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Development and Validation of High-Performance Liquid Chromatography-Tandem Mass Spectrometric Method for Simultaneous Quantification of Telmisartin in Human Plasma

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ABSTRACT
A simple, sensitive and selective method for the quantification of Telmisartan by using rapid high-performance liquid chromatography/positive electroscopy ionization tandem mass spectroscopy. The method consists of extraction with Acetonitrile followed by the analysis of the Extracted sample by liquid chromatography-mass spectroscopy (LC-MS/MS) in multiple reaction monitoring mode using electrospray ionization mode (ESI). Chromatography was performed on a C_{18} reverse phase column, Methanol: Acetonitrile: 10mM ammonium acetate (45:45:10) as a mobile phase. The assay exhibited a linear dynamic range of 10 to 700ng/ml for Telmisartan in human plasma. Stability assessment was also included. A run time of 2.5 min for each sample made it possible to analyse healthy volunteers participating in pharmacokinetics drug-drug interaction studies.

Keywords: LC-MS/MS, Human Plasma, Bioanalytical, Telmisartan, Validation.

INTRODUCTION

Telmisartan, 4-[(2-*n*-propyl-4-methyl-6-(1-methylbenzimidazole-2-yl)-benzimidazole-1-yl) methyl]-biphenyl-2-carboxylic acid, is a known drug against high blood pressure widely used in the treatment of hypertension. , which belongs to the group of angiotensin II receptor antagonists. [1-2] Other members of this group are, e.g. candesartan, eprosartan and valsartan. Telmisartan is highly selective for angiotensin II (AT1) receptors. It inhibits the angiotensin II receptor in a way that the effect of angiotensin II is blocked resulting in a decrease of blood pressure. [3] It undergoes minimal biotransformation in the liver to form the inactive telmisartan 1-*o*-acylglucuronide as its principal metabolite. [4] The long half-life and selectivity of telmisartan for angiotensin II receptors allows once daily dosing with minimal side effects.

Several methods to determine Telmisartan with HPLC or gas chromatography-mass spectrometry have been previously described. [5-15] The assays used relatively large plasma sample volumes (up to 2 ml) and either multiple-step liquid or solid-phase extraction procedures.

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In this report, we describe a highly sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method developed and validated for the quantification of Telmisartan in human plasma with simple single-step extraction method, minimum run time for chromatographic separation per each sample and minimum processed volume of plasma. It is essential to establish an assay capable of quantifying Telmisartan at lower concentrations. At the same time, it is expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of Telmisartan.

MATERIALS AND METHODS

Chemicals and Solvents

Telmisartan drug substance and Ticlopidine Hydrochloride (Internal Standard) was obtained from IDDS (Hyderabad, India). The chemical structures are represented in Fig. 1. HPLC-grade Methanol and Acetonitrile was purchased from JT Bakers. Ammonium Acetate was purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q water system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

LC/MS/MS instrument and conditions

The high-performance liquid chromatography (HPLC) SILHTC system (Shimadzu Corporation, Kyoto, Japan) is

equipped with LC-20 AD VP binary pump, a DGU20A3 Degasser, and a SIL-HTC auto sampler equipped with a CTO-10AS VP thermostated column. The chromatography [16-18] was on Thermo BDS Hypersil C18, (5 µm, 4.6 × 50 mm) at a temperature of 20°C. The isocratic mobile phase composition was a mixture of 45:45:10 ACN: MeOH: 10 mM Ammonium acetate, which was pumped at a flow rate of 0.8 ml/min. Mass spectrometric detection was performed on a TSQ Quantum Discovery MAX triple quadrapole instrument (Thermo Finnigan, USA) using the Multiple reaction monitoring (MRM) mode. A turbo electrospray ionization (ESI) interface in positive mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on LC Quan 2.5.6. Software package (Thermo).

