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Short communication

Development and validation of a rapid and sensitive liquid chromatography–tandem mass spectrometry method for benvitimod quantification in human plasma

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ABSTRACT

Benvitimod is a newly synthesized non-steroid small molecule being developed as a candidate drug for the treatment of inflammatory skin diseases. Here a rapid, sensitive and specific high performance liquid chromatography–tandem mass spectrometry (LC/ESI/MS/MS) method was developed for the determination of benvitimod in human plasma. The samples were alkalified with disodium tetraborate firstly, and then extracted by methyl tert-butyl ether. Fluorophenyl-benvitimod was used as internal standard (I.S.). Chromatographic separation was performed on an Ultra C_{18} column (150 mm × 2.1 mm, 5.0 μm). The mixed mobile phase delivered at 300 μl/min was CH_3CN/H_2O, 76.65:23.35 (v/v), containing 0.2 mmol/L NH_4COOH. Detection and quantitation was performed by electrospray ionization (ESI) and multiple reaction monitoring (MRM) in the negative ion mode. The most intense $[M-H]^-$ MRM transition of benvitimod at *m/z* 253.1→211.0 was used for benvitimod quantitation and the transition at *m/z* 270.9→229.2 was used to monitor I.S. The calibration curve was linear within the concentration range of 0.1–10.0 ng/mL ($r > 0.99$). The lower limit of quantification (LLOQ) was 0.1 ng/mL. The extraction recovery was above 80%. The accuracy expressed as relative error (RE) was less than 1.03%. The intra- and inter-day precisions were less than 11.81%. The freeze–thaw stability was also investigated and it was found that both benvitimod and the I.S. were quite stable. This method is especially useful for the pharmacokinetic study of benvitimod.

1. Introduction

Benvitimod (Fig. 1) is a newly synthesized non-steroid small molecule. It significantly inhibits the expression of pro-inflammatory cytokines such as interferon γ (IFN-γ), interleukin 2 (IL-2), and tumor necrosis factor α (TNF-α), inhibits the migration of peripheral blood monouclear cell (PBMC) towards leukotrine B4 (LTB4), inhibits the activities of T-cell including their infiltration processes, all these indicating that benvitimod may block the process in many autoimmune and inflammatory diseases [1–3]. Direct anti-inflammatory responses of benvitimod were also studied. When applied in the chemically induced ear inflammatory model, benvitimod exhibited a dose-dependent response by reducing both ear skin redness and thickness. When applied to experimental inflammatory bowel disease (IBD) models, benvitimod also shown significant efficacy [4,5].

Now benvitimod has been accepted for clinical trial in China and Canada. As a candidate drug for the topical, cream treatment of inflammatory skin diseases, it is essential to quantify the absorption and its passage into general circulation. Till now, there is not a method reported for the determination of benvitimod concentrations in biological samples. Recently, LC/ESI/MS/MS was used widely to measure concentrations of various drugs in biological fluids and tissues because of its high specificity and sensitivity. Therefore, in this work, we developed a LC/ESI/MS/MS method for the determination of benvitimod in human plasma samples. The method provides a highly sensitive and selective way to determine the level of benvitimod in human plasma with a lower limit of quantification of 0.1 ng/mL with only 0.2 mL of plasma. This method was validated and applied successfully to determine the plasma concentrations of benvitimod in patients with mild to moderate psoriasis treated with this drug.

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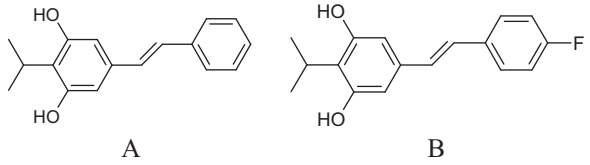

Fig. 1. The chemical structures of benvitimod and fluorophenyl-benvitimod (I.S.). A: 5-(2-phenylethenyl)-2-isopropyl-1,3-benzenediol; B: 5-[2-(4-fluorophenyl) vinyl]-2-isopropyl-1,3-benzenediol.

