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Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 1070–1074
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Evaluation of rapamycin chemical stability in volatile-organic solvents by HPLC

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Received 3 November 2005; accepted 2 February 2006
Available online 20 March 2006

Abstract

Rapamycin or sirolimus is a carboxylic lactone-lactam macrolide with a potent immunosuppressive activity. It can be successfully used to impregnate stents inserted in coronary arteries during surgical applications, preventing fatal infection and rejection adverse effect. The chemical stability of rapamycin in several organic-volatile solvents (acetone, chloroform, dichloromethane, hexane, ethyl alcohol, ethyl acetate, methyl alcohol, pentane and tetrahydrofurane) was established by HPLC–DAD-MS in reverse phase analysis. Results permitted to exclude rapamycin chemical degradation and to reveal a typical chemical isomerization, favoured in accordance to the solvent used. Two typical peaks appear, denominated β and γ, the time retention of which are, respectively, 11.3 and 15.0 min. Thanks to data recovered by NMR, spectrophotometric UV and mass analyses, it was possible to establish that both peaks correspond to two different isomeric forms of rapamycin. In addition, it was possible to establish that the relative percentage peak area varies according to the solvent and to the experimental time. The two isomeric forms are in equilibrium and each solvent concurs to differently displace this equilibrium versus one form rather than another, according to their both polarity index and aproticity.

Keywords: Rapamycin; Stability; HPLC; NMR

1. Introduction

The aortic stenoses interest a great number of persons. They affect the intima tunica of medium or large diameter arteries. In the final phase of pathology evolution, a plate can appear producing stenosis or complete artery obstruction. This problem can be efficiently resolved by the angioplasty, a surgical operation that consists on the introduction of a balloon through the artery, by the way of a catheter. Cycles of balloon compression and decompression allow to reducing the plate responsible for the stenosis. To avoid the collapse of the blood vessel tunica, a stent is released, performing a mechanical support. In spite of the immediate stenosis resolution, patients are frequently subjected to a restenosis during the 6 months following to the surgical operation. During the stent implant, as a consequence of the high expansion pressure, an injury can be produced both in the medium and intimate arterial tunica. The injury represents a further drive to the platelet aggregation, also favoured by the thrombo-genicity of the metallic surface of the stent. In order to reduce this effect, the possibility to combine the surgical event with an appropriate pharmacological therapy seems to be successful. In this aim, several devises has been studied where the stent releases drug after implant. During the clinical trials, best results have been reached with either paclitaxel [1] or rapamycin [2].

Rapamycin is a carboxylic lactone-lactam macrolide derived from an actinomycete (*Streptomyces hygroscopicus*), the chemical structure of which is given in Fig. 1 [3–5]. It has been shown to have potent immunosuppressive activity and to inhibit T-cell activation and proliferation [6–11]. As a consequence of its potent pharmacological properties, it is successfully used to impregnate stents inserted in coronary arteries during surgical applications, preventing fatal infection and rejection adverse effect [12,13].

Rapamycin potently inhibits downstream signaling from the mammalian target of rapamycin (mTOR) proteins which

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doi:10.1016/j.jpba.2006.02.009

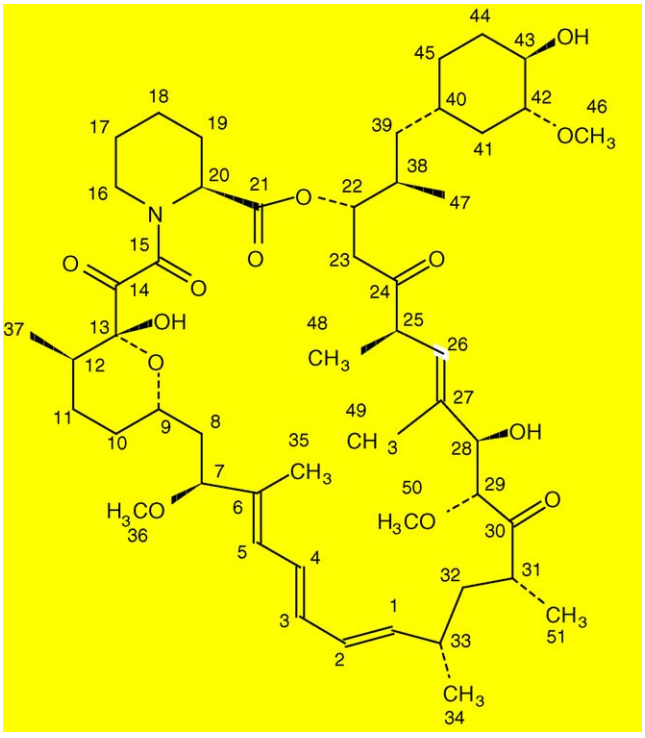

Fig. 1. Chemical structure of rapamycin ($C_{51}H_{79}NO_{13}$).