Sample preparation

Standard stock solutions of Telmisartan (10 mg/ml) and the IS (10 mg/ml) were separately prepared in Methanol. Working solutions for calibration and controls were prepared by appropriate dilution in 80:20 Methanol: water. The IS working solution (100 ng/ml) was prepared by diluting its stock solution with diluent (70:30) methanol: water. Working solutions (0.2 ml) were added to drug-free human plasma (9.8 ml) as a bulk, to obtain Telmisartan concentration levels of 10.827, 29.263, 69.674, 145.155, 290.309, 414.728, 552.970 and 700.213ng/ml, as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 13.430(LLOQ), 31.975(Low), 278.045 (medium) and 455.811 ng/ml (high), as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in Ria Vials (Tarson, 5 ml) and stored in a freezer at below -80°C until analyses. A plasma sample (0.100 ml) was pipetted into a 2 ml centrifuge tube, 50 µl of IS working solution (100 ng/ml) were added. After vortex mixing for 10 s, a 1.0 ml Acetonitrile was added and the sample was vortex-mixed for 10 s. Centrifuge the centrifuge tubes at 14000 rpm at 10°C for 10 min, transfer approximately 0.8 ml of supernatant to HPLC vials and a 10 µl aliquot was injected into the chromatographic system.

Bioanalytical method validation

A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and eight non-zero samples covering the total range 10–700 ng/ml, including the lower limit of quantitation (LLOQ). The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x^2$) least-squares linear regression [19-20] on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification. The within-batch precision and accuracy were determined by analyzing six sets of QC samples in a batch. The between batch precision and accuracy were determined by analyzing six sets of QC samples on three different batches. The QC samples were randomized daily, processed and analyzed in a position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations. Recovery of Telmisartan from the extraction procedure was determined by a comparison of the peak area of Telmisartan in spiked plasma samples (six each of low, medium and high QCs) with the peak area of Telmisartan in samples prepared by spiking extracted drug-free plasma samples with the same amounts of Telmisartan at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples (n = 6) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography. The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (-80°C), and to freeze/thaw stability studies. All the stability studies were conducted at two concentration levels (13.430 and 455.811 ng/ml as low and high values) with six determinations for each.

Table 1: Main working parameters of the tandem mass spectrometer

Parameters	Value
Spray voltage	4500
Sheath gas pressure	30
Auxiliary gas pressure	15
Capillary temperature	300
Tubelens offset	98 & 51(Analyte and IS)
Skimmer offset	0(Analyte) and -08 (IS)
Collision energy	45(Analyte) and 32 (IS)
Polarity	Positive
Mode of analysis	MRM
Ion transitions for	
Telmisartan, m/z	514.752±0.5/ 276.006±0.5
Ticlopidine, m/z	263.900±0.5/ 124.819±0.5

Table 2: Precision and accuracy data of back-calculated concentrations of calibration samples for Telmisartin in human plasma (SD: standard deviation)

Concentration Added (ng/ml)	Concentration found (mean±SD, n=6) (ng/ml)	Precision (%)	Accuracy (%)
10.827	10.8143 ± 0.42234	3.9	99.8
29.263	29.1427± 3.30960	11.4	99.6
69.674	70.8027 ± 0.97532	1.4	101.6
145.155	147.380 ± 2.67901	1.8	101.5
290.309	293.487 ± 6.890	2.3	101.1
414.728	426.758 ± 18.404	4.3	102.9
552.970	542.648 ± 9.565	1.8	98.1
691.213	661.546 ± 40.438	6.1	95.7

RESULTS AND DISCUSSION

Mass spectrometry

The analysis of Telmisartan from human plasma is of major interest in pharmaceutical research. Pharmacokinetic applications require highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by LC/MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique. The fill scan mass spectra's of Telmisartan and the IS are shown in Fig. 2. $[M-H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The collisionally induced dissociation (CID) mass spectrum of Telmisartan shows the formation of characteristic product ions at m/z 276.006. The CID mass

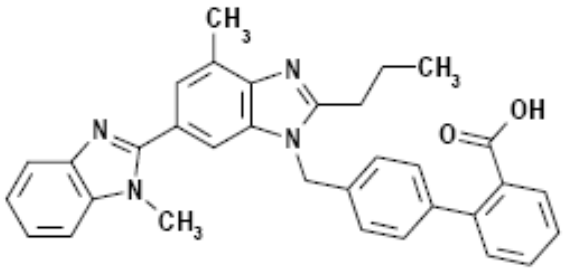
spectrum of the IS shows the formation of characteristic product ions at m/z 124.819. The most sensitive mass transition was from m/z 514.752 to 276.006 for Telmisartan and m/z 263.900 to 124.819 for the IS. LC/MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte. The parameters presented in Table 1 are the result of this optimization.

