OPTIMIZATION OF TOPICAL RAPAMYCIN: CHEMICAL, PHYSICAL AND MICROBIOLOGICAL STABILITY

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Abstract

Introduction: topical rapamycin has been established as an effective and safe therapy for facial angiofibromas in tuberous sclerosis. Different formulations have been tested for this skin disease, most using an ointment as a vehicle. Purpose: to improve the classical formulation of topical rapamycin and to determine the validity period of the proposed options based on chemical, physical and microbiological stability studies. Methods: four different 0.4% rapamycin formulations were prepared (ointment, emulsion, gel and liposomes). The stability studies for each formulation were: chemical (extraction with lipophilic solvents and high-performance liquid chromatography assay), physical (pH, uniformity, extensibility, absence of crystals, absence of phase separation and only for liposomal formulation was determined particle size, zeta potential and encapsulation efficiency) and microbiological (culture samples in blood-agar media) during 56 days. Results: only liposomes were chemically, physically and microbiologically stable after 8 weeks. Ointment, emulsion and gel formulations lost their chemical and physical stability before 56 days. Conclusions: this study describes a new four formulations to improve the previously treatment for facial angiofibromas in tuberous sclerosis. It also provides favorable stability data only for liposomes. However, more dermokinetic and clinical studies are needed to confirm that liposomes are most appropriate to ensure effectiveness, safety and high patient satisfaction.

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SHORT TITLE: TOPICAL RAPAMYCIN STABILITY

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KEY MESSAGE

Optimization and chemical, physical and microbiological stability of topical rapamycin for facial angiofibromas in tuberous sclerosis, allowing an improvement of therapy.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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ABSTRACT

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Methods : four different 0.4% rapamycin formulations were prepared (ointment, emulsion, gel and liposomes). The stability studies for each formulation were: chemical (extraction with lipophilic solvents and high-performance liquid chromatography assay), physical (pH, uniformity, extensibility, absence of crystals, absence of phase separation and only for liposomal formulation was determined particle size, zeta potential and encapsulation efficiency) and microbiological (culture samples in blood-agar media) during 56 days.

Results : only liposomes were chemically, physically and microbiologically stable after 8 weeks. Ointment, emulsion and gel formulations lost their chemical and physical stability before 56 days.

Conclusions : this study describes a new four formulations to improve the previously treatment for facial angiofibromas in tuberous sclerosis. It also provides favorable stability data only for liposomes. However, more dermokinetic and clinical studies are needed to confirm that liposomes are most appropriate to ensure effectiveness, safety and high patient satisfaction.

TEXT

INTRODUCTION

Tuberous sclerosis (TS) is an autosomal dominant disease, characterised by the multisystem formation of benign non-invasive tumors, called hamartoma and distributed in tissues and organs such as: brain, kidneys, lungs, heart, eyes and skin (1). The most prevalent skin condition in TS are facial angiofibromas (FA), which occur due to an alteration of the mTOR (mammalian target of rapamycin) pathway. This facial injuries suppose an aesthetic and psychological problem (2,3) and multiple treatments have been established for this patients. Physical treatment like radiofrequency, electrocoagulation, cryotherapy, dermabrasion and laser therapy (4) are invasive and painful, and require anesthesia. The interest of mTOR inhibitors focuses pharmacological treatment on everolimus and sirolimus, and recent publications place topical rapamycin (sirolimus) as the most appropriate alternative (5).

We found multiple studies with topical rapamycin in the literature, including four randomized clinical trials (6-9) and all of them provide favorable data of effectiveness and safety. However, high variability between studies in terms of concentration, vehicle, posology, duration of treatment and effectiveness assessment forces to study several formulations to choose the most appropriate.

Several authors have studied different formulations other than the classic formulation (an ointment based on petrolatum) with the aim of achieving a better appearance and consistency of the formulation in order to improve patient compliance with treatment.

Bouguéon et al (10) studied the physico-chemical stability of a cream formulation with a commercialized excipient called Excipial hydrocrème®. Additionally, they used diethylene glycol monoethyl ether P (Transcutol®) as a vehicle and demonstrated that rapamycin was ten times more soluble in Transcutol®(fully soluble, 20.2 mg/ml) compared to liquid paraffin (<2 mg/ml). Guyader et al (11) conducted a physico-chemical and microbiological stability study on a gel-type formulation. Ghanbarzadeh et al formulated rapamycin integrated into a liposomal solution explaining the metodology in development of rapamycin liposomas and additionally studied their chemical stability using a validated high-performance liquid chromatography (HPLC) method (12,13).

Among all the concentrations and dosages reported in the literature, 0.4% concentration, three times per week, it is interesting to avoid exposure to the drug every day and thus minimize possible cutaneus adverse events without compromising effectiveness (14,15).

In addition, the current legal regulations in Spain on magistral formulas establishes that the validity period of non-typified magistral formulas corresponds to the full duration of the treatment (16). Secondly, the Guide to Good Practice of Preparation of Medications in Hospital Pharmacy Services in Spain establishes a validity period of 30 days for ointments and creams, which can be increased if demonstrated with stability studies (17).

