

Validated LC-MS/MS Method for the Determination of Rosuvastatin in Human Plasma: Application to a Bioequivalence Study in Chinese Volunteers

Dujuan Zhang^{1,2}, Jing Zhang², Xiaoyan Liu², Chunmin Wei², Rui Zhang², Haojing Song², Han Yao², Guiyan Yuan², Benjie Wang², Ruichen Guo^{2*}

¹School of Pharmacy, Shandong University, Jinan, China, ²Institute of Clinical Pharmacology, Qilu Hospital of Shandong University, Jinan, China.
Email: *grc7636@126.com

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ABSTRACT

A sensitive and selective liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-MS/MS) method was developed and validated for the determination of rosuvastatin in human plasma using gliclazide as an internal standard (IS). Rosuvastatin and gliclazide in plasma were extracted with ethyl acetate, separated on a C18 reversed phase column, eluted with mobile phase of acetonitrile-methanoic acid (0.1%) (60:40, v/v), ionized by positive ion pneumatically assisted electrospray and detected in the multi-reaction monitoring mode using precursor \rightarrow product ions of m/z 482.1 \rightarrow 258.1 for rosuvastatin and m/z 324.2 \rightarrow 127.2 for IS, respectively. The calibration curve was linear ($r^2 > 0.99$, $n = 5$) over the concentration range of 0.1 - 60 ng/mL. The specificity, matrix effect, recovery, sensitivity, linearity, accuracy, precision, and stabilities were validated for rosuvastatin in human plasma. In conclusion, the validation results showed that this method was sensitive, economical and less toxic and it can successfully fulfill the requirement of bioequivalence study of rosuvastatin calcium tablets in Chinese healthy volunteers.

Keywords: Rosuvastatin, Bioequivalence, LC-MS/MS, Pharmacokinetics

1. Introduction

Rosuvastatin (**Figure 1**) is a hydroxyl-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitor used in the treatment of patients with dyslipidemia [1]. Rosuvastatin has been reported to reduce serum levels of low-density lipoprotein cholesterol [2], accompanied by increases in high-density lipoprotein cholesterol [3] and reductions in triglycerides [4]. The population pharmacokinetic study revealed that plasma exposure to rosuvastatin was significantly higher in Asian subjects than in White subjects living in the same environment [5].

In recent years, several methods have been reported for the quantification of rosuvastatin in plasma. Kumar TR et al published a HPLC-UV method for the determination of rosuvastatin in rat plasma [6]. Some LC-MS/MS methods were developed [7-9], but they adopted either ethyl ether as extraction solvent [7,8], which was

toxic and difficult to quantitate, or SPE [9], which was relatively expensive, especially for the bioequivalence study where a large number of samples were to be collected and treated. Simultaneous quantitations of rosuvastatin and fenofibric acid [10] or other coadministered drugs [11,12] in biological samples were also proposed by some literatures, using HPLC with ultraviolet detection [11,12] or LC-MS/MS [10], but the lowest detection limit of these methods was 0.6 ng/mL, not sensitive enough to meet the requirement for rosuvastatin determination in plasma. It was therefore necessary to develop a more optimized LC-MS/MS method for the quantitation of rosuvastatin in plasma.

In this paper, a sensitive and selective LC-MS/MS method has been developed and used to evaluate the bioequivalence of two rosuvastatin calcium tablets in healthy Chinese volunteers.

2. Experimental

2.1. Chemicals and Instrumentation

Rosuvastatin (lot no. 091201, purity 98.6%) was obtained from Shandong Dyne Marine Organism Pharmaceutical Co., Ltd. Gliclazide standard (**Figure 1**, lot no. 100269-9701) was obtained from National Institute for the Control of Pharmaceutical and Biological Products. Methanol, acetonitrile and ethyl acetate were all of HPLC grade, from J. T. BAKER. Methanoic acid and hydrochloric acid were analytical grade, from Sinopharm Chemical Reagent Co., Ltd. Blank human plasma was provided by Shandong blood center (P. R. of China). The test formulation (lot no. 100102) was manufactured by Shandong Dyne Marine Organism Pharmaceutical Co., Ltd. The reference formulation (lot no. GF550) was donated by IPR Pharmaceuticals, Inc.

