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Research Article

DEVELOPMENT AND VALIDATION OF BIOANALYTICAL METHOD FOR MACITENTAN IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

The present study describes a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the macitentan in human plasma using liquid liquid extraction technique. Method of macitentan has been developed and validated using macitentan D4 as internal standard. Electro spray ionization technique was used in this method. Analytes were recovered by liquid liquid extraction method and subsequently separated C18l column using 0.5% Formic Acid in water :Acetonitrile, 20:80v/v as a mobile phase, at a flow rate of 1.0 mL/min. Quantification of macitentan and macitentan D4 was performed using multi-reaction monitoring mode (MRM) in positive mode. The calibration curve was linear (r^2 > 0.99) over the concentration range of 1.00 to 500 ng/mL for macitentan. The intra-day and inter-day precisions were less than 15% and the accuracy was all within ±15% (at LLOQ level ±20%). The LC-MS/MS method was fully validated for all the other parameters such as selectivity, matrix effect, recovery and stability as well. In conclusion, the findings of the present study revealed the selectivity and sensitivity of this method for the macitentan in human plasma.

Keywords: Bioanalytical method; Macitentan; Human plasma and LC-MS/MS.

1. INTRODUCTION

Essential hypertension is the form of hypertension that by definition has no identifiable cause. It is the most common type of hypertension, affecting 95% of hypertensive patients, it tends to be familial and is likely to be the consequence of an interaction between environmental and genetic factors. Pre valence of essential hypertension increases with age, and individuals with relatively high blood pressure at younger ages are at increased risk for the subsequent development of hypertension. Hypertension can increase the risk of cerebral, cardiac, and renal events. Macitentan was approved in October 2013. It is indicated for patients with pulmonary arterial hypertension. Macitentan is an antagonist of endothelin receptors on blood vessels and smooth muscle, and, thus, blocks the stimulation of vasculature hypertrophy, inflammation, fibrosis, proliferation, and vasoconstriction[1-10].

Macitentan has a chemical formula $C_{19}H_{20}Br_2N_6O_4S$ and is a N-[5-(4-Bromophenyl)-6-[2-[(5-bromo-2-pyrimidinyl)oxy]ethoxy]-4-pyrimidinyl]-N'-propylsulfamide. Macitentan is administered orally, and it take about 8 hours for maximum plasma concentrations to be reached. Macitentan is >99% bound to plasma proteins, which are mainly albumin[1-10].

Qualitative and quantitative determination of macitentanl in the biological fluids is studied extensively using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and high performance liquid chromatography (HPLC). M.Purushothaman et al.[13] employed high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to quantify macitentan. Linearity range of this method is 0.997 to 1020.793 ng/mL and

Losartan was used as a internal standard which is limitation of this method. Yu L et al.[14] used high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to quantify macitentan and its metabolite with lower limit of quantification 1 ng/mL. Protein precipitation technique was used for extraction of macitentan.

In the light of above background, the present study was planned to develop and validate a run time efficient analytical method with minimum matrix effect and use of deuterated internal standard of macitentan in human plasma using a minimum sample volume. Further, the method was designed to be sensitive enough to quantify samples from a single dose study.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

Macitentan and macitentan metabolite 132577 were purchased from Clearsynth Labs, Mumbai, India. Macitentan D4 was purchased from Bioorganics, Mumbai, India. Human Plasma was purchased from Suptratech Laboratory, Ahmedabad, Gujarat, India. Methanol and acetonitrile of HPLC grade was purchased from Spectrochem Pvt. Ltd., Mumbai, India. Formic acid, Orthophosphoric acid, Dichloromethane and Di ethyl ether were purchased from Merck Millipore, Mumbai, India. Water was purified using a Milli-Q water purification system, Millipore Pvt. Ltd., Ahmedabad, Gujarat, India.

