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Short communication

Rapid determination of telmisartan in human plasma by HPLC using a monolithic column with fluorescence detection and its application to a bioequivalence study

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ABSTRACT

A rapid HPLC method using a monolithic column with fluorescence detection has been developed for determination of telmisartan in human plasma. Sample preparation was done by protein precipitation with acetonitrile and naproxen was used as IS. The compounds were detected by fluorescence detection, using an excitation wavelength of 300 nm and emission wavelength of 385 nm. Calibration curves of telmisartan were linear in the range of 1–200 ng/mL. The assay was high throughput, sensitive and precise, and it was successfully applied to a bioequivalence study of two formulations of telmisartan.

1. Introduction

Telmisartan,4-[(2-*n*-propyl-4-methyl-6-(1-methylbenzimidazole-2-yl)-benzimidazole-1-yl)methyl]-biphenyl-2-carboxylic acid, is a selective angiotensin II type 1 receptor (AT_1R) blocker, which belongs to the group of angiotensin II receptor antagonists [1]. It inhibits the angiotensin II receptor in a way that the effect of angiotensin II is blocked resulting in a decrease of blood pressure [2]. There are different mechanisms: increasing the activity of the sympathetic nervous system, causing a boosted sodium revertive resorption in the kidneys and promotion of the secretion of aldosterone in the adrenal glands [3–6]. The most recent clinical trials [7] demonstrated that telmisartan also has preventive roles against ischemic heart diseases in diabetic patients with a similar potency to angiotensin convertin genzyme inhibitor. Several studies recently suggest that the effects of telmisartan are mediated via not only blockade of AT_1R but also activation of peroxisome proliferators-activated receptor (PPAR)-γ [8,9].

A variety of methods have been developed for determination of telmisartan in biological samples including immunoassay [10], linear sweep polarography [11], HPLC with fluorimetric detection [12–17] and HPLC coupled with mass spectrometric detection (HPLC–MS/MS) [18–21]. In general, immunoassays lack specificity and cannot distinguish multiple analytes or active metabolites or degradation products from the parent compound. Although HPLC–MS/MS method can provide excellent sensitivity, it is not available for some laboratories because of its specialty requirement and financial reasons. Stangier et al. [12] reported a HPLC-fluorimetric method for determination of telmisartan in biological samples using a column switching system. The method had a good sensitivity with a lower limit of quantitation (LLOQ) of 1 ng/mL, but it required column switching devices. Torrealday et al. [13] established another HPLC-fluorimetric method to quantitate telmisartan in urine. In their study expensive solid phase extraction cartridges were used for sample pretreatment and the analytical run time was 5 min. Shen et al. [16] also developed a HPLC method with fluorimetric detection to determine telmisartan in human plasma. In their study liquid–liquid extraction (LLE) was used for sample pretreatment, thus it was time-consuming and required a large volume of organic solvent. In addition, the total run time was 7 min, so it was not quite suitable for high throughput analysis. Still other two HPLC-fluorimetric methods were reported by Tian et al. [14] and Zhou et al. [15]. Both methods needed a run time more than 10 min. Nie et al. [17] established a HPLC method using a novel sample pretreatment to determine several angiotensin II receptor antagonists in human plasma and urine. In this study a poly monolithic capillary was used for the in-tube solid-phase microextraction of analytes. All the reported methods have various limitations, including

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time-consuming sample clean-up, laborious extraction steps, and long chromatographic elution time for analytes.

This paper describes the development and validation of a rapid and simple HPLC method for the determination of telmisartan in human plasma using a monolithic column with fluorescence detection. The method gives a good sensitivity comparable with that obtained by HPLC–MS/MS but is free of matrix effect. A simple protein precipitation with acetonitrile ensured a high absolute recovery (>84%) and good purification from matrix interference. After full validation, the method was applied to a bioequivalence study of 40 mg telmisartan tablets (2 pills) versus 80 mg telmisartan tablets in 20 healthy Chinese volunteers.

2. Experimental

2.1. Reagents and chemicals

Telmisartan and naproxen (IS) were obtained from the National Institute for the Control of Pharmaceutical and Biological products (Beijing, PR China). The purities of telmisartan and naproxen were >99.5%. Telmisartan tablets 40 mg were from Green Valley Holding Co., Ltd (Shanghai, PR China) and telmisartan tablets 80 mg (Micardis®) were from Boehringer Ingelheim Company (Germany). Acetonitrile and methanol were HPLC grade and were purchased from Baker Company (USA). Other reagents were of analytical grade, and deionized (18.2 MΩ/cm) water was generated in-house using a Milli-Q System from Millipore (Bedford, MA, USA).