functions in a signaling pathway to promote tumor growth. These evolutionarily conserved protein kinases coordinate the balance between protein synthesis and protein degradation in response to nutrient quality and quantity. Rapamycin binds to the FK-506 binding protein (FKBP12, $K_d = 0.2$ nM) and the rapamycin/FKBP12 complex then binds to mTOR and prevents interaction of mTOR with target proteins in this signaling pathway. Rapamycin selectively inhibits the phosphorylation and activation of p70 S6 kinase (p70S6K, IC50 = 50 pM). It prevents the translational activation of IGF-II and inhibits later signaling events such as p110Rb phosphorylation, p34cdk1 kinase activation and cyclin A synthesis.

Because the poor water rapamycin solubility [14], the more frequently used surgical devises exploit the rapamycin dissolution in ethanol, which is then extemporary sprayed to the stent during the surgical event. The aim of the present study is to establish the chemical stability of rapamycin in additional organic-volatile solvents. Because the rapamycin ethanolic solutions are relatively stable, the hospital pharmacist has to prepare extemporary rapamycin solutions. Because there are some hospitals not equipped for this kind of preparation, the possibility to industrially prepare solution with greater chemical stability should be an important tool. In this scope, rapamycin solutions (4 mg/ml) were prepared in presence of 10 different solvents selected for the present study (acetone, chloroform, dichloromethane, hexane, ethyl alcohol, ethyl acetate, methyl alcohol, pentane and tetrahydrofurane) and analysed by HPLC–DAD-MS in reverse phase, after preparation and after 30 and 60 days at a temperature of 4 °C, the minimum shelf life for an industrial application.

2. Experimental

2.1. Materials

Twenty milligrams of rapamycin were kindly supplied by Galenica Senese (Siena, Italy) as one batch named Rap04. One received, it was immediately stored at a temperature of 4 °C. Volatile-organic solvents (acetone, chloroform, dichloromethane, hexane, ethyl alcohol, ethyl acetate, methyl alcohol, pentane and tetrahydrofurane) were all of analytical grade (Sigma–Aldrich Chemie, Stenheim, Germany). The water was the Lichrosolv for chromatography (Merck).

2.2. Methods

A rapamycin ethyl alcohol solution (4 mg/ml) was prepared and immediately analysed in order to establish the initial purity degree of the concerned batch.

At the same time (T_0), an appropriate amount of rapamycin accurately weighed was used to prepare solutions (4 mg/ml) in the several volatile-organic solvents. The solutions were stored at 4 °C in small glass vials, accurately closed by an elastomeric stopper and sealed by an aluminium cap, in order to avoid any solvent evaporation. Before the analysis, the volatile-organic solvent was kept to evaporate at room conditions under a nitrogen gas flux. An appropriate amount of ethanol was then added in order to restore the same initial concentration. The HPLC analyses were performed both after 30 and 60 days. At the same time intervals, an analysis of an ethanolic solution freshly prepared was repeated, in order to exclude any contemporary chemical degradation of the pure substance, stored as powder at 4 °C.

The HPLC analysis was performed by an HPLC 1090 Hewlett Packard Serie II, equipped with an auto-sampler, a diode array and a mass spectrometer (mod. 1100 MSD, with a ionization source ESI and quadrupole analyzer). The software was a LC/MSD Chemstation Rev. A.08.03.

The UV detector was used at the following wavelength: $\lambda 1 = 277$ nm; $\lambda 2 = 254$ nm; $\lambda 3 = 230$ nm; $\lambda_r = 550$ nm. The column was a Lichrocart 250-4-RP 18 5 um (Merk). The mobile phase was an 80:20 methyl alcohol:water. The injection volume was 10 μL and the speed flow was 1 mL/min. All samples are injected as an ethanolic solution.

The NMR spectra were acquired by a Varian Mercury Plus 400, equipped with a Varian ATB broadband probe.