2.2. Instrumentation

The LC-MS/MS system (Shimadzu Corporation, Japan) equipped with a LC-20AD solvent delivery system, a DGU-20A5R vacuum degasser, a CTO-20AC thermostated column oven, SIL-20AC auto sampler and coupled with a triple quadrupole mass spectrometer LCMS- API 4000 (MDS Sciex, USA). Data acquisition and processing were performed using analyst software (version 1.6.2; MDS Sciex, USA) and Watson LIMS (version 7.3; Watson, USA).

2.3. Chromatographic conditions

Chromatographic separations were achieved on cosmosil 5CI8-MS-II, 4.6 X 150mm column placed in thermostated column oven at 35°C using mobile phase consisting of 0.5% Formic Acid in water :Acetonitrile, 20:80 v/v at a flow rate of 1.0 mL/min with 70% spitting. Sample injection volume was 10 µL. Analytical run time was 4.0 min.

2.4. Mass spectrometric conditions

The mass spectrometer was operated in positive ion mode using electrospray ionization (ESI) source. Tuning parameters were optimized for Macitentan and macitentan D4 by infusing a solution containing 100 ng/mL of each analytes. Mass spectra of product ions are presented in Fig 1. The source dependent parameters maintained for macitentan were Gas 1 (Nebuliser Gas): 50 psig; Gas 2 (Heater Gas): 40 psig; ion spray voltage (ISV): 5500V, turbo heater temperature (TEM): 500°C; interface heater (Ihe): ON; collision activation dissociation (CAD): 6 psig and curtain gas (CUR): nitrogen: 30 psig. Quantification of analytes performed using multiple reaction monitoring of the transitions m/z 589.000/200.900 for macitentan and 593.000/204.900 for macitentan D4, with the dwell time of 200 ms per transition. Optimized collision energy (CE) of 21 was used for macitentan and macitentan D4.

2.5. Preparation of standard and quality control (QC) samples

The standard stock solutions of macitentan and macitentan D4 were prepared by dissolving 2 mg of each analytes in methanol to give final concentration of 1.00 mg/mL of each analyte. Macitentan stock solution was further diluted with methanol to give final concentration of 50.0 µg/mL. Further, solutions were diluted with methanol to achieve working standard solutions at the concentrations of 50.0, 100, 250, 500, 1250, 2500, 5000, 12500 and 25000 ng/mL for macitentan. A working solution of IS was prepared by diluting the standard stock solution of macitentan D4 in methanol to achieve a final concentration of 200 ng/mL. The working standard solutions (20 µL) were used to spike blank human plasma sample (980 µL) to build up the calibration curve of analyte and for quality control in validation studies [18]. The final concentrations in standard plasma samples were 1.00, 2.00, 5.00, 10.0, 25.0, 50.0, 100, 250 and 500 ng/mL for macitentan. The QC samples were prepared in the same way as the calibration samples. The plasma concentrations of QC samples were 1.00, 3.00, 30.0, 200 and 400 ng/mL for macitentan. All working solutions were stored at 2-8°C until analysis.

2.6. Plasma sample preparation

Aliquots of 300 µL of unknown plasma, blank samples, calibration curve standard (CCs) and quality control standard (QCs) samples were prepared in 5 mL eppendorf centrifuge tubes and 50 µL of IS solution was added. Subsequently, 100µL of Orthophosphoric acid (2%v/v) was added. 2.5 mL Di ethyl ether: Dichloromethane (80:20 v/v) was added and samples were extracted on extractor at 50 rpm for 20 minutes. Samples were centrifuged at

4000 rpm, at 10°C for 05 minutes. 2 mL of supernatant was collected in pre-labeled tubes and evaporated to dryness in an evaporator at 40°C under the gentle stream of nitrogen. The dried samples were reconstituted by addition of 100µL of mobile phase, loaded into autosampler and followed by injection of 10 µL of reconstituted samples into LC-MS/MS system.