2.2. Equipment

The HPLC system consisted of two Shimadzu LC-10ATvp pumps, a SCL-10Avp system controller and a SIL-10ADvp autosampler, a CTO-10ASvp column oven, an RF-10A_{XL} fluorescence detector and a Degasser (MetaChem technology). The data was collected and processed using Shimadzu CLASS-VP software.

2.3. Chromatographic conditions

Chromatographic separations were performed using a monolithic analytical column, Chromolith® (RP-18e 100mm × 4.6 mm, Merck, Germany). The oven temperature was set at 25 °C. The mobile phase consisted of acetonitrile–methanol–water–acetic acid (30:20:50:0.05, v/v) and was eluted at a flow rate of 3 mL/min. Fluorescence measurements were done at 300 nm excitation and 385 nm emission wavelengths. The chromatographic elution time for each analysis was only 2 min.

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

The stock solution of telmisartan of 1 mg/mL was prepared in methanol. Working standards of telmisartan were prepared from stock solution at 0.01–2 μg/mL in H_2O:methanol (25:75, v/v). A stock solution of 1 mg/mL for naproxen was prepared in acetonitrile and then was further diluted with acetonitrile to yield a working solution of 200 ng/mL. Calibration standards (1, 2, 5, 10, 20, 50, 100, 200 ng/mL, respectively) were prepared daily by spiking 10 μL of each working standard into 90 μL of human blank plasma. QC stock solutions (100 μg/mL) of telmisartan were prepared in methanol. QC samples of 2 (LQC, within three times of the LLOQ), 20 (MQC) and 160 ng/mL (HQC) were prepared by spiking 10 μL of diluted QC stock solutions into 90 μL of human plasma and were stored at −20 °C until analysis. All the spiked samples contained about 8% of methanol, but this had no influence on the whole procedure because the amount of methanol was quite small and the samples were later processed by protein precipitation. A total of 52 samples were analyzed together with one calibration set and one QC set.

2.5. Sample preparation

An aliquot (50 μL) of plasma was combined with 100 μL of IS working solution for protein precipitation. The mixture was vortexed for 1 min and centrifuged at 12,000 rpm for 10 min. Then 20 μL of the supernatant was injected into the HPLC system.

2.6. Study design

The bioequivalence of two tablets formulations of telmisartan 40 mg tablet (test formulation) of Green Valley Holding Co., Ltd versus 80 mg telmisartan tablet (Micardis®, standard reference formulation) was conducted in twenty Chinese healthy male subjects after they had been informed on the purpose, protocol and risk involved in the study [22]. All subjects gave written consent and local ethics committee approved the protocol. Volunteers enrolled in the study were 21–24 years old. The study was conducted in accordance with the current good clinical practices (GCP), International Conference on Harmonization (ICH) and FDA guidelines [22]. Heparinized venous blood samples, 0.3 mL, were collected before administration (0 h) and at 0.25, 0.5, 0.75, 1, 1.5, 2.0, 4.0, 8.0, 12.0, 24.0, 36.0 and 48.0 h, respectively, after dosing. Plasma was separated by centrifugation at 3000 rpm for 10 min and stored at −20 °C until analysis.

3. Results and discussion

3.1. Method development

3.1.1. Selection of internal standard

To select an appropriate internal standard, we have tried irbesartan, resveratrol, naphthalene and so on, but all failed due to the low response and poor stability (naphthalene) or even being interfered by endogenous matrix. Then naproxen was selected as the internal standard referring to Shen et al. [16], because of being a fluorescent compound, an appropriate retention time, and a good resolution from telmisartan under the chromatographic conditions in this study. There were no endogenous interferences in the regions where telmisartan and IS eluted. In addition, the stability of naproxen was acceptable all over the study.