2. Experimental

2.1. Chemicals and reagents

Benvitimod (Batch No: 070614) and fluorophenyl-benvitimod (I.S.) were supplied by Celestial Pharmaceuticals Co., Ltd (Shenzhen, PR China). The primary stock solutions (1 mg/mL) of benvitimod and I.S. were prepared separately in methanol:water (1:1). Working solutions of benvitimod and I.S. were obtained by diluting the stock solutions with methanol:water (1:1) to respective concentrations. All the standard solutions were stored at 4 °C and the volumetric flask's bouche was wrapped by parafilm membrane to prevent methanol evaporation when not in use. HPLC grade methanol, acetonitrile, ammonium formate, formic acid, sodium tetraborate and methyl tert-butyl ether were purchased from Fisher Scientific Co. (Waltham, USA). Blank (drug free) human plasma samples were obtained from healthy subjects. Distilled water was purified by a Derect-Q® ultrapure water system (Millipore, Bedford, MA, USA). Mobile phase used in LC/ESI/MS/MS was filtered using a 0.45 μm membrane filter provided by Ameritech Co. (Irvine, CA, USA).

2.2. Calibration standards and quality control (QC) samples

Routine daily calibration curves were prepared in drug free plasma. Appropriate volumes of working solutions and drug free human plasma were added to each test tube. Final concentrations of benvitimod were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 ng/mL, and final concentration of I.S. was 10 ng/mL. Similarly, QC samples that were run in each assay at concentrations of 0.2, 1.0 and 5.0 ng/mL were also prepared.

2.3. LC/ESI/MS/MS conditions and quantifications

High performance liquid chromatography was performed using an Agilent 1200 HPLC system consisting of a G1312A binary pump and a G1379B degasser and a G1329A autosampler and a G1316A column oven (Palo Alto, USA).

The tested compound was separated on a Resteck Ultra C_{18} column (150 mm × 2.1 mm i.d., 5.0 μm, Bellefonte, USA) maintained at 20 °C, which was the same with the ambient temperature of the laboratory. The mixed mobile phase delivered at 300 μl/min was CH_3CN/H_2O, 76.65:23.35 (v/v), containing 0.2 mmol/L NH_4COOH. The retention times of benvitimod and I.S. in plasma samples were approximately 1.9 min. Detection was carried out on an API 5500 Qtrap MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray interface (ESI) and operated in the negative ionization mode. Multiple reaction monitoring (MRM) at unit resolution was employed to monitor the transitions of the molecular ions ([M−H]) of benvitimod at *m/z* 253.1→211.0 and of I.S. at *m/z* 270.9→229.2. Optimized MS parameters were: curtain gas, gas 1 and gas 2 (nitrogen) 33, 37 and 55 p.s.i., respectively; dwell time 200 ms; ionspray voltage −4500 V; ion source temperature 600 °C; declustering potential (DP) −100 V for benvitimod and −140 V for I.S.; collision energy (CE) −25 V for benvitimod and −27 V for I.S. Data acquisition and analysis were performed by using the analyst software version 1.5.1(Applied Biosystems).

2.4. Sample preparation

To a 200 μL aliquot of human plasma in a 7 mL Eppendorf tube, 20 μL I.S. working solution and 100 μL sodium tetraborate (0.1 mM) and 3 mL methyl tert-butyl ether were added. The mixture was vortex-mixed for 10 min and centrifuged at 4000 rpm for 5 min. The centrifuged upper layer was carefully transferred in another 7 mL clean Eppendorf tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The dry residue was then reconstituted with 200 μL mobile phase, and vortex-mixing for 3 min, finally 10 μL solution was injected into the LC/ESI/MS/MS system.