2.7. Quantification

Quantitative analysis of macitentan was performed using macitentan D4 as an internal standard. Calibration curves were established with CCs prepared in plasma. Eight-point CCs constructed using peak area ratio of analytes to IS. Concentration of analytes in QCs and unknown samples were calculated by interpolation from the calibration curves.

2.8. Method validation

Method validation protocol was based on the recommendations of the United States Food and Drugs Administration (USFDA) guidelines[11] and EMEA guidelines[12].

2.8.1. Selectivity

The selectivity of method toward endogenous plasma matrix components was assessed by comparing the interfering signals in nine different batches of plasma (six were of K_3EDTA, and one each of lipidemic K_3EDTA, haemolysed K_3EDTA and heparinized plasma) against the signals of analytes and IS. Aliquots of plasma samples were used to prepare lower limit of quantification (LLOQ) and blank samples. Baseline noise kept < 20% of analyte response at this concentration level.

2.8.2. Linearity, accuracy and precision

The linearity of the method was assessed by processing an eight-point calibration curve over the concentration range of 1.00 to 500 ng/mL for macitentan in three consecutive runs. Calibration curves were constructed by fitting the analyte concentrations of the calibrators versus the peak area ratios of the analyte to IS. Each calibration curve was analyzed individually by least square weighted ($1/x^2$) linear regression. The inter- or intra-batch accuracy and precision were evaluated using six replicates of QC samples at LLOQ, lower (LQC), middle-2 (MQC-2), middle-1 (MQC-1) and higher (HQC) concentration levels for three consecutive analytical days. The concentration of QC samples was selected from the calibration curve range. The criteria for acceptability of the data included precision within 15% coefficient of variance (% CV) and an accuracy within ±15% relative error (% RE) of the nominal values. Limit of quantification was established by six replicates of 1.00 ng/mL in each three different runs.

2.8.3. Recovery

Recovery of the analytes after solid phase extraction was estimated at low, middle and high concentration levels by comparing mean peak-area of the extracted samples with mean peak-area of post-spiked extracted samples, which represent 100% recovery. Extraction recovery of IS was determined in the similar way using QC samples at medium concentration as a reference.

2.8.4. Matrix effect

Matrix effect was evaluated by comparing the mean peak area of analytes spiked in blank extracted plasma samples (respective analyte working solution was spiked at the time of reconstitution) (A) with corresponding mean peak area of analytes prepared in mobile phase (B).

$$\textbf{Matrix effect} = (A/B) \times 100$$

For a method to be free from relative matrix effect, the % coefficient of variance (CV) of normalized matrix effect should be less than 15%[12].

2.8.5. Stability

The present study also evaluated the stability studies of macitentan in plasma samples. The bench top (at ambient temperature), freeze-thaw (at -20°C), process stability (at room temperature and at 2-8°C) and long term stability (at -20°C) of each analyte was evaluated at LQC and HQC concentration level using six replicates at each concentration. Analyte was considered as stable if the % change is less than 15% as per USFDA guidelines[11]. Freeze-thaw cycle included thawing of samples at room temperature for 90 min and then refreezing at -20°C. Concentrations of stability samples and freshly prepared samples were calculated and stability was shown as the percentage mean change in calculated concentration. Samples for long term stability study, were kept at -20°C, processed and then compared with new freshly prepared solutions.

3. RESULTS AND DISCUSSION

3.1. Optimization of the mass spectrometric condition

For optimum detection and simultaneous quantification of macitentan with IS in human plasma, it was necessary to adjust chromatographic conditions and mass

spectrometric parameters as well. The mass spectrometric parameters were tuned in both the positive and negative ionization mode for all analytes. Macitentan and macitentan D4 showed prominent peak in the positive ionization mode.
Optimization of ionization voltage, interface temperature, curtain gas, GS1, GS2, and CAD gas flow are of utmost importance in order to minimize ion suppression and to increase sensitivity. The results of the present study showed ionization voltage, interface temperature, curtain gas, GS1, GS2, and CAD gas flow above 5500, 500°C, 30.0, 50.0, 40.0 and 6.00, respectively, and augmented the intensity of the analyte. A dwell time of 200 ms for macitentan and macitentan D4 was sufficient and no cross talk was found between all multiple reaction monitoring.