3. Results and discussion

The volatile-organic solvents selected for the present study are listed in Table 1. The selection took into account three different parameters: the boiling temperature, i.e. the volatility, the polarity index and the classification in accordance to the ICH Topic Q 3C Step 4 (17 July 1997) that describes the residual solvent limit allowed for a daily drug administration. The volatility should be important to favour the rapid removal of the solvent after the extemporary spreading of the rapamycin solution just before the surgical use, and thus to reduce the residual stent solvent amount. The polarity was taken into account because

Table 1
List of the solvent used for the present study

Solvent	Bowling temperature (°C)	Polarity index	Class
Acetone	56	5.1	3
Chloroform	61	4.1	2
Dichloromethane	41	3.1	2
Hexane	69	0.0	2
Ethyl alcohol	78	5.2	3
Ethyl ether	35	2.8	3
Ethyl acetate	77	4.4	3
Methyl alcohol	65	5.1	2
Pentane	36	0.0	3
THF	65	4.0	3

Their bowling temperature and polarity index are indicated. The affiliation class according to the ICH Topic Q 3C Step 4 (17 July 1997) is also given.

Table 2
Typical rapamycin peaks revealed by HPLC from an ethanolic solution of rapamycin Rap04 batch at the time 0 (T_0)

	α (9.5)	β (11.3)	γ (15.0)	β + γ
Rap04				
T_0	1.24	95.71	2.81	98.52
T_{30}	1.14	95.84	2.68	98.52
T_{60}	1.16	95.83	2.70	98.53

The retention times are indicated in bracket. HPLC analysis was repeated both at T_{30} and at T_{60}.

it influences the rapid dissolution of the rapamycin during the extemporary preparation. Rapamycin is practically insoluble in water and contains no functional groups that are ionisable in the pH range of 1–10 [14]. The solubility in the ethyl alcohol is less then 90 mg/ml [14]. Rapamycin was soluble in the concentration of 4 mg/ml in all the organic solvents used during the present study. In Fig. 2, the chromatogram of a rapamycin ethanolic solution performed at the time 0 is given. Three typical peaks appear, designed as α, β and γ, the retention times of which are given in Table 2. Both the UV and mass spectra are identical for the three peaks (Figs. 3 and 4), suggesting that they are closely related.

Rapamycin solutions prepared in different solvents were then analysed. The percentage area of the peaks for each chromatogram is reported in Table 3. Results were evaluated with

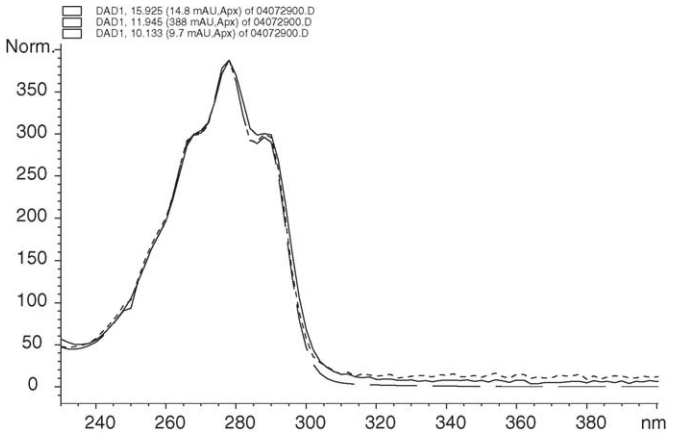

Fig. 3. UV spectra for the three-rapamycin typical peaks. From their superposition, it is possible to establish the chemical identity of the compared species.

the respect to that obtained at both T_{30} and T_{60} for an ethanolic solution freshly prepared from rapamycin powder (Table 2). It must be noted the complete superposition of different ethanolic solutions prepared at different time intervals that concurs to exclude chemical degradation of substance.

Concerning the α-peak, its intensity remains quite constant, whatever the solvent was used. This slight peak, the molecular weight and the UV spectra of which are identical to that of rapamycin, should corresponds to isomeric specie. The fact that it remains quite constant, whatever the solvent and time, suggests that it participate only in negligible manner in the equilibrium process with the other forms.

An opposite result is concerned with both the β- and γ-peaks. In fact, it is known from the literature that rapamycin occurs in solution as a mixture of two conformational isomers about the peptide bond [15–18] in a *trans*:*cis* ratio dependent on the solvent (90:10 DMSO, 80:20 $CDCl_3$). Since also the presence of a structural isomer in equilibrium with rapamycin has been suggested [19], we compared the NMR spectra (^1H and ^{13}C) of our sample with the reported spectra of the conformational and structural isomers of rapamycin, concluding that only the two conformational isomers were detectable by NMR. On this basis, we can suppose that peaks β and γ represent the two conformational isomers of rapamycin. Their equilibrium in solution

