International Journal of ChemTech Research
CODEN(USA): IJCRGG ISSN : 0974-4290
Vol.4, No.4, pp 1543-1552, Oct-Dec 2012

Development and Validation of method for the determination of related substances of Tacrolimus in Tacrolimus Capsules and Degradation Studies

Qingping Shi*[1,2], Jianchun Li[2], Feng Ding[1]

[1]Department of Pharmacy, the First Affiliated Hospital of Bengbu Medical College, Bengbu 233004,China

[2]College of Pharmacy, Bengbu Medical College, Bengbu 233004,China.

Corres. author: sir_shi@126.com, Tel: +86-0552-3086246.

Abstract: The present study describes a simple and stability-indicating reverse phase high-performance liquid chromatography (RP-HPLC) method for the determination of tacrolimus (FK506) and its related substances in tacrolimus capsules and degradation studies. The chromatogram of KF506 and its isomers named isomer I(IS-I), isomer II(IS-II) and other unknown related substances were found. Successful separation of the drug from the related substances and its isomers were achieved on a KromasiL100-5 C_{18} column (250×4.6mm, with a diameter of 5μm) and detector of UV at 215nm, 1.0ml/min as a flow rate, and 20 μl as an injection volume. For the RP-HPLC method, acetonitrile- water- phosphoric acid (700: 300: 0.2, v/v/v) was used as mobile phase and the column temperature was 60℃. Accuracy satisfactory by % recovery obtained in the range of 97.36%-101.42%, the linearity results for KF506 and impurities in the specified concentration calibration curves was linear with a coefficient of variation (*r*) not less than 0.99. An accelerated degradation study was carried out in FK506 capsules under the conditions including acid, base, peroxide, heat and photolytic degradation. The proposed RP-HPLC assay was found to be specificity, linearity, and precision, intermediate precision, and accuracy, stability in the assay solution and robustness.

Keywords: Tacrolimus; Reversed phase high performance liquid chromatography;Related substances; Forced degradation; Isomers.

1. Introduction

Tacrolimus (also called FK506) was among the first macrolide immunosuppressants discovered, preceded by the discovery of rapamycin (sirolimus) on Rapa Nui [1,2]. Although it is produced by a type of bacterium called Streptomyces tsukubaensis, it was isolated from soil fungus initially [3,4,5]. In 1994, FK506 was firstly approved by the Food and Drug Administration for liver transplantation. Later, it has been further used for the treatment of organ transplants including kidney, heart, small bowel, pancreas, lung, trachea and skin[6,7].

Organic impurities in drug substances can arise during the manufacturing process and storage. Thus, the criteria for their acceptance of certain limits are based on pharmaceutical studies or known safety data [8]. Several methods have been reported for the analysis of KF506 and related substances in drug products [9,10]. High performance liquid chromatography (HPLC) using UV detection has been proposed for the separation of its

isomers and other unknown related substances [11,12,13,14,15,16]. However, no validated stability-indicating reversed phase HPLC (RP-HPLC) method has been used for the separation and quantitative analysis of KF506 in its pharmaceutical forms and its related substances including IS-I and IS-II. In addition, drug impurities generally appeared due to various environmental factors such as being exposure to acid, base, peroxide, heat and photolytic conditions. Therefore, stress testing can help to identify the degradation of the drugs, which will provide important information about intrinsic stability of the drug product.

In this study, a rapid and validated RP-HPLC method was developed to separate KF506 and its isomers. The limit of detection (LOD), limit of quantification (LOQ) and sensitivity of the method was tested in accordance with Chinese Pharmacopoeia (CP) and ICH guidelines [17,18].

2. Experimental and Methods

2.1 Reagents, Materials and Instrumentation

Acetonitrile was obtained from Anhui Sinikang Medicine Technology Ltd (Hefei, China). All other chemicals of analytical grade were gained from local sources unless specified. The instrument used was a Shimadzu LC-10Avp HPLC system consisting of a pump, a UV detector (Suzhou Shimadzu, China) and N2000 data analysis software. KromasiL 100-5 C_{18} HPLC columns (250×4.6mm i.d., 5μ particle sizes) were used for the analysis.

2.2 Preparation of standard solution

Weigh and transfer accurately about 100.0 mg FK506 into a 100ml volumetric flask. Then the drug was dissolved by the diluent solution consists of acetonitrile and water (50:50, v/v), and then dilute up to the mark with the diluent solution. Precisely measure amount of 1 ml, 3 ml, 5 ml, 7 ml and 10 ml the above FK506 solution, and then dilute them to a 100ml volumetric flask using the diluent solution, respectively.

2.3 Preparation of sample solution

Twenty capsules of KF506 were powdered and the capsule powder equivalent to about 2.5mg of KF506 was accurately weighed into a 25 ml volumetric flask. Then about 15ml the diluent solution consists of acetonitrile and water (50:50, v/v) was added and sonicated for 5 min and the volume was made up to 25 ml with water and the solution was mixed thoroughly to achieve a concentration of 100 μg/ml of KF506. The solution was centrifuged for 10 minutes at 3000 rpm and filtered through 0.45 micron cellulose acetate filter.

2.4 Method validation

The RP-HPLC method was validated for specificity, system suitability, linearity, LOD, LOQ, precision, accuracy, solution stability and robustness according to CP and ICH guidelines.