3.2. Optimization of the sample preparation and chromatographic conditions

One of the key fundamental steps in the development of an analytical method is sample preparation. Sample preparation procedure should be quick, easy to proceed and should require least amount of reagents with maximum recovery of analytes. In this regards, literature review revealed the use of protein precipitation technique for the extraction of macitentan. However, we employed liquid liquid extraction as the sample preparation method and it yielded cleaner sample, least matrix effect and desired recoveries of the analytes. Interestingly, the proposed method was developed with less plasma and injection volume, which may lead to better acceptability of the method. To develop rugged liquid liquid extraction method, several trials of different organic solvent were evaluated with changes made in buffer and reconstitution solution. Better response, least matrix effect, less interference and high recovery were obtained using organic solvent Di ethyl ether: Dichloromethane (80:20 v/v) and Orthophosphoric acid (2%v/v) as buffering agent. There was no sign of interference from any endogenous or exogenous plasma matrix and IS did not alter analyte recovery, sensitivity and/or ion suppression as well.
Macitentan D4 was chosen as the internal standard for this method. Chromatographic conditions were optimized to achieve good sensitivity and peak shape for macitentan and IS, as well as a short chromatographic run time with proper retention of the peak. In this study, we tried Gemini C18, Waters symmetry C18, Cosmosil C18 columns with various mobile phases such as methanol, acetonitrile, formic acid, ammonium acetate and ammonium carbonate. The Cosmosil 5CI8-MS-II, 4.6 X 150mm was selected as it gave better sensitivity with mobile phase 0.5% Formic Acid in water :Acetonitrile, 20:80v/v as its give highest sensitivity and better peak shape Low injection volume of 10 µL reduced overloading of column with analytes, thereby ensuring more numbers of analyses on the same column. The quantification of macitentan was done using LC-MS/MS. Chromatograms of STD BL, STD ZERO, STD1, and LLOQ of macitentan and macitentan D4 are presented in Fig.2.

3.3. Selectivity

Fig. 2 depicts that there was no interference peaks observed from endogenous compounds at retention time in any of the samples of macitentan and macitentan D4 extracted from plasma. The responses of drug substances in blank plasma were less than 2.21% for macitentan, respectively, at LLOQ of 1.00 ng/mL. Typical retention time of macitentan and macitentan D4 was 2.80 min, respectively.

3.4. Linearity, accuracy and precision

Usually the least square method can create relatively large errors at the levels with low concentrations, as in case with proposed developed method. The proposed method utilized relatively low concentration range i.e. 1.00 to 500 ng/mL for macitentan. To overcome above mentioned problem, the concept of weighted calibration curves was applied and calculation was made using weighting factors [$1/x^2$] and the calibration curve was calculated. The results indicated that the weighted data for calibration curve was more accurate in the experimentation and the application of weighted factor was the best choice for proposed method. A typical equation of the calibration curve on a validation batch was as follows: $y = 0.0260x + 0.0011$ ($r^2 = 0.9968$) for macitentan. The present bio-analytical method provided lower limit of quantitation and good range of linearity. The proposed method can detect lower concentration up to 5% C_{max} of macitentan and upper concentration was more than two times of the C_{max} of drugs. Good linearity was obtained with aforementioned concentration ranges with a correlation coefficient (r^2) greater than 0.995. Table 3 summarizes the results for intra- and inter-day precision and accuracy for macitentan measured by QCs. To validate the accuracy and precision of the developed method, three concentrations of QCs in six replicates were utilized. The results showed that the intra- and inter-day accuracy within 15% for macitentan. In context to this, the present LC-MS/MS method for simultaneous assessment of macitentan was found to meet the

accepted limits for accuracy and precision experiments[11].