3. Results and discussion

3.1. Optimization of the method

The LC/ESI/MS/MS with negative ionization and the MRM mode provided a highly selective method for the determination of benvitimod and the I.S. The ESI negative ion mode was chosen for ion product since there are two hydroxy groups in the structure of benvitimod. The ion source temperature was set at 600 °C which was to enhance the sensitivity. As the $[M-H]^-$ MRM transition at *m/z* 253.1→211.0 for benvitimod and *m/z* 270.9→229.2 for I.S. were the most intense ones, thus were used as the quantifier (see Fig. 2). Various mobile phase combinations of ammonium formate (0.2 mmol/L) in water and methanol or acetonitrile were investigated to optimize sensitivity, speed and peak shape. The results demonstrated that acetonitrile gave a better response than methanol and ammonium formate (0.2 mmol/L) in water and

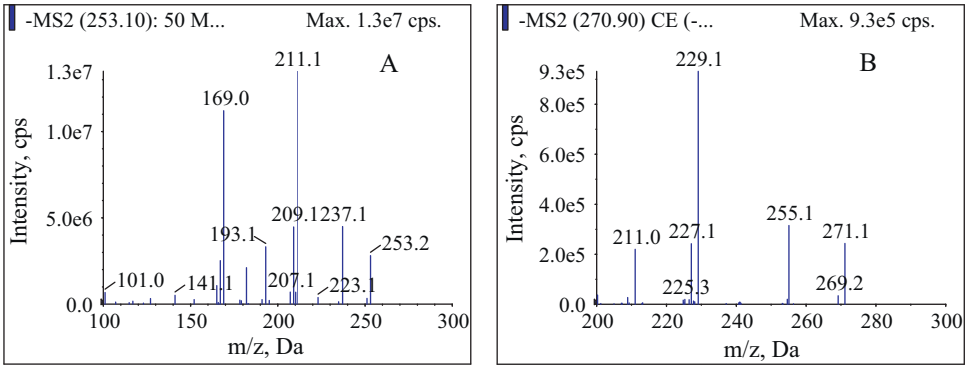

Fig. 2. MS2 of benvitimod and fluorophenyl-benvitimod (I.S.) A: 5-(2-phenylethenyl)-2-isopropyl-1,3-benzenediol; B: 5-[2-(4-fluorophenyl) vinyl]-2-isopropyl-1,3-benzenediol.

Table 1
Extraction recoveries, intra- and inter-day precision and accuracy of benvitimod in human plasma ($\bar{x} \pm$ SD, $n = 5$).

Concentration (spiked)	Concentration (found)	Accuracy RE (%)	Precision RSD (%)	
			Intra-day	Inter-day
0.20	0.20 ± 0.01	−1.03	3.71	8.52
1.00	1.00 ± 0.05	0.01	4.85	1.84
5.00	4.86 ± 0.32	−0.27	5.17	11.81

Table 2
Stability of benvitimod in human plasma ($\bar{x} \pm$ SD, $n = 3$).

Concentration spiked	Before-preparative (24 h at room temperature)		Post-preparative (24 h at room temperature)		Long-term (6 months at −80 °C)		Three freeze–thaw (−80 °C and room temperature)	
	Measured	RE%	Measured	RE%	Measured	RE%	Measured	RE%
0.20	0.19 ± 0.01	−2.67	0.20 ± 0.01	1.00	0.20 ± 0.01	2.62	0.21 ± 0.01	−7.00
1.00	1.04 ± 0.03	4.00	0.99 ± 0.03	−0.77	0.86 ± 0.01	1.23	0.98 ± 0.03	−1.70
5.00	5.02 ± 0.05	0.47	5.12 ± 0.03	2.47	5.24 ± 0.18	3.35	5.02 ± 0.14	0.47

acetonitrile improved the efficiency of ionization and peak shapes, at the same time, increased the sensitivity. In some published reports [6–9], Chuan Li et al. had discovered a so-called "LC-electrolyte effects" to increase the sensitivity by adding a low concentration of HCOOH or NH_4COOH to the mobile phase. In the current study, we also demonstrated that enhanced ESI signal intensity caused by electrolyte-modification of the LC mobile phase was associated with improvement in the lower limit of quantification, indicating that the LC-electrolyte effect solidly improved the analytical sensitivity.