Table 3: Precision and accuracy of the method for determining Telmisartin concentrations in plasma samples (SD: standard deviation)

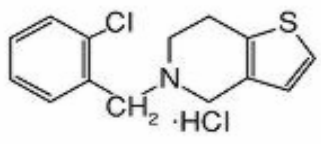
Between- batch precision (n=6)				With in batch precision (n=6)		
Concentration added (ng/mL)	Concentration found (mean±SD) (ng/ml)	Precision (%)	Accuracy (%)	Concentration found (mean±SD) (ng/ml)	Precision (%)	Accuracy (%)
456.200	480.8809 7 ± 4.236905	0.9	105.4	477.534 ± 27.65129	5.5	104.7
278.282	291.7491 0 ± 2.973445	1.0	104.8	295.0435 ± 12.92095	4.8	106.0
32.002	33.60227 ± 1.300334	3.9	105.0	34.4938 ± 2.31343	6.7	107.8
13.441	15.06417 ± 0.863554	5.7	112.1	15.2477 ± 0.72715	4.8	113.4

Table 4: Stability of Telmisartin in human plasma

Sample Concentration (ng/ml) (n=6)	Concentration found (ng/ml)	Precision (%)	Accuracy (%)
Short –term stability for 15 h in plasma			
456.200	481.7943	5.6	99.2
32.002	32.3928	7.5	94.7
Freeze-thaw cycles			
456.200	505.216	4.6	104.3
32.002	2.31484	6.5	104.8
Autosampler stability for 48 h			
456.200	464.7928	1.6	95.7
32.002	33.8708	5.0	99.0
Long term stability for 24 days in plasma			
456.200	454.6214	1.8	99.7
32.002	32.9106	4.8	102.84

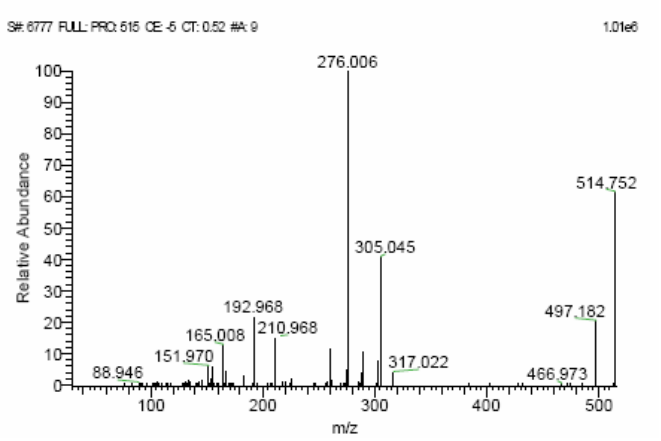


(a) Telmisartan

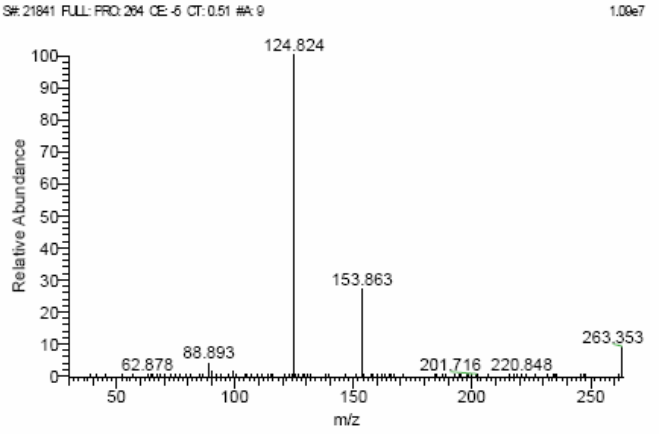


(b) Ticlopidine Hydrochloride

Fig. 1: Chemical Structures for Telmisartan and IS (Ticlopidine Hydrochloride)