3.5. Recovery

The % recovery was determined by comparing the mean peak area in extracted samples with freshly prepared un-extracted samples at three different concentrations. The % recovery of macitentan was found to be 68.04, 72.09, 71.52% respectively, at three different concentrations. Mean recovery for the macitentan D4 was 77.79% (Table 4). In bioanalytical method development the extent of recovery is not considered as an issue provided that the method yields sensitivity, precision and accuracy [11].

3.6. Matrix effect

Two QC concentrations of each tested analyte and IS were utilized in the test with six different sources of human plasma. Table 5 depicts that there was no significant ion suppression or enhancement observed for all the analytes and IS under the present experimental conditions.

3.7. Stability

The stability study of macitentan in plasma has been evaluated. The bench top (at ambient temperature), freeze-thaw (at -20 °C), process stability (06 hr at room temperature and 163 hr at 2-8 °C) and long term stability (at -20 °C) study of each analyte was evaluated at LQC and HQC concentration level using six replicates at each concentration. Analyte was considered as stable if the % change is less than 15% as per USFDA guidelines[11]. The bench top stability, process stability and freeze-thaw stability for macitentan were assessed at different conditions of temperature and time. Frozen samples were allowed to thaw at room temperature for 90 min and then refrozen at -20 °C. Concentrations of stability samples and freshly prepared samples were calculated and stability was shown as the percentage mean change in calculated concentration. Long term stability was performed for 47 days. Samples were kept at -20 °C for 47 days, processed and then compared with new freshly prepared solutions. Results of stability experiments were given in table 6.

3.8. Study Sample analysis

This method has been successfully applied for the estimation of macitentan. Incurred sample reanalysis results were also found within acceptance criteria (Fig.3). Comparison of two formulations are given in Fig.4.

4. CONCLUSION

A simple, rapid, sensitive and selective LC-MS/MS method was developed and validated for the estimation of macitentan in human plasma using macitentan D4 as an internal standard. The present method has advantage of low processing volume (300µl), shorter run time (4 min) and minimum matrix effect in comparison to available stereo isomers method. To the best of our knowledge, the findings of the present study provide strong scientific evidence for accuracy and precision of quantification of macitentan in human plasma. This method may have application to characterize the clinical pharmacokinetic studies of macitentan in humans.

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Table1: Intra-day and inter-day variation for macitentan in six replicates (n=6) at each concentration

Concentration (ng/mL)	Intra-day		Inter-day	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
1.000	105.60	5.05	99.20	10.89
3.000	104.50	3.32	100.27	4.26
30.000	95.83	1.99	98.79	2.88
200.000	98.27	1.96	99.23	1.98
400.000	91.27	1.92	93.10	2.66

Table 2: Recovery values of macitentan and macitentan D4 (n=6)

	Concentration (ng/mL)	Recovery
Macitentan	3.000	71.52
	200.000	72.09
	400.000	68.04
Macitentan D4	200.000	77.09

Table 3: Matrix factor for macitentan at high and low concentration (n=10)

Concentration (ng/mL)	ISTD normalized matrix factor	RSD (%)
3.000	1.002	1.56
400.000	1.013	0.92

Table 4: Stability studies of macitentan in human plasma at high and low concentration (n=6)

Storage Conditions	Concentration (ng/mL)	%Accuracy	RSD (%)
Bench top	3.000	100.17	2.66
	400.000	97.73	2.03
Wet extract stability (2 to 8°C)	3.000	100.20	2.93
	400.000	98.02	2.71
Freeze thaw	3.000	94.73	5.14
	400.000	96.54	1.33
Dry extract	3.000	96.27	8.41
	400.000	97.09	2.32
Long term in matrix	3.000	98.03	2.58
	400.000	95.74	3.14