3.2. Validation of the method

3.2.1. Specificity

Drug-free plasma was prepared and analyzed by the developed method. Fig. 3A shows the representative chromatograms of a drug-free plasma sample. No endogenous peaks are present at the retention times (t_R) of benvitimod or that of the I.S., demonstrating that the developed LC/ESI/MS/MS method is highly selective. Moreover, that no analyte-interfering peaks were observed in all the samples collected before drug administration also supports the high selectivity of this method.

3.2.2. Calibration curve and lower limit of quantitation (LLOQ)

Calibration curves for benvitimod were constructed in the concentration range of 0.1–10.0 ng/mL ($n = 8$, each point was the mean of two experimental measurements) and calculated by plotting peak area ratio (y) of benvitimod and I.S. versus benvitimod concentrations (x, ng/mL). Regression equations were obtained through weighted least-square linear regression analysis ($w = 1/x^2$). The typical equation was $y = 0.108x + 0.112$ ($r = 0.9992$). The LLOQ validated was 0.1 ng/mL (S/N > 5, shown in Fig. 3B). Determinations at LLOQ were found accurate (RE = 6.0%) and precise (RSD = 2.91%) for benvitimod. These features are important for clinical situations when blood volume is restricted and yet high assay sensitivity is required.

3.2.3. Extraction recovery, accuracy and precision

In this study, we utilized a liquid–liquid extraction method to prepare the blood sample. The extraction recoveries of analyte were determined at three different concentration levels by comparing the peak areas of analyte obtained from the quality control samples ($n = 5$) after extraction to those obtained from the reference samples which were prepared by adding the analyte to the post-extracted blank plasma samples at the same concentrations. The corresponding extraction recoveries at three quality control concentrations (0.2, 1.0 and 5.0 ng/mL) were 99.10 ± 9.72%, 93.73 ± 11.03%, 89.00 ± 18.47%, respectively.

The intra-assay precision was assessed by measuring the concentration of benvitimod in five aliquots of three different quality control samples extracted and analyzed on a single day. Inter-assay precision and accuracy was determined from the results of those different quality control samples which were extracted and analyzed fivefold on three consecutive days. All the results are summarized in Table 1. As can be seen, the accuracy expressed as relative error (RE) was less than 1.03%. The intra- and inter-day precisions were less than 5.17% and 11.81%, respectively.

3.2.4. Matrix effects

The matrix effect (ME, i.e. potential ion suppression or enhancement effects of co-eluting and undetected matrix components in plasma) was investigated. It was evaluated by comparing the peak area of benvitimod and I.S. spiked in post-extracted blank plasma samples to that of benvitimod spiked in mobile phase at equivalent concentration. If the ME < 85% or >115%, an endogenous matrix effect is implied. In this study, the matrix effect of the assay was evaluated at three quality control concentrations (0.2, 1.0 and 5.0 ng/mL) of benvitimod. The blank plasmas used in this study were from six different batches of healthy human blank plasma. The precision in peak area ratio among the different plasma was calculated as an indicator of the inter-lot matrix variability.

As a result, the ME of nominal quality control samples at concentrations of 0.2, 1.0 and 5.0 ng/mL were 97.95 ± 14.06%, 108.76 ± 12.25% and 111.32 ± 15.56%, respectively. The inter-lot variation of benvitimod in peak area ratio (RSD) was 14.35%,

Table 3
Dosage regimen of benvitimod to patients with mild to moderate psoriasis.

Cohort (number)	Concentration of the cream (%)	Dosing frequency	Dosage (mg/d)	Number of cases	
				Test group	Placebo group
1 (1–12)	0.5	b.i.d.	10	8	4
2 (13–24)	1.0	b.i.d.	20	8	4
3 (25–36)	1.5	b.i.d.	30	8	4