Determination of rosuvastatin in human plasma was performed with an Agilent 1200 series HPLC and an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA).

2.2. LC-MS/MS Conditions

The chromatographic separation was achieved on a Diamonsil C18 column (150 mm × 4.6 mm, 5 μm) at 30°C maintained with a thermostated column oven. The mobile phase consisting of acetonitrile-methanoic acid (0.1%) (60:40, v/v) was employed at a flow rate of 0.8 mL/min. The injection volume was 20 μL.

Mass spectrometric analysis was performed in the positive ion MRM mode by monitoring ion transitions at m/z 482.1 → 258.1 for rosuvastatin and m/z 324.2 → 127.2 for IS (**Figure 2**), with spray gas pressure of 350 Pa, protective air of nitrogen gas at a flow rate of 9 L/min, dwell time of 200 ms, capillary voltage of 4000 V, fragment electric voltage of 140 V for rosuvastatin and 100 V for IS, and collision energy of 40 eV for rosuvastatin and 20 eV for IS. All data were acquired employing Agilent 6410 Quantitative Analysis version analyst data processing software.

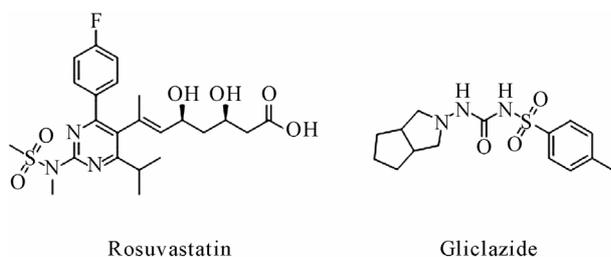


Figure 1. The chemical structure of rosuvastatin and gliclazide.

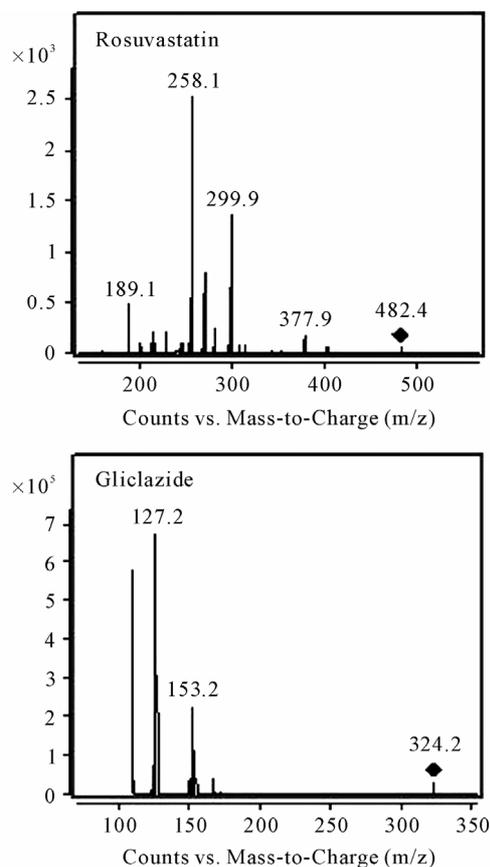


Figure 2. Product ion spectrum of rosuvastatin and gliclazide (IS).

2.3. Preparation of Standard Solutions, Calibration and Quality Control Samples

Rosuvastatin standard was accurately weighed and dissolved in methanol achieving concentration of 0.1 mg/mL stock solution, and further diluted with mobile phase obtaining 1, 10, 100, 1000 ng/mL working solutions, respectively. The stock solution of IS of 1 mg/mL was diluted with mobile phase to obtain the working solution of 10 ng/mL. All were stored in the refrigerator (4°C), and equilibrated to room temperature before use (approximately 15 minutes).

Rosuvastatin calibration of 0.1, 0.5, 2, 5, 10, 20 and 60 ng/mL and quality control (QC) of 0.2, 5 and 50 ng/mL in blank human plasma were prepared by spiking with rosuvastatin and IS working solutions and used for method validation, standard curve or quality control during run of analysis, and all were disposed with the established procedure.