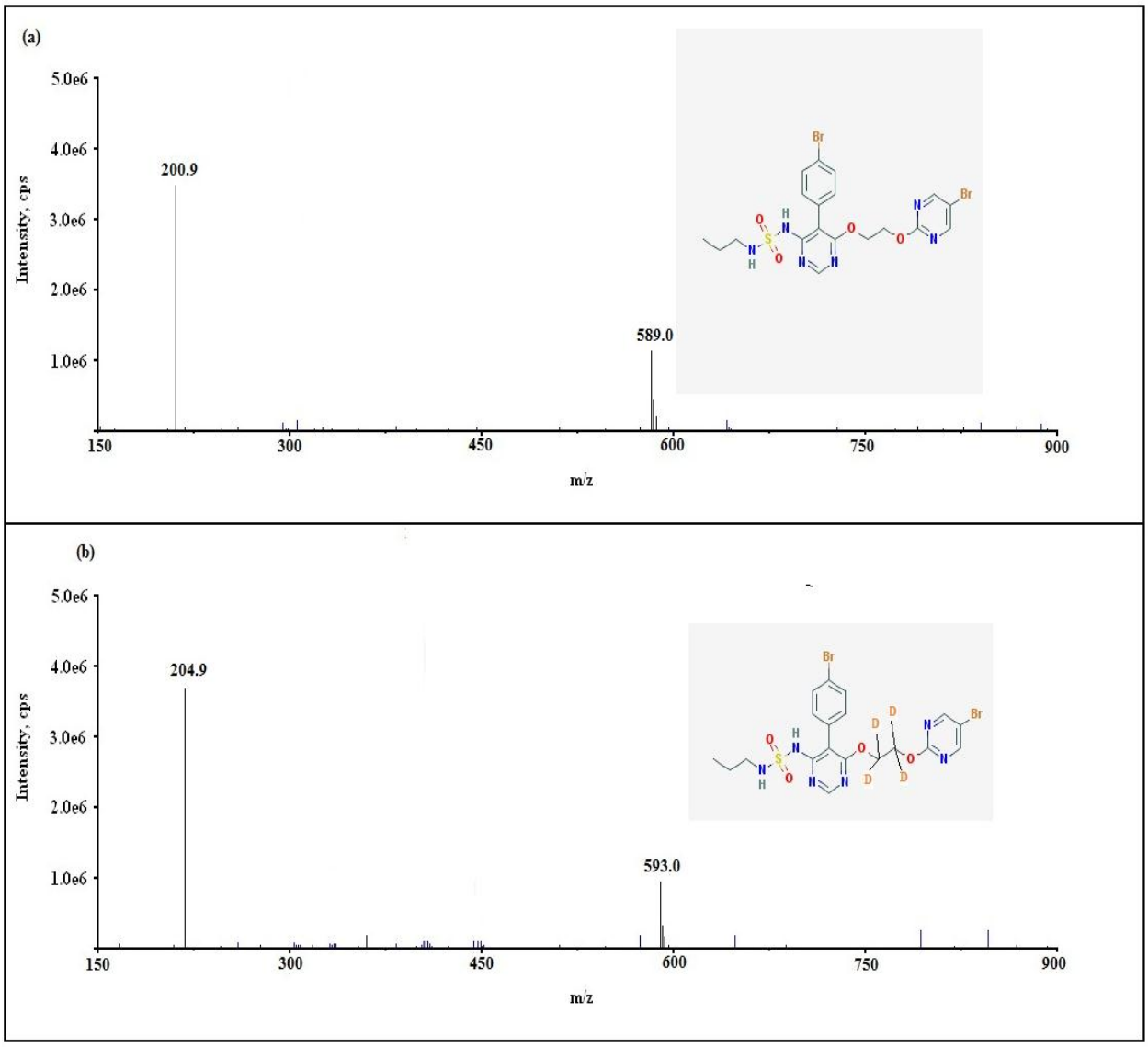

Fig. 1: Spectrum of product ion scans of macitentan (a) and macitentan D4

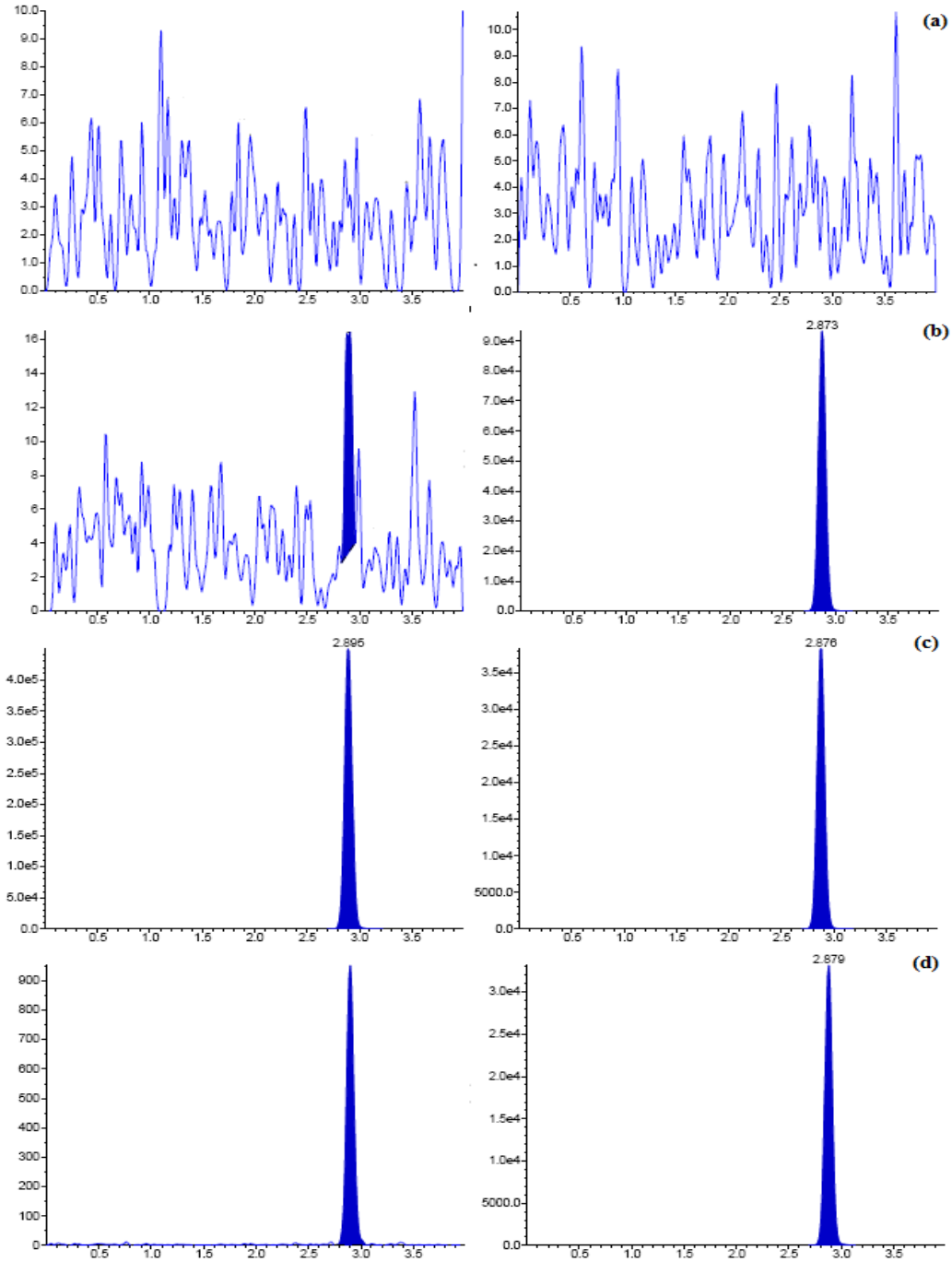

Fig. 2: Representative MRM Chromatogram of macitentan at standard blank (a), standard zero (b), upper limit of quantification (ULOQ) (c) and lower limit of quantification (LLOQ) (d) level in human plasma