2.5 Statistical analysis

The %RSD value, the linear regression analysis by the method of least squares and other statistical analysis were calculated using Excel program.

3. Results and discussion

3.1 Optimization of chromatographic conditions

During method development, many HPLC columns were evaluated for the peak shape, retention time and resolution of KF506, IS-I and IS-II peaks. To obtain the selectivity for substances, trials were conducted using ctadecyldimethyl-silane (C_{18}) and octyldimethylsilane (C_8) stationary phases with changing pH, flow rate, composition of mobile phase solution of different column and temperature. IS-I, IS-II, KF506 and other unknown related substances was eventually separated using a KromasiL100-5 C_{18} HPLC column (150mm×4.6mm, 5μm particle sizes). The chromatograms recorded using these columns have been presented in Figures 1 to 2.

Acetonitrile, water and phosphoric acid were selected as the mobile phases. An asymmetry peak shape was obtained as the column temperature was 50℃. Whereas, a symmetry peak shape was obtained as the column temperature was 60℃. The optimized wavelength was 215nm as the pharmaceutical excipients in KF506 capsules did not interfere with each other at this wavelength (Figure 3, Figure 4 and Figure 5). The system suitability parameters were achieved using different test mobile phases and column temperatures (Table 1). The standard and spiked sample chromatograms obtained by using the selected column and the mobile phase has been presented in Figures 3 and 4, respectively. The retention times of KF506, IS-I and IS-II are about 12.0, 7.0 and 10.0 min, respectively. The relative retention time of KF506 was 1.71 and IS-II was 1.43 with respect to IS-I peak. The tailing factor for the peaks was not more than 1.0 and the number of theoretical plates of the peak of KF506 was more than 2000. The peaks of KF506, IS-I and IS-II were spectrally pure as calculated by the software on the

basis of the purity angle and purity threshold. For the RP-HPLC, acetonitrile- water- phosphoric acid (700: 300: 0.2, v/v/v) was used as mobile phase and the column temperature was 60℃. The wavelength was set at 215nm using a KromasiL100-5 C_{18} HPLC column (150mm×4.6mm, the diameter of the particle was 5μm). The flow rate was kept at 1mL/min. The sample injection volume was 20 μl.

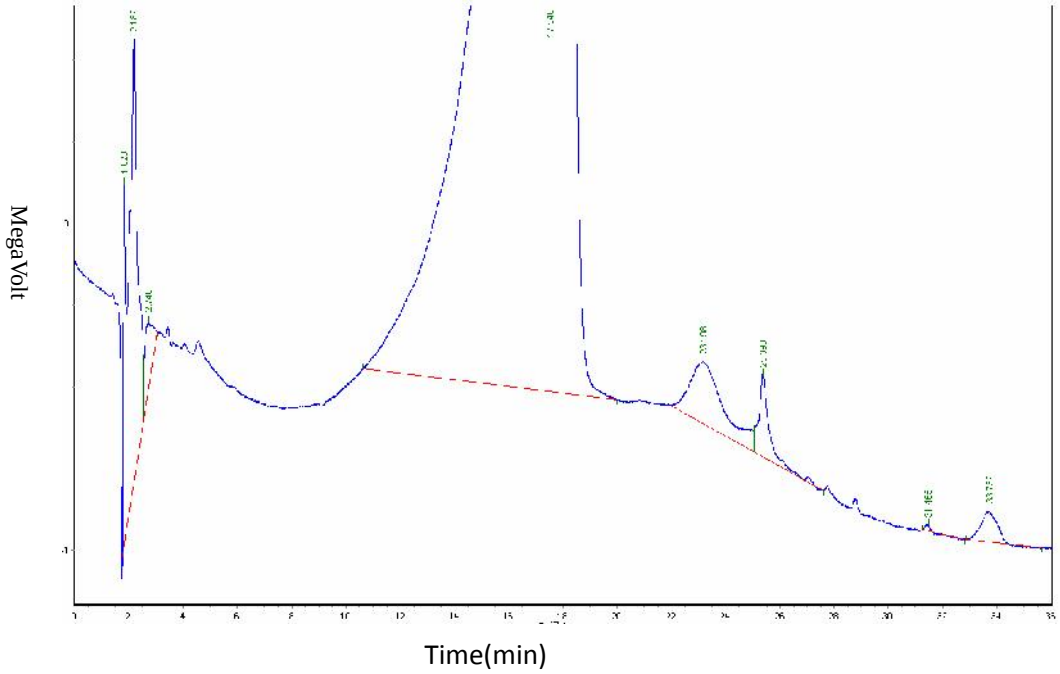

Figure1 Standard chromatogram in C_8 column 250 × 4.6 mm, 5 μ.