2.4. Plasma Disposition

30 μL of the IS (10 ng/mL gliclazide in mobile phase)

was mixed with 0.5 mL plasma sample, then 100 μ L hydrochloric acid (0.1 mol/L in water) and 4 mL ethyl acetate were added, vortex-mixed for 2 min, and centrifuged at 5000 rpm for 5 min. The organic phase was transferred to a clean tube and evaporated to dryness under gentle stream of nitrogen gas at 40°C. The residue was reconstituted with 100 μ L mobile phase, and 20 μ L was injected onto the LC-MS/MS for analysis.

2.5. Method Validation

The method was validated for specificity, matrix effect, recovery, sensitivity, linearity, precision, accuracy, and stability according to the US Food and Drug Administration (FDA) guidelines for the validation of bioanalytical methods [13].

The specificity of the method was evaluated by comparing chromatograms of blank plasma, rosuvastatin and IS standard, blank plasma spiked with rosuvastatin and IS, plasma sample from a volunteer after administration of rosuvastatin calcium tablet.

Blank biological samples were extracted and then spiked with rosuvastatin at three concentration levels (0.2 ng/mL as low, 5 ng/mL as medium and 50 ng/mL as high) and IS in five replicates, respectively, to evaluate the matrix effects of plasma. The corresponding peak areas were then compared to those of standard solutions, and peak area ratio is defined as the matrix effect.

The extraction recoveries were evaluated by comparing peak areas of analytes in spiked biological samples with those of samples to which the analytes had been added after extraction. Five replicates of each QC level were disposed with the established extraction procedure.

The lower limit of quantization (LLOQ) was evaluated by analyzing five replicates of spiked plasma samples at the concentration of 0.1 ng/mL.

The calibration curve was prepared by analyzing spiked calibration samples at 7 different concentration levels on each day of analysis, typically described by equation $y = ax + b$, where y corresponds to the peak-area ratio and x to the concentration ratio of rosuvastatin to IS. The linearity of calibration curve was assessed by linear regression with a weighting factor of the reciprocal of the concentration squared ($1/x^2$).

Accuracy and precision were assessed by the determining of QC samples with five replicates for each concentration level on the same day or on three consecutive days. Precision was expressed by coefficient of variation (RSD) and accuracy by relative error (RE).

The stabilities of rosuvastatin in plasma samples at different concentrations were examined under different study conditions; *i.e.* storing at -20°C for 45 days. Freeze/thaw stability was determined after freezing (-20°C) and

thawing (25°C) QC samples for two cycles. Stability of post-extracted samples in the HPLC auto-sampler at room temperature for 7 hours was also observed.

2.6. Bioequivalence Study Design

The study was approved by the Ethics Committee of Qilu Hospital, Shandong University according to Declaration of Helsinki. Twenty healthy young male Chinese volunteers with age of (24.6 ± 1.4) years, weight of (64.9 ± 7.5) kg, height of (173.7 ± 4.9) cm and body mass index (BMI) of (21.5 ± 1.9) kg/m^2 were screened to participate and signed Informed Consent Form. 10 mg rosuvastatin tablet was orally administered with 200 mL of warm water after an overnight fast (10 hours). Standard meals or a given amount of water were provided 4 hours after administration in the study, and no coffee, smoke, chocolate or other food was allowed. All volunteers were under supervision of physicians for any possible adverse events.

Blood samples were collected before and 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0, 36.0, 48.0, 72.0 hours after administration. Blood was centrifuged immediately at 5000 rpm for 5 min, and plasma was transported into two EP tubes equally, clearly labeled and stored at -20°C pending analysis. All plasma samples of 20 volunteers were determined and the pharmacokinetic parameters including the maximum plasma concentration (C_{max}), time to maximum plasma concentration (T_{max}), half-life of drug elimination during the terminal phase ($t_{1/2}$), area under the plasma concentration-time curve from 0 to last measurable time (AUC_{0-t}) or from 0 to infinity ($\text{AUC}_{0-\infty}$) were estimated using non-compartmental model method. C_{max} and T_{max} were obtained directly from the measured data. $T_{1/2}$, AUC_{0-t} and $\text{AUC}_{0-\infty}$ were calculated using *Drug and Statistical Software-Version 2.0* (DAS 2.0, P. R. of China).