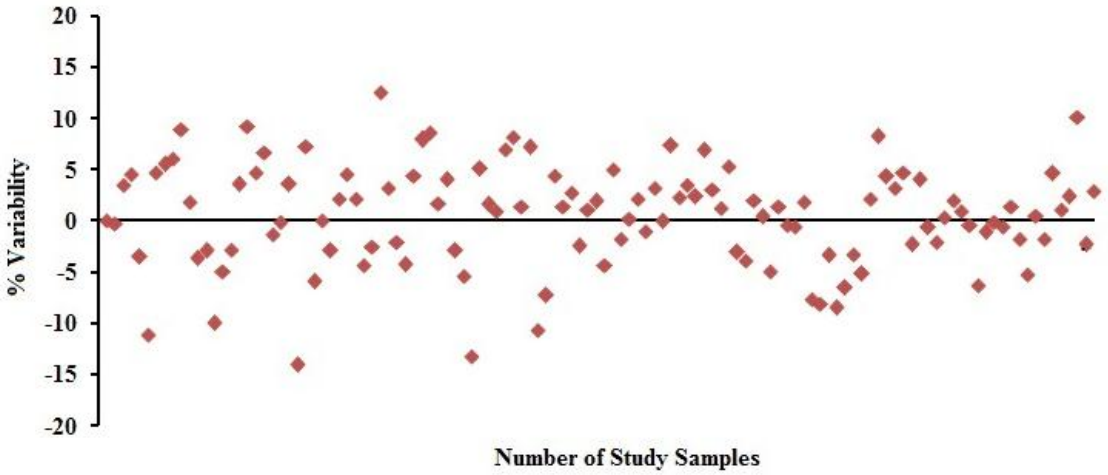

Fig. 3: Incurred sample reanalysis results

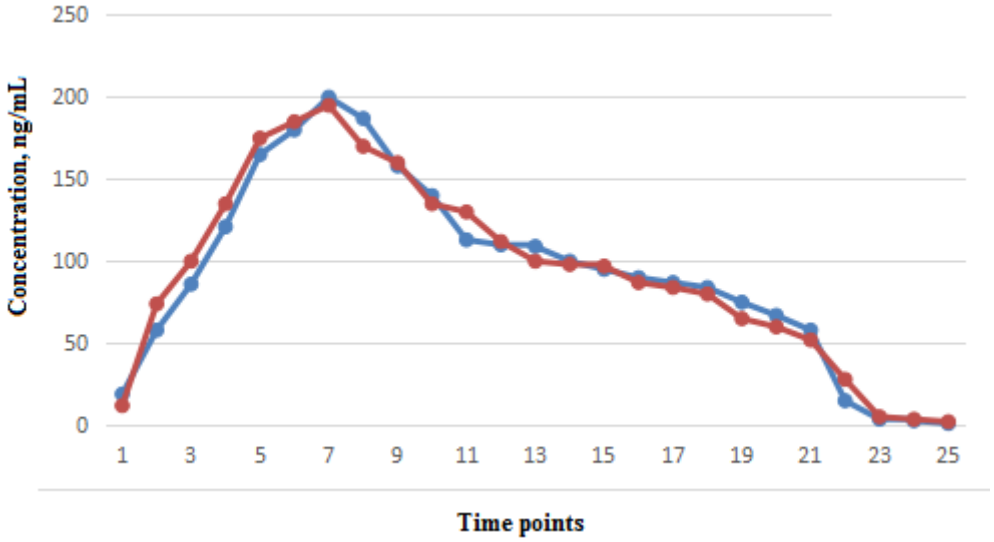

Fig. 4: Comparison of Subject sample profile of two formulations

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