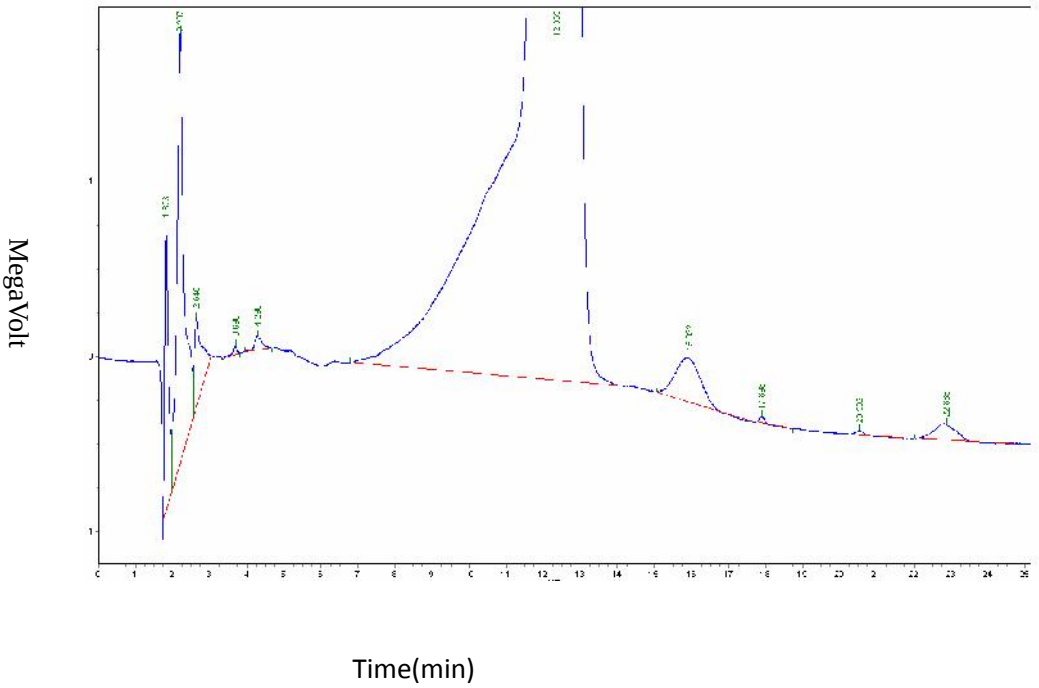

Figure 2. Standard chromatogram in C_{18} column 250 × 4.6 mm, 5 μ, using acetonitrile-water（65：35）as mobile phase, the column temperature at 60℃.

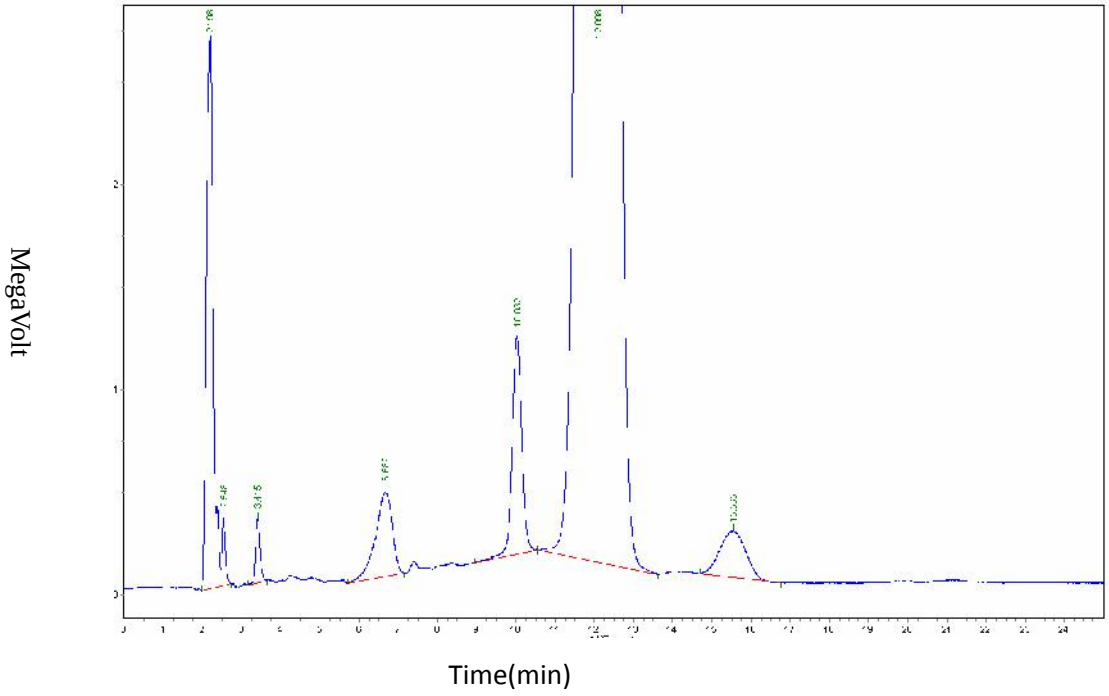

Figure 3. Standard chromatogram in C_{18} column 250 × 4.6 mm, 5 μ, using acetonitrile- water- phosphoric acid(700：300：0.2 v/v/v), the column temperature at 50℃.