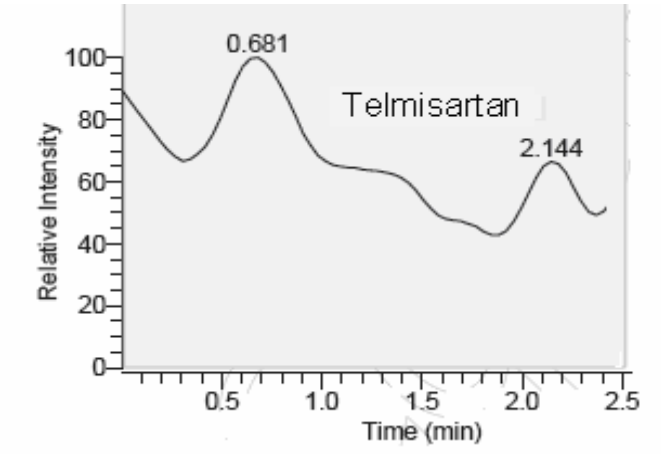


(a) Telmisartan full scan mass spectra

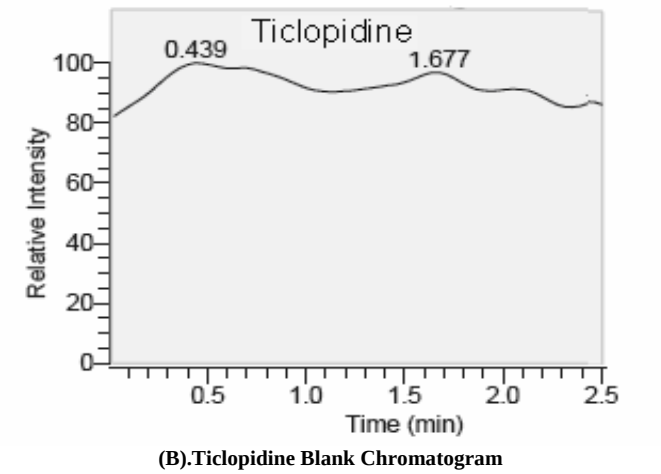


(b) Ticlopidine full scan mass spectra

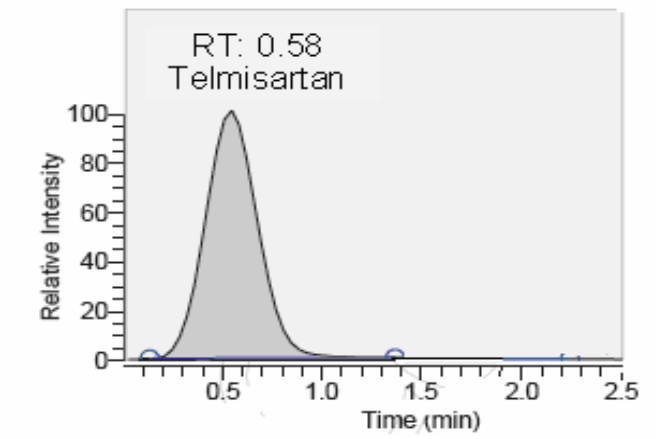
Fig. 2: Full- scan ion mass spectra's for Telmisartan and Ticlopidine



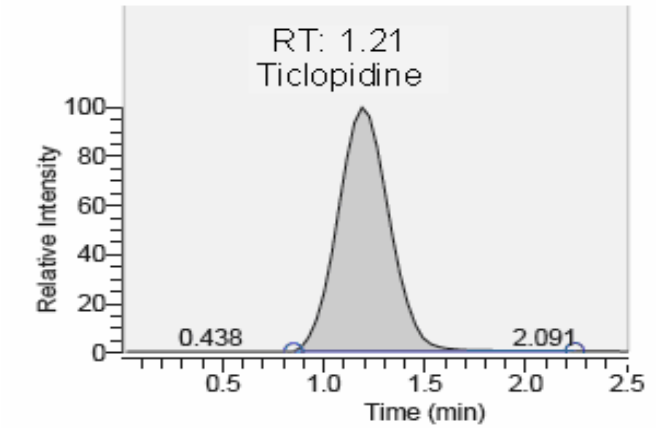
(A).Telmisartan Blank Chromatogram



(B).Ticlopidine Blank Chromatogram



(C).Telmisartan Chromatogram at LOQ Level



(D).Ticlopidine Chromatogram at LOQ Level

Fig. 3: MRM chromatograms for Blank [with out Drug (A) and IS (B)], Telmisartan(C) and the IS (D) at LOQ Level resulting from analysis

Method development

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of ACN: MeOH: 10 mM Ammonium acetate (45:45:10) could achieve this purpose and was finally adopted as the mobile phase. The proportion of organic solvent eluted the analyte and the IS at retention times of 0.58 and 1.21 min, respectively. A flow rate of 0.4 ml/min produced good peak shapes and permitted a run time of 2.5 min. Extraction with Acetonitrile was used for the sample preparation in this work. Extraction with Acetonitrile can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC/MS/MS analyses. An Extraction with Acetonitrile was found to be optimal, which can produce a clean chromatogram for a blank plasma sample. The average absolute recoveries of Telmisartan from spiked plasma samples was 98.5 ± 2.0 % and the recovery of the IS was 107.4 ± 1.8% at the concentration used in the assay (100 ng/ml). Recoveries of the analytes and IS were good, and it was consistent, precise and reproducible. Therefore, the assay has proved to be robust in high-throughput bioanalysis.