3. Results

3.1. Method Validation

3.1.1. Specificity

Typical MRM chromatograms of blank plasma, rosuvastatin and IS standard, blank plasma spiked with rosuvastatin and IS, plasma from a volunteer after administration of rosuvastatin calcium tablet spiked with IS were shown in **Figure 3**. Retention times of rosuvastatin and IS were 3.0 and 5.1 min, respectively, and overall run time was within 6 min. No significant interfering peak was observed around the rosuvastatin and IS during analysis.

3.1.2. Matrix Effect and Extraction Recovery

The mean matrix effect values of rosuvastatin at three concentrations of 0.2 ng/mL, 5 ng/mL and 50 ng/mL and

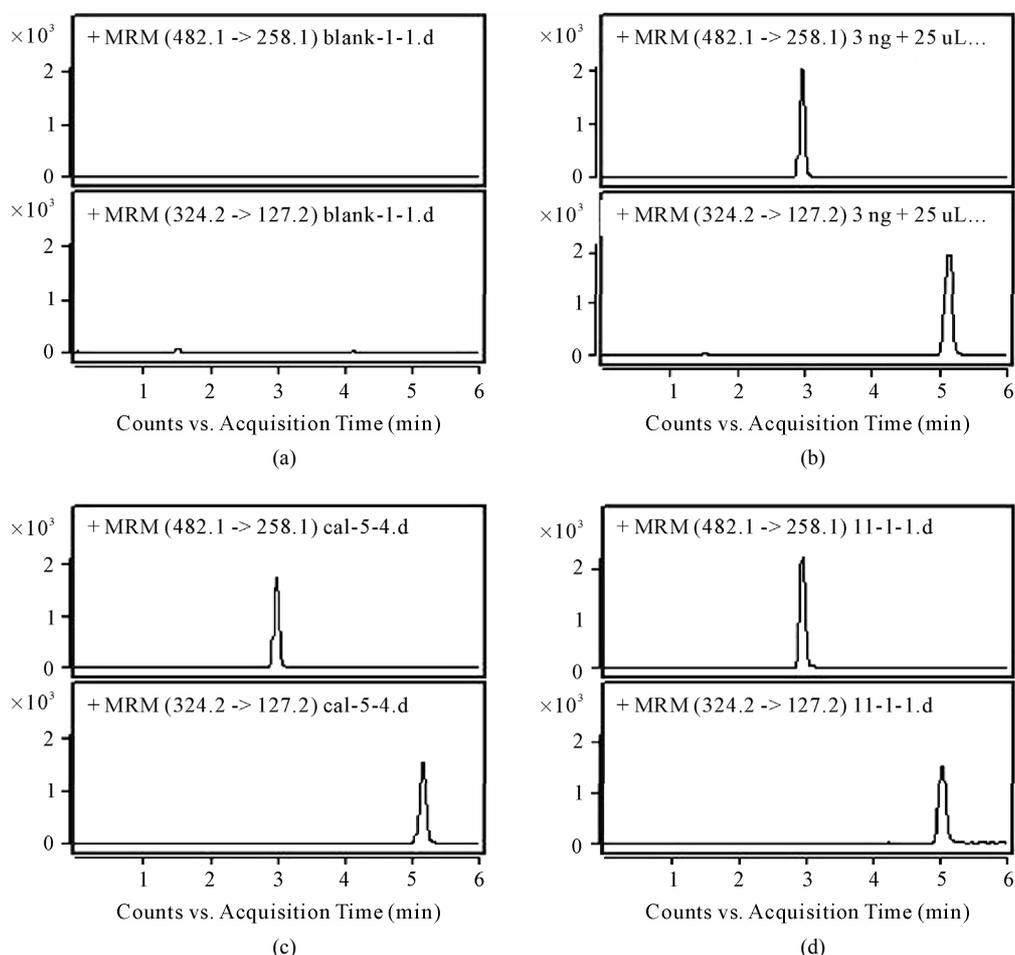


Figure 3. Typical chromatograms of rosuvastatin and IS in human plasma: blank plasma sample (a); rosuvastatin and IS standard (b); a blank plasma sample spiked with rosuvastatin (3 ng/mL) and IS (c); a plasma sample of a volunteer at 0.5 hours after administration of 10 mg rosuvastatin calcium tablet (d).