3.1.2. HPLC conditions

The chromatographic conditions were optimized through several trials to achieve good resolution and symmetric peak shapes for both analyte and IS, as well as a short run time. It was found that a mixture of acetonitrile–methanol–water–acetic acid (30:20:50:0.05, v/v) was appropriate. Owing to the use of the monolithic column, much faster separations are possible as compared with traditional chromatographic columns packed with porous particles. Accordingly, the chromatographic elution step was undertaken within 2 min while the run time was about 6 min to obtain acceptable resolution using a traditional C18 column (100mm × 4.6 mm, 5 μm, Dikma Technologies, Beijing, China). Fluorescence detection was used to get high sensitivity. The excitation and emission wavelengths of 300 and 385 nm, respectively were set according to the fluorescence spectra of telmisartan, and fortunately, the IS also has strong response under the above conditions (Fig. 1).

3.2. Method validation

The method was validated according to the guidelines of the main regulatory agencies [23–25]. The validation experiments and results obtained are described below.

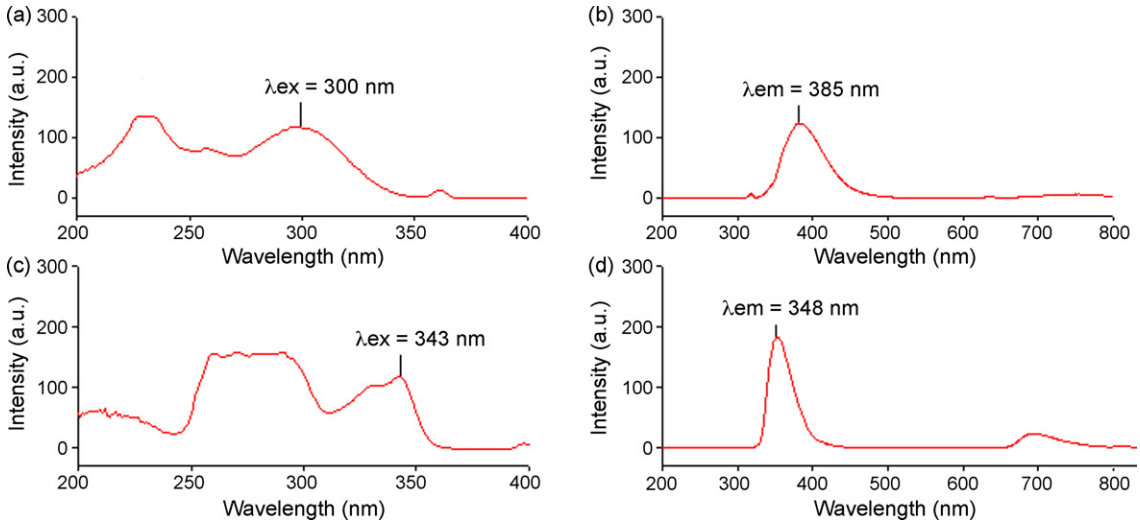

Fig. 1. The fluorescence spectra of telmisartan and naproxen (IS) at the concentrations of 2 and 25 ng/mL, respectively in the mobile phase. (a) Excitation spectrum of telmisartan; (b) emission spectrum of telmisartan; (c) excitation spectrum of naproxen; (d) emission spectrum of naproxen.

3.2.1. Selectivity

The resulting chromatograms were essentially free from endogenous interferences of all six lots of plasma. The peak shape and the resolution between telmisartan and IS were satisfactory and suitable. Typical chromatograms are shown in Fig. 2.

3.2.2. Linearity

The linearity of assay for the test compounds was evaluated with a total of five calibration standards. Calibration curves consisted eight concentrations of telmisartan spiked in human plasma: 1, 2, 5, 10, 20, 50, 100 and 200 ng/mL, respectively. The concentrations of the calibration standards and the study samples were back-calculated using linear regression with $1/x^2$ weighing. The linear regression model was selected based on the goodness of the fit to the data when compared with other regression models. The mean regression equation of five standard curves was $y = (0.09054 \pm 0.0017)x + (0.1473 \pm 0.05473)$, where y presented the peak area ratio of telmisartan to the IS and x was the plasma concentration of telmisartan. The precisions (% CV) of the slope and intercept were 1.88% and 3.71%, respectively. The calibra-