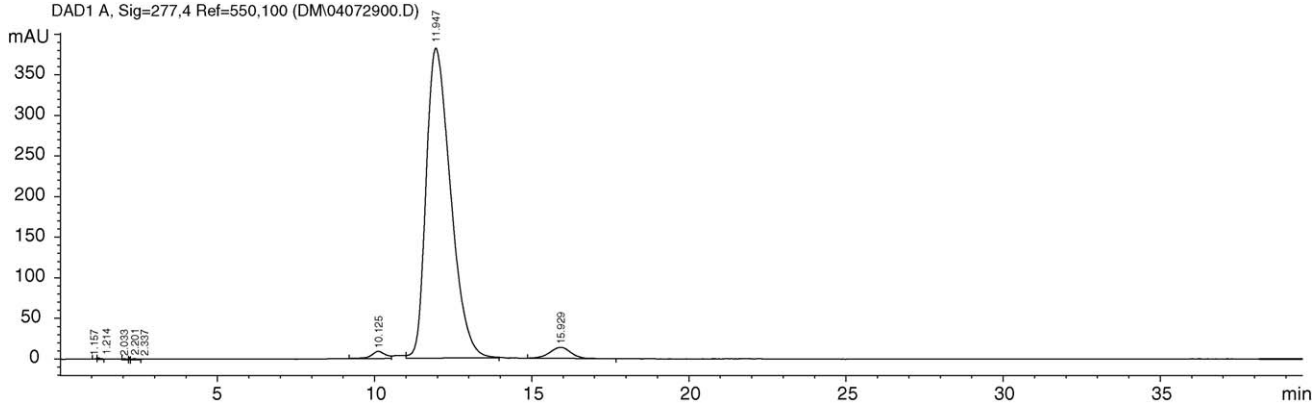

Fig. 2. Chromatograms of an ethanolic solutions of rapamycin (4 mg/ml) freshly prepared.