IS at 3 ng/mL were $(96.41 \pm 6.14)\%$, $(99.75 \pm 1.43)\%$, $(98.35 \pm 3.01)\%$, and $(88.93 \pm 3.39)\%$, respectively. It demonstrated no matrix significantly affected the determination of rosuvastatin in human plasma.

Mean extraction recoveries of rosuvastatin at three QC levels and IS were $(85.73 \pm 6.62)\%$, $(83.99 \pm 1.52)\%$, $(79.67 \pm 4.13)\%$, and $(75.98 \pm 1.34)\%$, respectively. The results were shown to be consistent, precise and reproducible.

3.1.3. Calibration Curve and LLOQ

The calibration curves showed a good linearity in the concentration range of 0.1 - 60 ng/mL with correlation coefficient ($r^2 > 0.99$) and the LLOQ for rosuvastatin was proved to be 0.1 ng/mL with RSD and RE were 4.5% and -5.6%, respectively.

3.1.4. Accuracy and Precision

The results of intraday and interday precision and accu-

racy were shown in **Table 1**. Both precision values (RSD) were less than 7.7%. Intraday and interday accuracy (RE) ranged from -10.1% to -4.3% and -9.5% to -2.8%, respectively.

3.1.5. Stability

The results of stability of rosuvastatin were shown in **Table 2**. The data demonstrated that rosuvastatin was stable under the indicated conditions for the bioequivalence study. The stock solutions of rosuvastatin and IS were comparable to the freshly prepared ones after storage at -20°C for days and two freeze-thaw cycles.

3.2. Application to Bioequivalence Study

The validated method was applied to the bioequivalence study of rosuvastatin in human plasma after a single oral administration of 10 mg rosuvastatin calcium tablet to 20 healthy male volunteers. The mean plasma concentration-time profile and main pharmacokinetic parameters

Table 1. Interday and intraday precision and accuracy for the determination of rosuvastatin in human plasma (n = 5).

Nominal Conc. (ng/mL)	Intraday			Interday		
	Mean ± SD	RSD (%)	RE (%)	Mean ± SD	RSD (%)	RE (%)
0.2	0.19 ± 0.01	7.7	-4.3	0.19 ± 0.01	6.3	-2.8
5	4.50 ± 0.16	3.6	-10.1	4.52 ± 0.18	4.1	-9.5
50	46.35 ± 0.99	2.1	-7.3	47.05 ± 3.34	7.1	-5.9

Table 2. Stability of rosuvastatin at various conditions (n = 5).

Condition	Nominal Conc. (ng/mL)	Mean ± SD	RSD (%)	RE (%)
Fresh samples	0.2	0.21 ± 0.01	7.0	3.4
	5	4.76 ± 0.29	6.2	-4.7
	50	48.84 ± 3.39	6.9	-2.3
-20°C, 45 days	0.2	0.19 ± 0.01	6.4	-3.6
	5	5.28 ± 0.27	5.2	5.6
	50	45.81 ± 3.28	7.2	-8.4
Two freeze-thaw cycles	0.2	0.19 ± 0.02	10.4	-5.8
	5	5.06 ± 0.17	3.4	1.2
	50	52.52 ± 3.88	7.4	5.0
Post-extracted samples, 25°C, 7 h	0.2	0.21 ± 0.01	4.6	5.1
	5	4.59 ± 0.19	4.2	-8.2
	50	48.26 ± 1.24	2.6	-3.5

were shown in **Figure 4** and **Table 3**, respectively.

Bioequivalence of test and reference was evaluated by calculating 90% confidence intervals (90% CI) for the test/reference ratio of logarithmic transformed C_{max} , AUC_{0-t} and $AUC_{0-\infty}$. The 90% confidence intervals (90% CI) for the test/reference ratio of logarithmic transformed C_{max} , AUC_{0-t} and $AUC_{0-\infty}$ were shown in **Table 4**, and all were within the bioequivalence acceptance range 80% - 125% adopted by US-FDA [14]. The two rosuvastatin calcium tablets are bioequivalent.

