.02
16	02 / II / 020	335.565	361.391	348.478	7.41
17	03 / I / 008	3572.916	3365.365	3469.141	-5.98
18	03 / I / 012	3242.747	3420.542	3331.645	5.34
19	03 / I / 021	468.291	491.968	480.130	4.93
20	03 / I / 022	214.316	213.116	213.716	-0.56
21	03 / II / 006	3924.709	3100.400	3512.555	-23.47
22	03 / II / 007	4518.131	3891.392	4204.762	-14.91
23	03 / II / 022	249.541	209.604	229.573	-17.40
24	03 / II / 023	163.996	128.357	146.177	-24.38
25	04 / I / 007	3335.021	3321.079	3328.050	-0.42
26	04 / I / 011	3339.249	3507.108	3423.179	4.90
27	04 / I / 019	1072.730	1158.220	1115.475	7.66
28	04 / I / 020	381.138	400.302	390.720	4.90
29	04 / II / 009	2032.321	2302.294	2167.308	12.46
30	04 / II / 012	2079.133	1994.308	2036.721	-4.16
31	04 / II / 019	662.633	789.707	726.170	17.50
32	04 / II / 020	277.824	321.136	299.480	14.46
33	05 / I / 007	2817.893	2854.185	2836.039	1.28
34	05 / I / 008	2740.465	2691.639	2716.052	-1.80
35	05 / I / 020	388.346	373.503	380.925	-3.90
36	05 / I / 021	187.789	187.384	187.587	-0.22
37	05 / II / 010	2213.004	2535.508	2374.256	13.58
38	05 / II / 011	2210.087	2186.241	2198.164	-1.08
39	05 / II / 020	567.237	565.742	566.490	-0.26
40	05 / II / 021	179.722	187.670	183.696	4.33
41	06 / I / 011	3248.921	3607.874	3428.398	10.47
42	06 / I / 012	3009.303	2865.491	2937.397	-4.90
43	06 / I / 020	440.952	426.658	433.805	-3.30
44	06 / I / 021	175.673	157.904	166.789	-10.65
45	06 / II / 011	3508.409	3167.121	3337.765	-10.23
46	06 / II / 013	3462.987	3653.528	3558.258	5.35
47	06 / II / 020	448.842	459.760	454.301	2.40
48	06 / II / 021	189.901	178.460	184.181	-6.21
49	07 / I / 008	2950.533	2797.557	2874.045	-5.32
50	07 / I / 010	2915.308	2782.023	2848.666	-4.68

Sample ID xx/yy/zz whereas xx denotes Subject number; yy: Period number; ZZ: Time point number.

Table 3: Incurred subject reanalysis result of Haemolysis samples for Doxycycline.

Analyte	Treatment	C_{max} (ng/mL)	T_{max}	AUC_t	AUC_{inf}	K_{el}	$T_{1/2}$(h)
Doxycycline	Test	3323.86	3.10	75168.89	77838.59	0.03	19.63
	Reference	3493.27	3.02	80037.67	82619.92	0.03	19.42

Table 4: Summary of statistics for target parameters, Test vs. Reference, following a single dose of Doxycycline 150 mg Delay release tablet to thirty six volunteers under fasting conditions.

plasma. Result shown in Table 4, with initial run while Sample Analysis compare with Incurred subject re analysis to demonstrate method reproducibility as 96% ISR samples meet acceptance criteria for Erythromycin Ethylsuccinate, whereas the acceptance criteria for ISR is 67% samples should meet + or - 20% difference. The test and reference formulation behavior was resulted as bioequivalence.

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