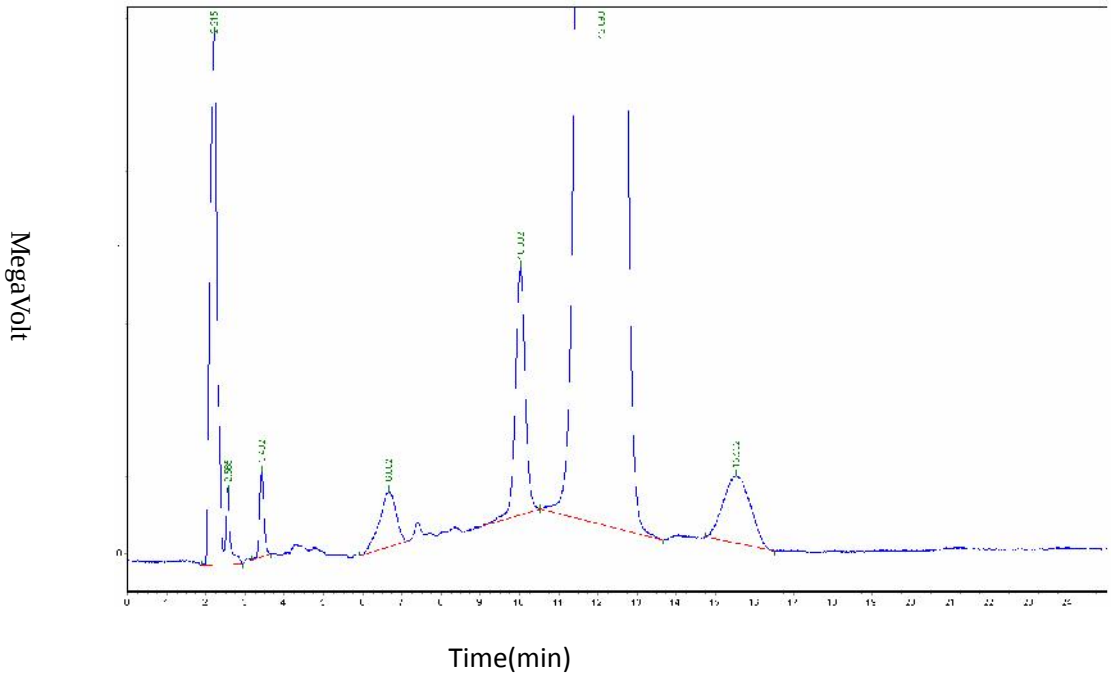

Figure 4. Standard chromatogram in C_{18} column 250 × 4.6 mm, 5 μ, using acetonitrile- water- phosphoric acid(700：300：0.2 v/v/v), the column temperature at 60℃.