4. Discussion

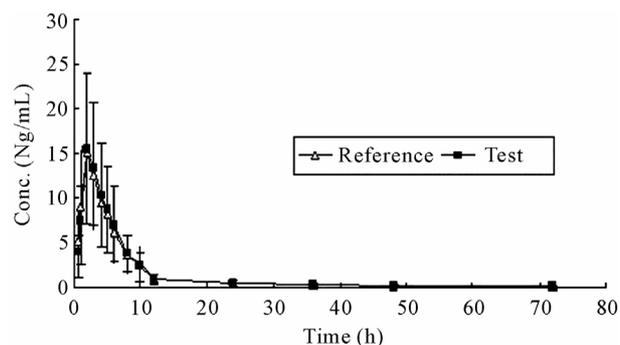
Mass spectrometric detection was carried out on an Agilent 6410 triple quadrupole instrument equipped with an ESI source operated in the positive ion mode. During optimization of the mass spectrometric parameters, strong and stable signals of rosuvastatin and IS can be observed in the form of their $[M + H]^+$ molecular ions with mass to charge ratios of m/z 482.1 and m/z 324.2, respectively. Each of the precursor ions was subjected to

Table 3. Main pharmacokinetic parameters of rosuvastatin after a single dose of 10 mg rosuvastatin calcium tablet test and reference (n = 20, mean ± SD).

Parameter	Test	Reference
$t_{1/2Z}$ (h)	18.30 ± 11.34	16.74 ± 12.70
T_{max} (h)	2.55 ± 0.51	2.50 ± 0.61
AUC_{0-t} (ng·h/mL)	95.15 ± 46.68	91.89 ± 44.34
$AUC_{0-\infty}$ (ng·h/mL)	99.10 ± 48.24	95.24 ± 45.65
C_{max} (ng/mL)	17.52 ± 8.04	16.53 ± 7.20

Table 4. The 90% Confidence Interval (90% CI) for the test/reference ratio of C_{max} , AUC_{0-t} and $AUC_{0-\infty}$.

Parameter	Mean Ratio (%)	90% CI (%)	P-Value
C_{max}	105.1	99.4 - 111.1	0.137
AUC_{0-t}	103.2	96.2 - 110.8	0.441
$AUC_{0-\infty}$	103.8	96.8 - 111.4	0.368

**Figure 4. Mean (±SD) plasma concentration-time curves of rosuvastatin calcium after a single oral administration of 10 mg rosuvastatin calcium tablet test and reference (n = 20).**

collision-induced dissociation to determine the resulting product ion. Different collision energy (CE) values (20, 30, 40 eV) for rosuvastatin were tested, and at higher CE (40 eV), a major fragment ion at m/z 258.1 was formed, giving a considerably better response and a higher signal. Fragmentation of IS produced the most abundant product ion at m/z 127.2. Therefore, the ion transitions m/z 482.1 → 258.1 and 324.2 → 127.2 were selected for MRM of the rosuvastatin and the IS, respectively.

An ideal IS used to guarantee high accuracy of LC-MS/MS assay should track the analyte during the extraction with almost the same recovery of the analyte. It was also eluted close to the analyte on the column and compensated for potential inconsistent response for matrix effects. Several compounds were investigated such as pravastatin sodium, fluvastatin sodium, lorazepam, losar-

tan potassium, and gliclazide. Significant interfering peaks were observed for pravastatin sodium, fluvastatin sodium and lorazepam during analysis. Different responds were detected for losartan potassium when spiked with purchased blank plasma and plasma samples from volunteers. Gliclazide, with suitable retention time, acceptable matrix effects, and extraction recoveries was selected as internal standard.

Protein precipitation (PPT), liquid-liquid extraction (LLE) and solid-phase extraction (SPE) were tried to obtain a simple and excellent plasma preparation procedure. PPT was easy to dilute the sample and failed to sufficiently remove endogenous interference. The SPE column for solid-phase extraction is relatively expensive for a great quantity of samples. LLE method with various extraction solvents, including chloroform, ethyl acetate, n-hexane, dichloromethane, n-hexane-dichloromethane-isopropanol (20:10:1, v/v/v) was investigated and evaluated for acceptable extraction recoveries and matrix effect, and ethyl acetate with no-concentration-dependent extraction recovery and acceptable matrix effect was adopted.

In conclusion, the developed method was simple, specific, sensitive, and successfully applied to bioequivalence study of rosuvastatin calcium tablets. The two rosuvastatin calcium tablets were bioequivalent.

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