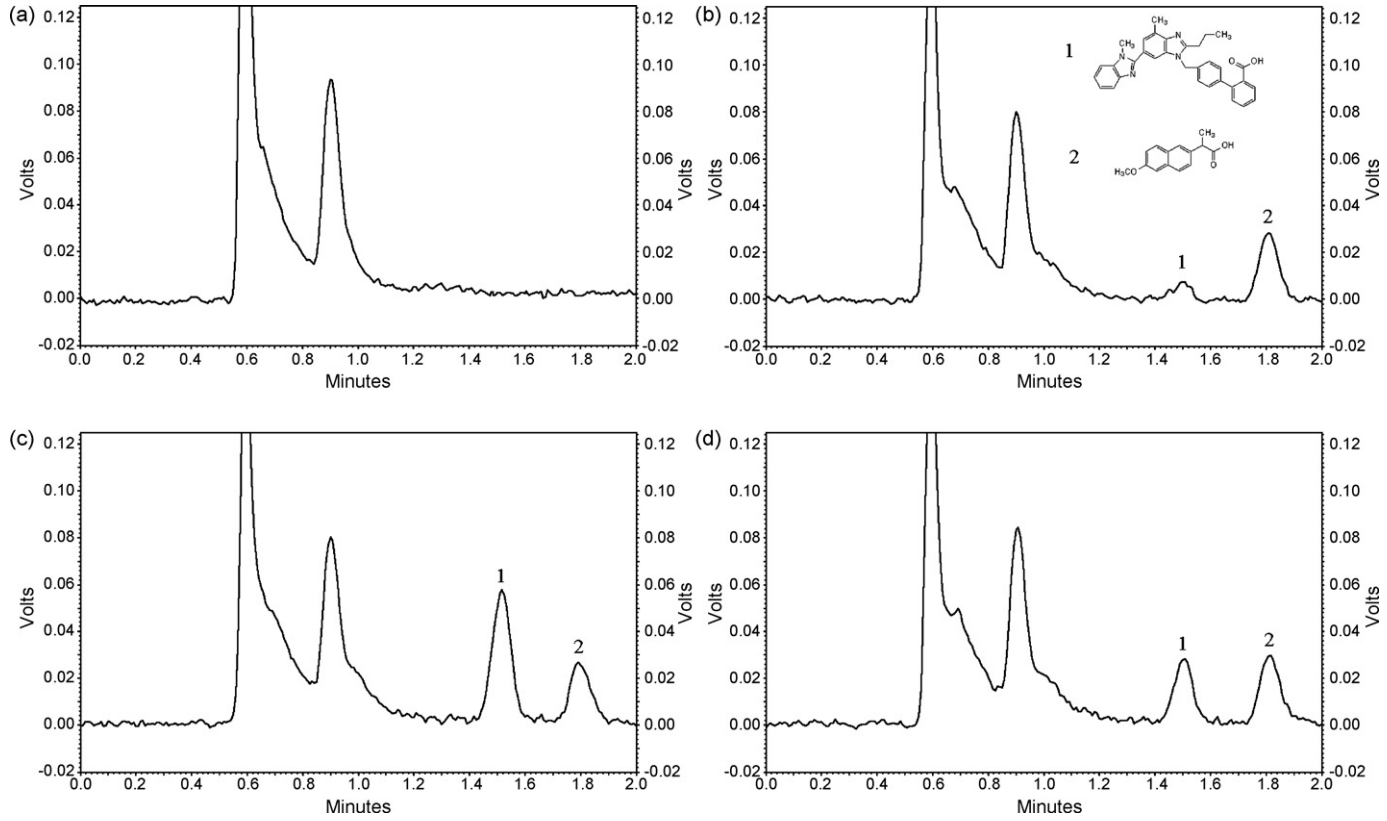

Fig. 2. Chemical structures of (1) telmisartan and (2) naproxen (IS), and representative chromatograms: (a) double blank plasma; (b) blank plasma spiked with 1 ng/mL telmisartan and 200 ng/mL of IS; (c) blank plasma spiked with 20 ng/mL telmisartan and 200 ng/mL of IS; (d) plasma sample collected from a subject 24 h after receiving a 80 mg oral administration of telmisartan. The assayed concentration of telmisartan in this sample was 10.2 ng/mL. (1) telmisartan; (2) naproxen (IS).

Table 1
Intra- and inter-day accuracy and precision of telmisartan ($n = 5$).