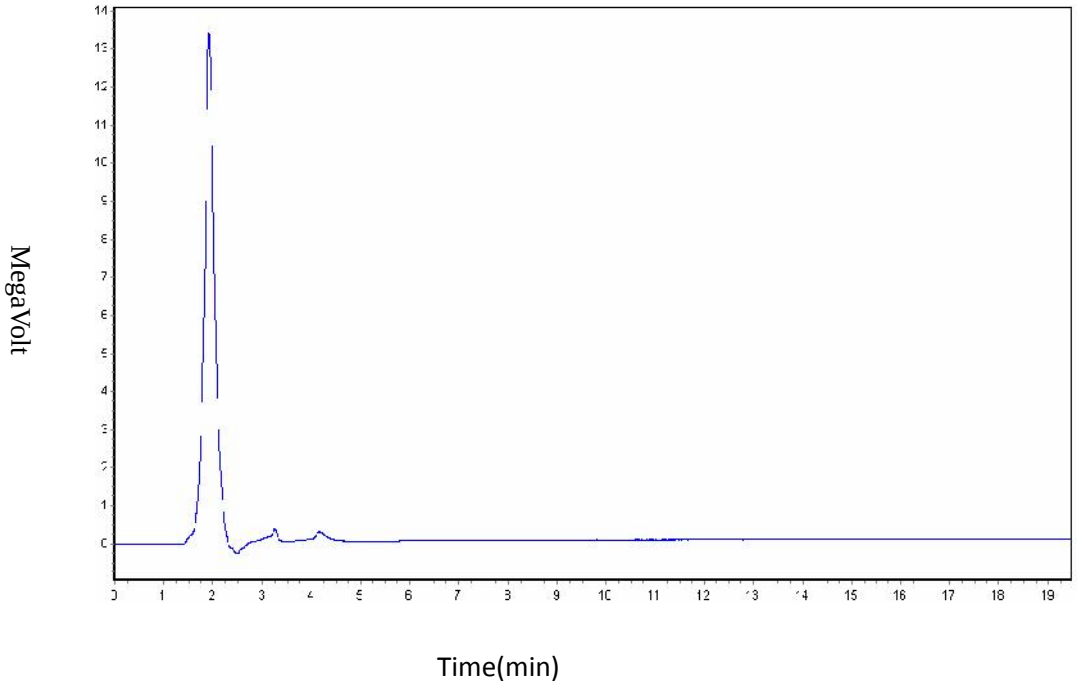

Figure5 Chromatogram of blank sample solution

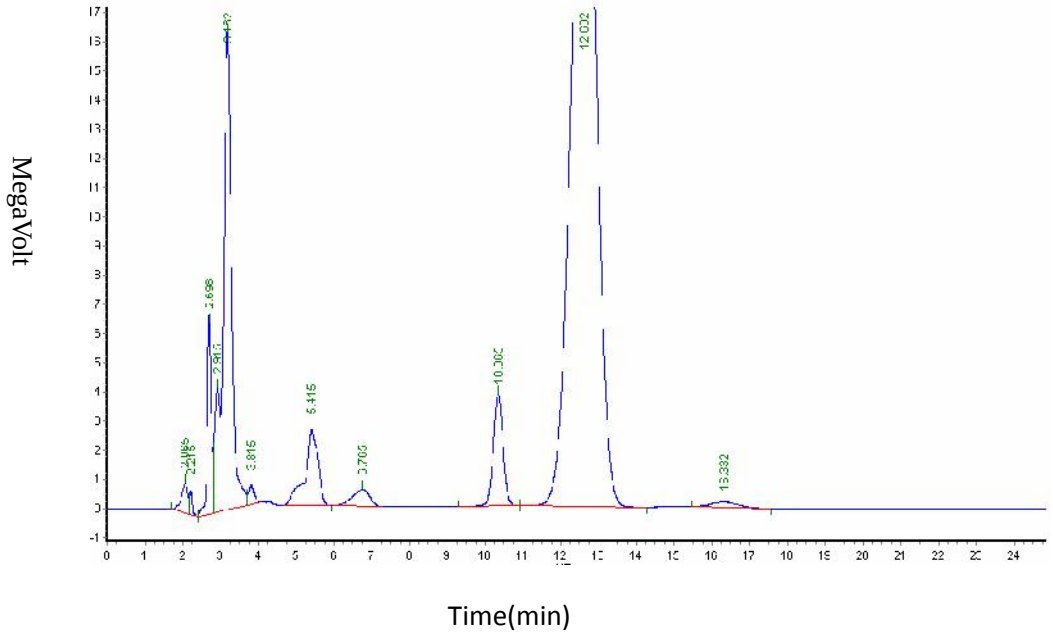

Figure6 Chromatogram of acid degradation

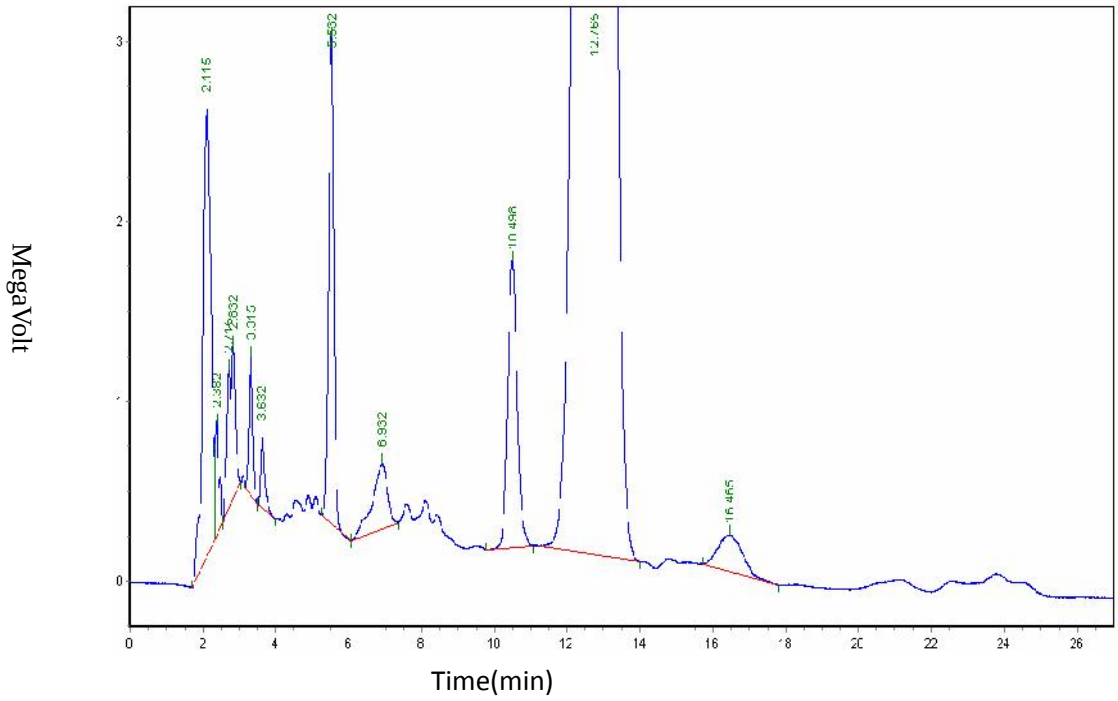

Figure 7 Chromatogram of thermal degradation

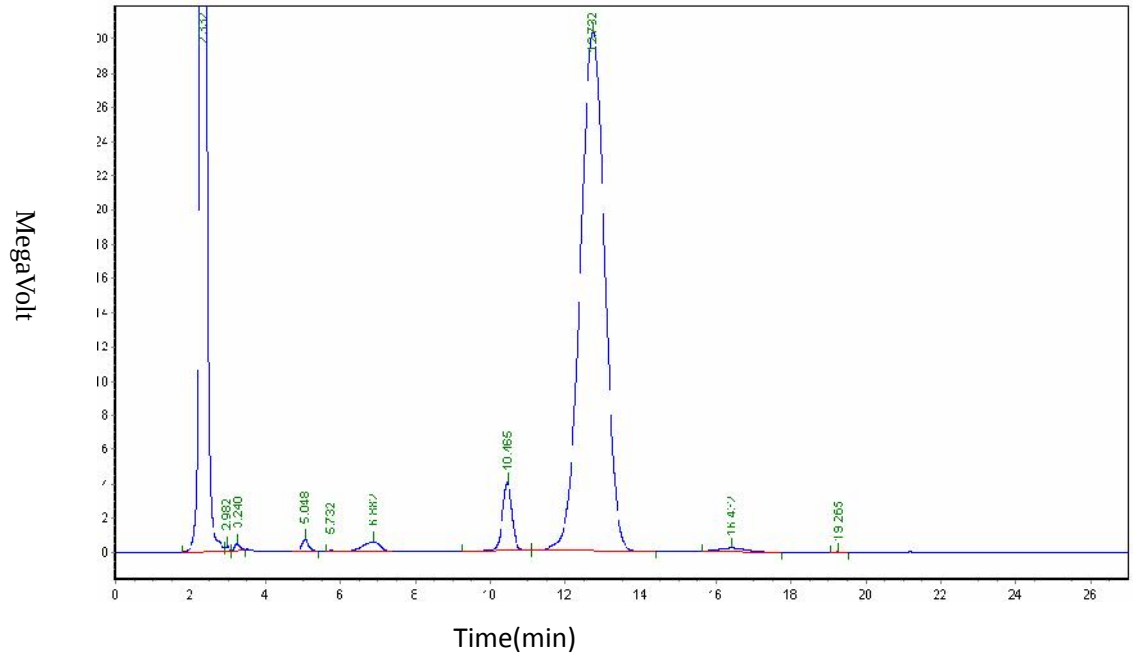

Figure8 Chromatogram of peroxide degradation

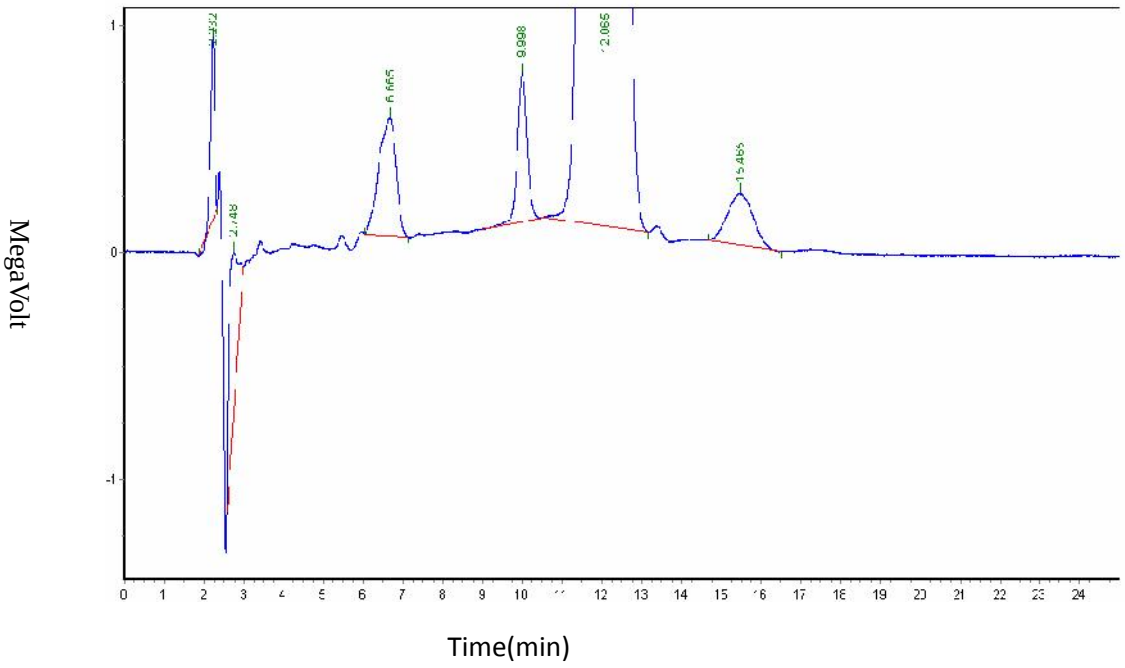

Figure9 Chromatogram of photolytic degradation

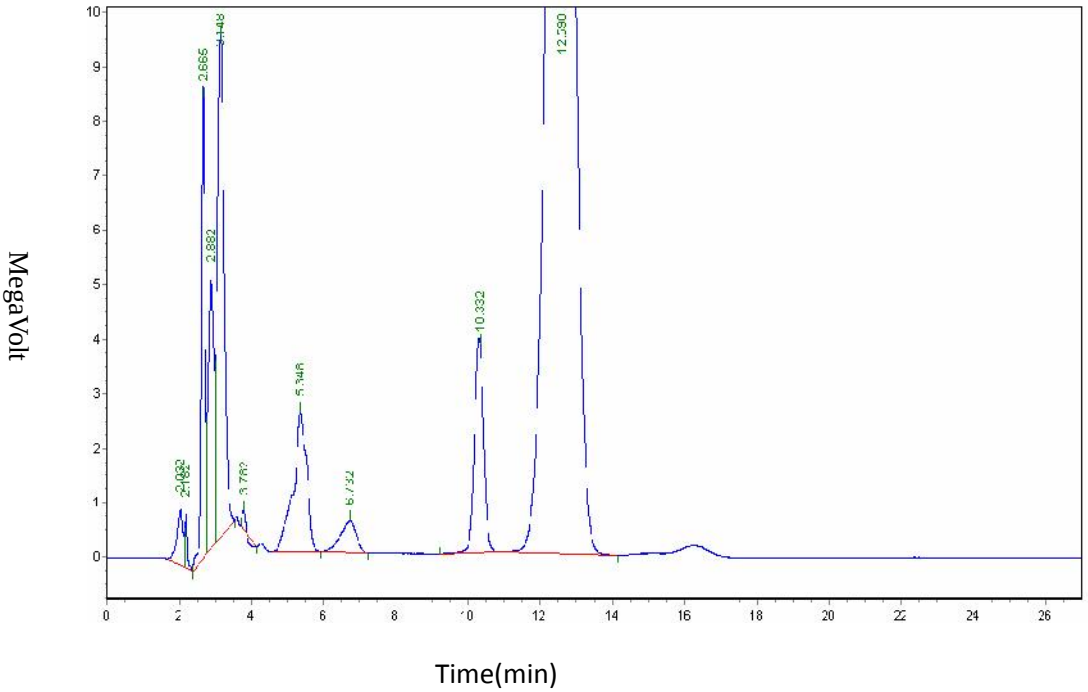

Figure10 Chromatogram of alkali degradation

Tab1e1- the system suitability parameters using different test mobile phase and different column temperature

chromatographic conditions		system suitability parameters														
mobile phase	column temperature	Retention time			Theoretical -plates			separating -degree			trailing factor			Asymmetry factor		
		IS-I	IS-II	KF506	IS-I	IS-II	KF506	IS-I	IS-II	KF506	IS-I	IS-II	KF506	IS-I	IS-II	KF506
acetonitrile-water（65：35）	50℃	2.182	2.740	17.548	242.14	414.77	2015.61	0.785	0.863	11.974	1.02	2.491	0.709	1.407	3.793	0.700
acetonitrile-water（65：35）	60℃	2.215	2.793	9.365	183.28	104.88	2607.52	1.197	0.710	2.343	1.094	2.438	0.726	1.399	4.012	0.708
acetonitrile-water（70：30）	60℃	2.207	2.640	12.365	1076.0	4600.9	2015.12	1.484	1.150	9.246	1.224	2.686	1.575	1.448	4.020	0.918
acetonitrile-water-phosphoric acid（700：300:0.1）	50℃	3.548	10.515	12.907	4712.1	8002.2	1048.15	4.028	17.490	1.968	1.246	1.075	0.936	1.411	1.067	0.860