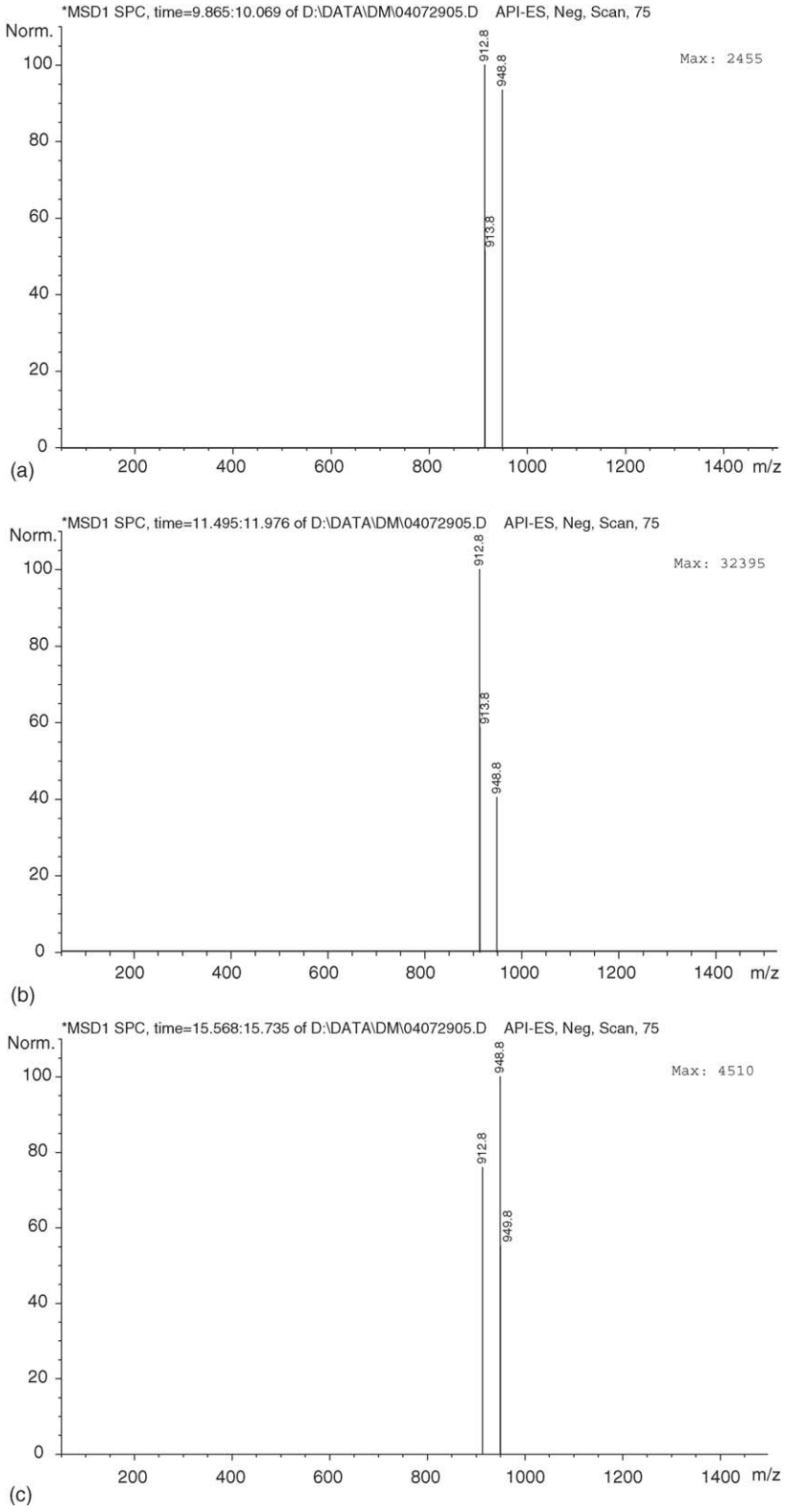

Fig. 4. Mass-spectra for the three-rapamycin typical peaks. (a) α-peak, (b) β-peak and (c) γ-peak. The spectrum of negative ions shows two peaks that respectively correspond to species $[M-H]^-$ (*m/z* 912.8) and $[M+Cl]^-$ (*m/z* 948.8). The three species (α, β and γ) do not give positive ionization because in the three isomers there are not basic centres able to form adducts with ions H^+, Na^+ and K^+.

Table 3
% peak area for chromatograms of rapamycin solutions in several volatile-organic solvents, stored at 4 °C for both 30 and 60 days

	α	β	γ	β + γ
Acetone				
T_{30}	1.14	91.77	6.33	98.10
T_{60}	1.19	92.13	6.32	98.45
Ethyl alcohol				
T_{30}	1.91	91.97	5.73	97.70
T_{60}	2.53	91.41	5.34	96.75
Chloroform				
T_{30}	1.14	93.33	5.10	98.43
T_{60}	0.80	96.22	2.83	99.05
Dichlorometane				
T_{30}	0.81	93.54	5.37	98.91
T_{60}	0.92	96.20	2.76	98.96
n-Hexane				
T_{30}	1.48	92.85	4.88	97.73
T_{60}	0.76	96.08	2.79	98.87
Ethyl ether				
T_{30}	1.94	92.05	5.12	97.17
T_{60}	2.32	93.55	3.76	97.31
Ethyl acetate				
T_{30}	1.28	90.91	5.98	96.89
T_{60}	1.33	92.43	4.30	96.73
Methyl alcohol				
T_{30}	1.03	90.12	5.90	96.02
T_{60}	2.05	89.71	5.75	95.46
THF				
T_{30}	1.90	90.88	6.04	96.92
T_{60}	1.86	91.56	5.79	97.35
Pentane				
T_{30}	1.98	93.23	4.24	97.47
T_{60}	0.39	90.19	6.35	96.54

is significantly affected by the solvent. Considering the ethanol solution, immediately after preparation, the preferred specie at the equilibrium is that relative to the β-peak, but after 28 days, and also after 53 days, the ethanol favours the equilibrium displacement toward the isomer relative to the γ-peak. The sum of the two peaks always remains constant, and this fact lead to confirm that these two isomeric forms are implied in an isomeric equilibrium. The equilibrium displacement towards the γ-form occurs within 24 h (β = 92.53 and γ = 4.93).

From Table 3, it is possible to observe that acetone, ethyl ether, ethyl acetate, methyl alcohol, THF and pentane can also displace the equilibrium towards the γ-form, in the same manner that ethyl alcohol. The other solvents, chloroform, dichloromethane, *n*-hexane displace the equilibrium towards the β-form. It was also possible to observe that the addition of chloroform to the solid in which the ethyl alcohol displaced towards the γ-form draw back. All these equilibrium observations permit to definitely exclude the degradation of rapamycin in the considered solvents, for the considered experimental time.

4. Conclusion

The analysis protocol lead to conclude about the chemical purity of the rapamycin at the time t_0 and to exclude any chemical degradation in the considered volatile-organic solvents.

In all chromatograms, it is possible to detect the presence of two main different peaks designed as β and γ, the time retentions of which are 11.3 and 15.0 min. Thanks to NMR, UV and mass analysis it was possible to conclude that the peaks correspond to the isomeric forms of rapamycin rather than chemical degradation products. The relative percentage of the peak area varies according to the solvent and to the time interval. In particular it was possible to establish an equilibrium between the β- and γ-forms. The equilibrium ratio of the two forms depends to the polarity and the proticity of the solvents.

Acknowledgements

Authors gratefully acknowledge the Galenica Senese (Monteroni d'Arbia, Siena, Italy) for the financial support.

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