The purpose of this study is to improve the classic formulation of topical rapamycin for FA in TS and determine the validity period of the proposed formulations based on chemical, physical and microbiological stability.

METHODS

Materials and reagents

Rapamycin powder (active substance) was provided by Acofarma (Madrid, Spain). The rest of excipients used in the preparation of the formulations were supplied by Guinama (Valencia, Spain). All reagents and solvents were provided by Scharlab (Valencia, Spain).

Formulation and preparation

Galenic optimization of the topical formulation of rapamycin at 0.4% based on its excipients was performed. The most widely used classical formulation in the literature is petrolatum as a vehicle. However, other options were formulated based on previous studies.

Four diferent formulations were developed in biological safety cabinet, all of them with a rapamycin concentration of 0.4% and with differences in their excipients. The resulting formulations were: ointment, emulsion, gel and liposomes (Table 1). Each formulation was packaged in a bottle protected of ambient light and stored in a cold room at $5^{o}C \pm 3^{o}C$.

Chemical stability

Equipment

An Agilent Technologies 1100 HPLC system (Agilent Technologies Inc., Waldbronn, Karlsruhe, Germany) with a quaternary pump, micro-vacuum degasser, autosampler, thermostated column compartment, diode array detector and Agilent Technologies Chemstation for LC 3D Software was used for the analysis.

Chromatographic conditions

To determine the validity period of each formulation according to its chemical stability a HPLC method was developed and validated. A C18 Kromasil column (150 mm x 4.6 mm, 5 μm) was used. The mobile phase consisted of a mixture of acetonitrile and water (75:25 v:v). The flow rate was 1 ml/min. The sample

injection volume was 10 µL, the column temperature was 50ºC and the analysis time was 15 min. Rapamycin detection was processed at 280 nm.

Method validation

The analytical methods were validated according to ICHQ2R1 (International Consensus on Harmonization, 2015) (18). Two calibration curves were developed: one using a mixture of acetonitrile, hexane and water for inyection (WFI) as diluent (for chemical stability of ointment, emulsion and gel formulations), and the other using methanol (for chemical stability of liposomes). The interday and intraday precision and accuracy of the methods was established using six concentration levels (0.025, 0.05, 0.075, 0.1, 0.125 and 0.15 mg/ml) in duplicate on three different days. The least squares method was used to evaluate linearity calculating a regression line between concentrations and peak areas of the chromatogram.

Rapamycin extraction

For the extraction of rapamycin from ointment, emulsion and gel formulations 0.100 g of cream was introduced in a glass tube with 4 ml of a mixture of acetonitrile, hexane and WFI and stayed in the vortex for 10 seconds. The samples were then centrifuged at 3500 rpm for 10 minutes. The resulting supernatant was removed and 1 ml was aliquoted for HPLC analysis. For the extraction of rapamycin from liposomes 0.100 g of cream was introduced in a glass tube with 4 ml of methanol. The samples stayed in the vortex for 10 seconds, 50 µL was aliquoted and after 1/20 dilution with methanol was analyzed with HPLC method.

Rapamycin determination

Rapamycin quantity per weighed formulation quantity (Q, mg/g) and percentage content of remaining rapamycin (%CR) in each formulation was determined by triplicate at times (t) = 0, 2, 7, 14, 21, 28, 42 and 56 days. T_{90} was established when %CR was [?] 90%.

Physical stability

All pertinent procedures to establish the physical stability of the formulations were carried out following the specifications of National Drug Formulary (19) and the Guide to Good Practice of Preparation of Medications in Hospital Pharmacy Services (17).

pH, uniformity, extensibility, absence of crystals and absence of phase separations were evaluated on a transparent surface according to 3 levels: level 1, the least favorable and level 3, the most favorable, at t=0 days for each formulation.

Only for liposomes was determined mean particle size, zeta potential and encapsulation efficiency (EE%) by triplicate at t=0 days. Mean particle size and zeta potential was determined using particle size analyzer which uses laser diffraction method (Malvern Instruments, Worcestershire, UK). The EE% were determined via ultra-filtration, using Amicon® ultra-centrifugal filters (Merck Millipore, Ireland) with a molecular weight cut off of 10,000 Daltons. An aliquot of 500 µL of the liposomal formulation was added to the sample reservoir and centrifuged for 15 min at 14,000 g. Then 50 µL was aliquoted by duplicate and after 1/20 dilution with methanol was analyzed with HPLC method to determine the concentration of free drug in the filtrate. The following equations were used for the calculations(13,20):

Where Wt and Wf represent the total amount of the drug and the free amount of the drug, respectively.

Microbiological stability

Culture samples in blood-agar media of each formulation were incubated at 37°C by duplicate at t=28 and 56 days, according to microbiological control instructions of National Drug Formulary.