acetonitrile-water-phosphoric acid（700：300:0.1）	60℃	6.682	10.032	12.098	1221.3	8802.4	2215.38	5.748	4.774	2.412	0.788	1.009	0.990	0.626	1.021	0.988
acetonitrile-water-phosphoric acid（700：300:0.2）	50℃	6.515	9.865	11.932	1135.8	8400.7	2154.7	5.681	4.729	2.408	0.818	1.020	0.987	1.261	1.049	0.979
acetonitrile-water-phosphoric acid（700：300:0.2）	60℃	6.682	10.032	12.098	1248.9	8462.8	2179.2	5.752	4.774	2.485	0.862	0.988	0.987	0.717	0.987	0.975

Table2- Linearity, LOD, LOQ and accuracy dataof KF506 and the two isomers

drug	linear equation	r	range of concentration	LOD	LOQ	Accuracy %Recovery(n=3)
KF506	Y=14328X+16944	0.9992	10.20μg/mL～100.20μg/mL	0.128μg/mL	10.20μg/mL	98.53～99.57
IS-I	Y=12718X-917.54	0.9990	0.930μg/mL～9.303μg/mL	0.128μg/mL	0.930μg/mL	97.36～99.95
IS-II	Y=10735X+657.07	0.9992	0.590μg/mL～5.903μg/mL	0.128μg/mL	0.590μg/mL	98.64～101.42

3.2 Method validation

3.2.1 Specificity

To evaluate the ability of the proposed method to separate FK506 form the isomers and its related substances, degradation study was carried out under stress conditions including heat (100℃, 4h), acid (1.0M HCL,4h), base (1M NaOH, 4h), oxidation (3.0% H_2O_2 RT, 4h) and photolytic degradation (4500Lx, 4h). The chromatogram of the forced degradation studies are given in Figure 6 to 10. The blank sample solution had no interference with KF506 and its isomers under the chromatographic conditions (Figure 5). The study showed that the baseline separation was achieved between the three tautomeric isomers and their degradation products. This study proved the specificity and stability-indicating of the method.