QC sample	Nominal concentration (ng/ml)	Mean measured concentration (ng/ml)	Accuracy (%)	Precision (CV %)
Intraday				
LQC	2.00	2.13	106.6	4.4
MQC	20.0	20.4	101.9	2.0
HQC	160	174	108.5	3.3
Interday				
LQC	2.00	2.00	99.6	9.7
MQC	20.0	21.6	107.9	5.9
HQC	160	177	110.9	4.5

tion curve was linear over the concentration range of 1–200 ng/mL with a mean correlation coefficient of 0.9996. The linear range was smaller than those obtained using LC–MS/MS methods [18–20]. However, it was the same as that in Stangier's study which was also done with HPLC-fluorimetric method [12]. The indication may be ascribed to the high flow rate of the method which greatly affected the linearity in fluorescence detection when the concentration above 200 ng/ml existed.

3.2.3. Sensitivity

The LLOQ of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within 20% of nominal and precision not exceeding 20% CV, was 1 ng/mL for telmisartan. The reproducibility of LLOQ was determined by examining five LLOQ samples independent from the standard curve, and the accuracy and precision was 101.5% and 8.29%, respectively. The precision of the ratios of the raw peak areas of telmisartan to those of IS was 3.19%. A typical chromatogram of an LLOQ sample is shown in Fig. 2b. The quantitative data suggested that the LLOQ of 1 ng/mL met the criteria in spite of the fact that the S/N ratio was not so high.

3.2.4. Precision and accuracy

The intra-day accuracy and precision of the assay were determined by analyzing replicates ($n = 5$) containing telmisartan at three different concentration levels. The inter-day accuracy and precision were determined by analyzing three concentrations of QC samples, five times at each concentration. Table 1 presents the intra- and inter-day accuracy and precision for each of the QC samples. The accuracy at high concentrations seems to be a little poor, but it still met the criteria, and it was in the linear range. Perhaps this could explain why the linear range was small.

3.2.5. Recovery

To investigate extraction recovery, a set of samples ($n = 5$ at each concentration in unique lots of plasma) was prepared by spiking telmisartan into plasma at 2, 20, and 160 ng/mL, respectively. Each of the samples (50 μL) was vortex-mixed with 100 μL of IS (200 ng/mL), then processed using the procedure described previously. A second set of plasma samples was processed and spiked post-extraction with the same concentrations of telmisartan and IS that actually existed in the pre-extraction spiked samples. Extraction recovery for each analyte was determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples to those of the samples spiked after extraction. Mean extraction recoveries of telmisartan at concentrations 2, 20, 160 ng/mL were 84.50%, 89.35% and 86.42%, respectively, and the extraction recovery of the IS was 78.70%.

3.2.6. Stability

The stability of telmisartan was studied under various conditions. The mean values and standard deviations of the ratios between the concentrations found and initial concentrations were used for stability evaluation. Telmisartan had an acceptable stability in human plasma at room temperature for 8 h, at −20 °C for 1 month, in the autosampler at room temperature for 24 h after protein precipitation and after three freeze-thaw cycles with the accuracy of 96.2–97.1%, 93.5–96.8%, 98.0–101.2% and 99.0–99.5%, respectively, at the three concentrations studied.