Table 4
Plasma concentration of benvitimod from 36 patients with mild to moderate psoriasis.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Day-1																		
0.0 h																		
0.25 h																		
0.5 h					0.155													
0.75 h				3.04														
1.0 h					1.18													
1.25 h																		
1.5 h															0.1			
2.0 h	0.193			0.228											0.134			
3.0 h														0.116	0.14			
4.0 h										0.493								
8.0 h											0.377							0.348
12.0 h																		
Day-7																		
0.0 h																		0.167
Day-14																		
0.0 h							0.112											0.115
Day-21																		
0.0 h														1.41				
Day-28																		
0.0 h																0.226		
Day-35																		
0.0 h											0.125							
Day-42																		
0.0 h																		
0.25 h							0.141							0.486				
0.5 h							0.156											
0.75 h					0.116													
1.0 h															0.15			
1.25 h																		
1.5 h																		
2.0 h																		
3.0 h																		
4.0 h																		
8.0 h																		
12.0 h				0.154			0.179											

	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Day-1																		
0.0 h																		
0.25 h		0.134							0.122									0.131
0.5 h													0.173		1.01			
0.75 h																		
1.0 h									1.21									0.168
1.25 h									0.107									
1.5 h									0.111					0.759				0.204
2.0 h																		0.164
3.0 h									0.133									0.139
4.0 h									0.731			0.161		0.124				0.127
8.0 h									0.146		0.59							0.135
12.0 h								0.205					0.187					0.218
Day-7																		
0.0 h				0.352							0.107							0.227
Day-14																		
0.0 h								0.176	0.116		0.381	0.154						0.351
Day-21																		
0.0 h								0.423	0.103		0.535							
Day-28																		
0.0 h								0.116				0.131		0.154				
Day-35																		
0.0 h																		
Day-42																		
0.0 h			0.145						0.113		0.109							
0.25 h			0.107						0.208									
0.5 h			0.118						0.102									
0.75 h								0.142										
1.0 h																		
1.25 h			0.144					0.132										
1.5 h													0.395					
2.0 h								0.112										
3.0 h																		
4.0 h			0.111					0.12	0.149									
8.0 h																		
12.0 h																		