3.2.2 Precision

The system precision was validated by injecting a concentration of 50μg/ml of KF506 standard solutions to prove the consistency of the signal output from the HPLC system. The method precision was assessed by calculating RSD (%) for the results obtained from six individual injections using standard and sample solutions to prove the consistency of the method. RSD (%) of three tautomeric forms (IS-I, IS-II and KF506) for method precision were less than 3.5%, which are no more than the acceptance criteria: % RSD not more than 5.0.

3.2.3 Linearity

The linearity of response for each known drug was determined in the concentration range of the limit of quantitation to about 150% of specification limit for each known drug and substances. Acceptance criteria squared correlation coefficient was not less than 0.99 (**Table2).**

3.2.4 Limit of detection and limit of quantification

The Limit of detection (LOD) and limit of quantification (LOQ) were determined from the slopes of linear regression curves. The LOD and LOQ for IS-I, IS-Ⅱ and KF506 were determined by injecting a series of dilute solutions. The results were depicted in **Table 2.**

3.2.5 Accuracy

The accuracy of the method was verified by calculating the recovery of IS-I, IS-Ⅱ and KF506 at three different concentrations of 80, 100 and 120% at the specific limit. The results presented in **Table 2** showed the recovery ranges from 97.36% to 101.42%, indicating the accuracy of the method was acceptable.