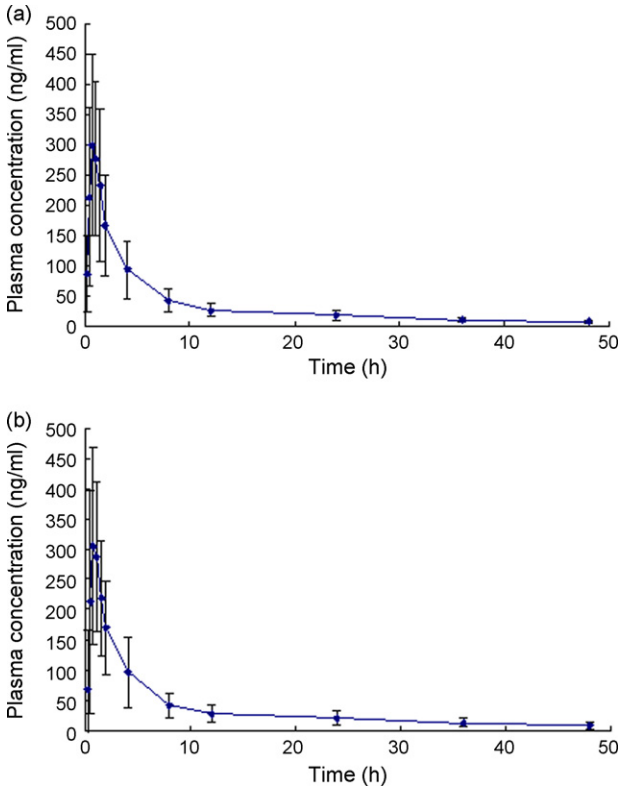

Fig. 3. Mean plasma concentration vs. time graph of telmisartan after administration of test and reference formulations to healthy, adult, male human subjects. (a) Test formulation; (b) reference formulation.

3.2.7. Sample dilution

To investigate the ability to dilute and analyze samples containing telmisartan at concentrations above the assay upper limit of quantitation, a set of plasma samples were prepared containing telmisartan at a concentration of 800 ng/mL, and placed in a −20 °C freezer overnight prior to analysis. After thawing, a 20 μL aliquot was withdrawn for analysis ($n = 5$), diluted with 80 μL of control human plasma, vortex for 30 s, then treated as described in Section 2.5. The accuracy of the test was 98.3% with a good precision (CV = 2.8%).

3.3. Application of the assay

This method was applied to a bioequivalence study of two telmisartan formulations. A representative chromatogram from

Table 2
Mean pharmacokinetic parameters and 90.0% confidence interval for telmisartan, after the administration of an oral dose of 80 mg of test and reference formulations to healthy volunteers.

Pharmacokinetic parameters	Reference formulation (mean ± SD)	Test formulation (mean ± SD)	Confidence limit 90.0%
[a]C_{max} (ng/ml)	375.3 ± 154	377.7 ± 142.6	92.8–114.4
[b]T_{max} (h)	0.9 ± 0.31	0.91 ± 0.37	–
[c]$t_{1/2}$ (h)	20.1 ± 5.23	19.4 ± 3.55	–
[d]MRT (h)	19.4 ± 6.11	18.2 ± 3.17	–
[e]AUC_{0-t} (h/ng/ml)	1693.9 ± 701.4	1623.2 ± 570.2	93.6–102.6
[f]$AUC_{0-\infty}$ (h/ng/ml)	1942.8 ± 874.1	1831 ± 625	93.8–103.0

[a] The maximum plasma concentration.
[b] The time to reach C_{max}.
[c] The elimination half-life.
[d] Mean residence time.
[e] Area under the plasma concentration-time curve from time zero to the last sampling time.
[f] Area under the plasma concentration-time curve from time zero to infinity.

a post-dose sample is provided in Fig. 2d. The mean plasma concentration–time profiles of telmisartan after a single oral dose of 80 mg of either formulation are shown in Fig. 3. To determine the pharmacokinetic parameters of the two formulations, the concentration–time data were analyzed by non-compartmental methods using the Bioavailability Program Package (BAPP, Version 2.0, Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University). The pharmacokinetic parameters of the two telmisartan formulations are shown in Table 2, and the relative bioavailability of the test formulation was 98.5 ± 11.5%. T_{max} and $t_{1/2}$ in this study were similar to the reported data [14,15,19–21]. C_{max} and AUC were comparable with the data obtained by Li et al. [19] and Chen et al. [20], but were both lower than the data provided by Tian et al. [14], Zhou et al. [15] and Zhang et al. [21] in spite of the fact that the six studies were all carried out on Chinese population. The RE% of pharmacokinetics parameters in different papers were large as well as in ours. Those phenomenons can only be explained through the individual difference of telmisartan pharmacokinetics. The means and standard deviations of the parameters for the two formulations were similar, indicating that the pharmacokinetics of the two telmisartan formulations are similar. The 90% confidence intervals for the ratios of test drug to reference drug in terms of AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} were within the range 80.0–125.0%, which is the range accepted by FDA [22].

4. Conclusion

In this paper, a rapid, high throughput, sensitive and accurate HPLC method using a monolithic column with fluorescence detection was investigated for the determination of telmisartan in human plasma. The method was capable of estimating accurately telmisartan down to 1 ng/mL in human plasma with an analytical time of only 2 min. The disadvantage of the method was that the linearity domain was not high enough, so some samples needed dilution and reanalysis. After full validation it was successfully applied to a bioequivalence study.

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