Choosing the appropriate internal standard is an important aspect to achieve acceptable method performance, especially with LC/MS/MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled internal standards for all analytes should be used, but these are not commercially available. Therefore, we opted for Ticlopidine Hydrochloride commercially available. In addition its retention behavior is similar to that of the target analyte. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. All validation experiments in this assay were performed with matrices obtained from different individuals. As all data all within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

Assay performance and validation

The eight-point calibration curve was linear over the concentration range 10–700 ng/ml. The calibration model was selected based on the analysis of the data by linear regression with intercepts and weighting factors (1/x, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighting factor. Linear regression equation for the calibration curve is y =m x + c here y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.9997±0.0004; Table 2 summarizes the calibration curve results.

The selectivity of the method was examined by analyzing six blank human plasma extract, no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Excellent sensitivity was observed for a 10 µl injection volume. The MRM chromatograms obtained for an extracted from blank plasma sample and extracted from plasma sample at LOQ level are depicted in Fig. 3.

The matrix effect of the method was performed by processing six blanks from each screened matrix obtained from six different sources and spiked the aqueous solution at low and high quality control level in triplicate for each of the processed blank samples with internal standard. Injected the extracted samples with six injections of unextracted low and high quality control samples. No significant matrix effect in screened plasma lots was observed.

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 10.827 ng/ml in human plasma. The mean response for the analyte peak at the assay sensitivity limit (10.827 ng/ml) was ten-fold greater than the Mean response for the peak in eight blank human plasma samples at the retention time of the analyte. The between-batch precision at the LOQ QC was 5.7%, and the between-batch accuracy was 112.1% (Table 3). The within batch precision was 8.5% and the accuracy was 105.1 for Telmisartan. The middle and upper quantification levels of Telmisartan ranged from 456.200 to 32.002 ng/ml in human plasma. For the between-batch experiments the precision ranged from 0.9 to 3.9 % and the accuracy from 100.3 to 105.1 % (Table 3). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria (≤15%). The upper concentration limits can be extended with acceptable precision and accuracy by a fourfold dilution with control human plasma. These results suggest that samples with concentrations greater than the

upper limit of the calibration curve can in this way be assayed to obtain acceptable data.

Stability studies

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above, and the results are given in Table 4. These results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in the auto sampler for 48 h was also assessed. The results indicate that solutions of Telmisartan and the IS can remain in the auto sampler for at least 48 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time (Table 4). The data representing the stability of Telmisartan in plasma at two QC levels over three freeze/thaw cycles are given in Table 4. These tests indicate that the analyte is stable in human plasma for three freeze/thaw cycles, when stored at below -80°C and thawed to room temperature. Table 4 also summarizes the long-term stability data for Telmisartan in plasma samples stored for a period of 24 days at below -80°C. The stability study of Telmisartan in human plasma showed reliable stability behavior, as the means of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of Telmisartan in plasma samples at below -80°C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 6-8 h, 72 h, and under refrigeration (-4°C) for 21 days (data not shown). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

In summary, a method is described for the quantification of Telmisartan in human plasma by LC/MS/MS in positive ESI mode using multiple reaction monitoring and fully validated according to commonly accepted criteria. [16-20] The current method has shown acceptable precision and adequate sensitivity for the quantification of Telmisartan in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. The desired sensitivity of Telmisartan was achieved with an LOQ of 10.827 ng/ml, which has within- and between-batch coefficients of variance (CVs) of 8.5% and 5.7%, respectively. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results. The simplicity of the assay and use of rapid Acetonitrile extraction and sample turnover rate of 2.5 min per sample make it an attractive procedure in high-throughput bioanalysis of Telmisartan. The validated method allows quantification of Telmisartan in the 10.827–700.213 ng/ml range.

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