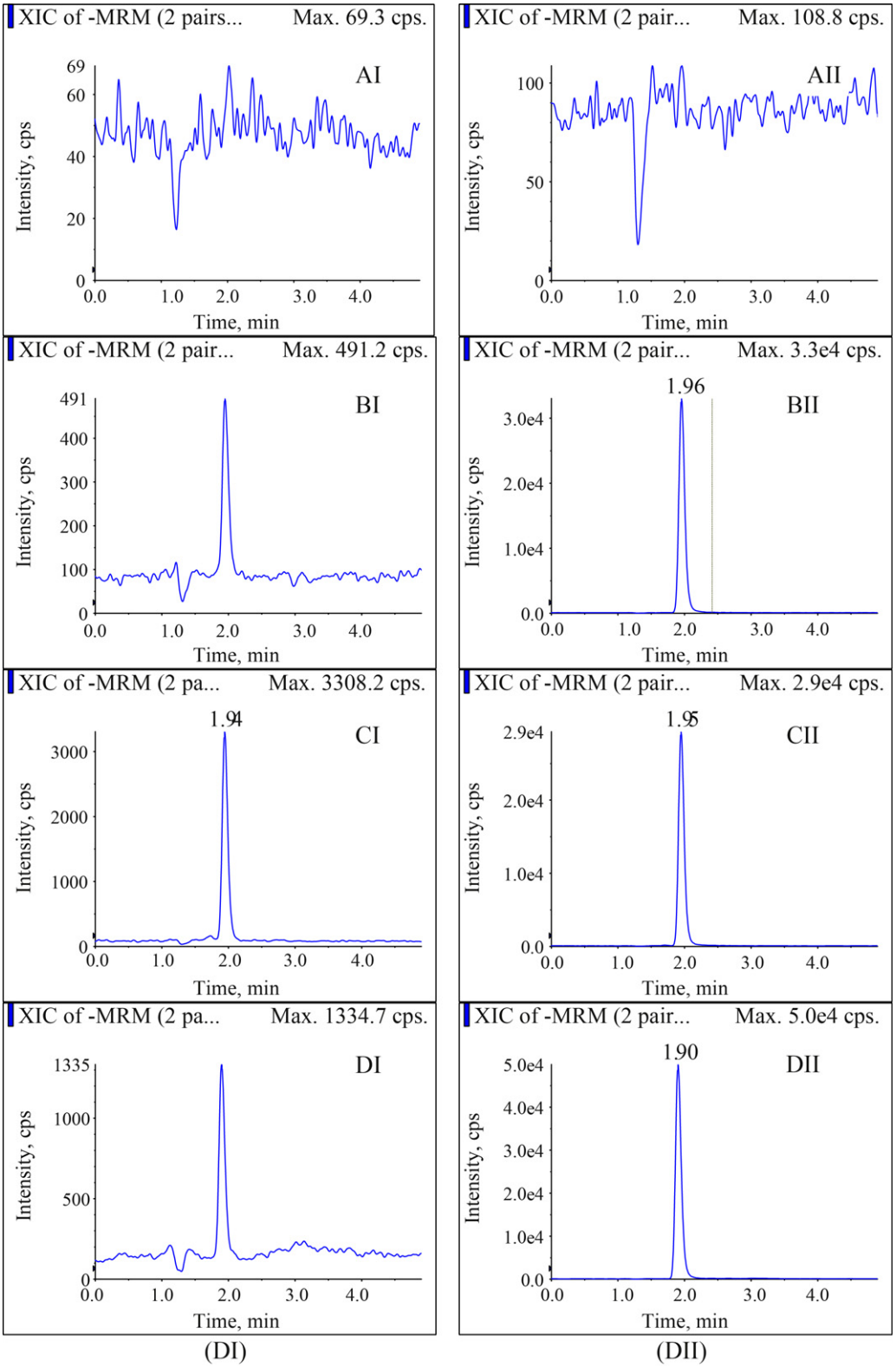

Fig. 3. MRM chromatograms of benvitimod (I) and I.S. (II) obtained from human plasma samples: (A) blank plasma; (B) blank plasma spiked with 0.1 ng/mL standard solution (LLOQ); (C) blank plasma spiked with 2 ng/mL standard solution; (D) plasma sample from a patient with mild psoriasis after treatment with benvitimod cream (1.5 h after dosing, determined concentration of 0.206 ng/mL).

11.26% and 13.98%, respectively. All the results were well within the acceptable limit, it indicated that the analysis of benvitimod was not interfered by endogenous substances in plasma.

3.2.5. Stability

Quality control plasma samples (0.2, 1.0 and 5.0 ng/mL)) shown good stability in plasma when placed in the short-term (24 h) room temperature, three freeze/thaw cycles and stored at −80 °C

for 6 months. In addition, processed quality control samples of benvitimod (i.e. ready for LC/ESI/MS/MS analysis) was also found stable when placed in the autosampler at 4 °C for 24 h. All the results are summarized in Table 2. The results indicate that, taking account of the analytical variability, the stability of processed sample was acceptable.

3.3. Pharmacokinetic study

The method described above was applied to a double-blind, randomized, placebo controlled study of 36 patients with mild to moderate psoriasis. In this study, the patients were divided into four cohorts, treated for 42 days with benvitimod cream or vehicle cream (see details in Table 3). Blood samples were drawn at different time points (0, 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0, 3.0, 4.0, 8.0, and 12.0 h on the first and the 42nd day, 0 h on the 7th, 14th, 21st, 28th and 35th day) after dosing. The clinical pharmacokinetic study was approved by the Ethics Committee of Peking University People's Hospital. All patients gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

As shown in Table 4, benvitimod was not detected in most plasma samples, demonstrating the concentration of benvitimod in plasma was very low. However, due to the sensitivity and selectivity of this method we did find traces of benvitimod in certain time points. Our results confirmed the low absorption of this drug and its passage into general circulation was negligible in most subjects.

4. Conclusion

A LC/ESI/MS/MS method has been developed for the determination of benvitimod in human plasma. The assay described here has been found rapid and sensitive in application, and no significant interferences caused by endogenous compounds are observed. This simple and sensitive assay is suitable for the quantification of benvitimod in human biological samples. Project supported by Peking University People's Hospital Research and Development Funds, RDB2010-12.

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