3.2.6 Robustness

When the chromatographic conditions varied, the chromatograph was recorded. The selected parameters were mobile phase composition (±2% of gradient composition), flow rate (±10%), wavelength (±5nm) and column temperature (±2°C). System suitability requirements of tailing factor, number of theoretical plates and resolution were within the specified limits even with the altered method parameters indicating that the method is robust and that slight changes the flow rate of mobile phase and column temperature do not affect the performance of the method.

3.2.7 Solution stability

The stability of the standard and sample solutions was monitored at 0 h, 1 h, 2 h, 4 h, 6 h, 8 h, 12 h and 24 h. The results indicated no significant difference was noted in the area of the peaks compared to the initial peak area (within ±2% for KF506 and its tautomeric isomers). All this demonstrated the test sample was stable at room temperature for 24 hours.

4. Conclusions

The RP-HPLC method developed for the estimation of IS-I, IS-II, KF506 and its related substances in the capsules is very useful tool for monitoring of the quality of KF506 and its pharmaceutical forms. The method validation tests show that this is a stability indicating robust method which can be used for checking the quality of the manufactured capsules as well as for stability studies of the pharmaceutical capsules. The proposed RP-HPLC assay was found to be specificity, linearity, and precision, intermediate precision, and accuracy, stability in the assay solution and robustness.

Acknowledgment

The authors wish to thank for their support of Department of Pharmacy, Bengbu Medical College and permitting this work to communicate for publication.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

QPS: Study design and conception, development and validation the assay of HPLC; data analyses, drafting the manuscript; JCL and FD: Development and validation the assay of HPLC; data analyses. All authors contributed to writing of the final manuscript; All authors read and approved the final manuscript.

References

[1] Kino T, Hatanaka H, Hashimoto M, Nishiyama M, Goto T, Okuhara M, Kohsaka M, Aoki H, Imanaka H (1987)."FK-506, a novel immuno suppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics." *J Antibiot (Tokyo)* 40 (9): 1249–1255.

[2] Lauerma AL, Granlund H, Reitamo S. Use of the newer immunosuppressive agentsin dermatology. Bio Drugs 1997,8:96-106 .

[3] Pritchard D (2005). "Sourcing a chemical succession for cyclosporin from parasites and human pathogens.". *Drug Discov Today* 2010 (10): 688–691.

[4] Bieber T. Topical tacrolimus (FK 506): A new milestone in the management of atopic dermatitis. *J Allergy Clin Immunol*.1998 ,102:555-557 .

[5] Ponner, B, Cvach, B (Fujisawa Pharmaceutical Co.): Protopic Update 2005.

[6] Alaiti S, Kang S, Fiedler VC, et al. Tacrolimus (FK506) ointment for atopic dermatitis: A phase I study in adults and children. *J Am Acad Dermatol*. 1998,38:69-76.

[7] Griffith JP, Kim JL, Kim EE, et al. X-ray structure of calcineurin inhibited by the immunophilin - immunosuppressant FKBP12-FK506 complex. *Cell*.1998;82:507-22.

[8] International Conferences on Hormonization Q3A (R2): Draft Revised Guidance on impurities in New Drug Substances, 2006.

[9] Chen Yue. HPLC determination of tacrolimus and its related substances. *Chinese Journal Pharmcy Anal* 2008,28(2):309-313.

[10] Y. Namiki, A. Fujiwara, N. Kihara, et.al. Determination of the immunosuppressive drug tacrolimus in its dosage forms by high-performance liquid chromatography, *Chromatographia* 1995;40(5):253-258.

[11] Xu Xin-jun, Liu Gaolin,Deng Yul in, *et. a1*. Pharmacokinetics of tacrolimus, a novel potent immunosuppressant in healthy chinese volunteers. *Chin J Antibiot* 2002;27(1):59.

[12] Zhang Yi, Wang Zhi-Yu, Wang Zhao-qin, *et. a1*. Comparision for monitoring methods of tacroliums in human plasma. *Chin Pharm* 2003;38(5):386.

[13] Li Guoqin, Zeng Su.Determination of Related Substances of Tacrolimus by HPLC with Gradient Elution.*Chinese Pharmaceutical Journal* 2008;43(07):551-553.

[14] Uwe Chirstians, Gudrun Schmidt, Augustinus Bader, *et. a1*. Identification of drugs inhibiting the *in vitro* metabolism of tacrolimus by human liver microsomes. *Br J Clin Pharmacol* 1996;41:187-190.

[15] Shiraga T. Matsuda M, Nagase K, *et. a1*.Metabolism of FK506, a potent immunosupressive agent, by cytochrome p450 3A enzymes in rat, dog and human liver microsomes. *Biochem Pharmacol* 1993; 47: 727-735.

[16] Karanam BV. Vincent SH, Lee Chiu SH. KF506 Metabolism in human liver microsome: Investigaions of the involvement of cytochrome P450 isozymes other than CYP3A4. *Drug Metab Dispos* 1994;22:811-814.

[17] International Conferences on Hormonization Q2 (R1) Validation of analytical Procedures: text and methodology, 2005.

[18] Chinese Pharmacopoeia, the third method in appendix of 2005 edition.

*****