RESULTS

Chemical stability

Method validation

Calibration curve for chemical stability of ointment, emulsion and gel formulations demonstrated linearity with a coeficient of determination (r^2) of 0.9998. The limit of detection (LD) and quantification (LQ) were 0.003 and 0.009 mg/ml, respectively. Linearity was determined too for chemical stability of liposomal formulation with a coeficient of determination (r^2) of 0.9958, LD= 0.011 mg/ml and LQ= 0.038 mg/ml.

Rapamycin determination

Table 2 shows the rapamycin quantity per weighed formulation quantity (mg/g) and %CR in each formulation and standard desviation (SD) at times (t) = 0, 7, 14, 21, 28, 42 and 56 days.

T_{90} was reached during the sampling period for the ointment, emulsion and gel formulations: T_{90}=56, 14 and 21 days, respectively. Only liposomes were stable for 56 days.

Physical stability

Table 3 expose the results of the physical stability parameters for the four formulations. The ointment formulation obtained a level 1 score in extensibility property, due to its high petrolatum content, similar to the classical formulation. It should be noted that the emulsion formulation resulted in a pre-separation of phases at t=14 days and a definitive breakdown of the emulsion at t=21 days, therefore it was assigned a level 1 score in absence of phase separations property. The Score of level 1 in the absence of crystals property for the gel formulation, was awarded by the presence of white particles. The mean particle size of prepared liposomes determined a good quality result of the analysis with a polidispersity index value <0.2, indicating a homogenous dispersion. The EE% turned out to be very favorable for the liposomal formulation, with a high load of rapamycin per liposome formed.

Microbiological stability

Cultures samples were negative in blood-agar media for each formulation at t=28 and 56 days (Table 3).

DISCUSSION

This study concludes that only one of the four proposed formulations, liposomes, maintain their chemical, physical and microbiological stability throughout all the sampling time, awarding them a validity period of 56 days.

For the ointment and gel formulations, the chemical stability was the determining factor for not reaching the validity period of 56 days, with T_{90} = 56 and 21 days, respectively. In the case of the emulsion formulation, the physical stability was very obviously compromised after pre-separation of phases at t=14 days and a definitive breakdown of the emulsion at t=21 days. All formulations were microbiologically stable throughout the sampling period. Considering all stability studies, the validity period for each formulation was: ointment=42 days, emulsion=7 days, gel=14 days and liposomes=56 days.

Rapamycin is a highly apolar active substance. This stability study confirms that rapamycin is more comfortable among lipophilic excipients, such as petrolatum and liposomes. In hydrophilic vehicles it tends to precipitate and lose its physical and chemical stability, as in the case of emulsion and gel formulations.

Regarding excipients, transcutol allows a greater solubility of rapamycin in the vehicle (10), thus avoiding possible precipitations of the active principle and constituting a significant improvement in the formulation.

The liposomal formulation has certain advantages over the ointment formulation: it has a better appearance, more comfortable and better extensibility. Historically, some patients complained that classic ointment was difficult to apply (21) and these enhanced organoleptic properties of liposomal formulation would help increase compliance with treatment and patient satisfaction with topical therapy.

Additionally, liposomes have been studied as carriers of molecules for immunosuppressive therapies and chemotherapy (22,23). Liposome-encapsulated rapamycin has been shown to have the ability to reach the drug's site of action more quickly, with antiproliferative properties on T cells (23,24).

Therefore, liposomes exert their influence on the stratum corneum of the skin, improving the penetration of the active substance and providing increased bioavailability of rapamycin. This property makes liposomes particularly effective for cosmetic and pharmaceutical use.

In these therapies, the role of the hospital pharmacists is key in the preparation of the formulations, since rapamycin was added to NIOSH list as a hazardous drug in 2014 (25), which results in greater control in the development of topical formulations (26). In addition, pharmaceutical care has special relevance in the process of dispensing, administration and monitoring, promoting the supervision and control of these patients.

After galenic improvement and confirmation of stability liposomes, more dermokinetic and clinical studies are needed to ensure effectiveness, safety and high patient satisfaction.

STATEMENTS

CONFLICTS OF INTEREST STATEMENT

The authors declare no conflict of interests.

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AUTHOR CONTRIBUTIONS

None

STATEMENT OF HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human and animal subjects performed by any of the authors.

LIMITATIONS

The limitations of this paper lie in what there is significant variability in chemical stability results for all formulations. This is due to the fact that during the preparation of the formulations the correct homogenization of the excipients is very difficult.

In any case, this article provides important new stability data, allowing support in therapeutic decisions for other authors and clinicians.

Table 1. Composition of rapamycin formulations

Formulation	Component	Quantity
Ointment	Rapamycin	0.4 %
	Transcutol®	10 %
	Lanolin	10 %
	Shea butter	20 %
	Vitamin E	1 %
	Petrolatum	q.s. 20 g
Emulsion	Rapamycin	0.4 %
	Transcutol®	10 %
	W/O autoemulsifiable base	20 %
	WFI	q.s. 20 g
Gel	Rapamycin	0.4 %
	Transcutol®	10 %
	Hydroxypropyl methyl cellulose	2 %
	WFI	q.s. 20 g
Liposomes	Rapamycin	0.4 %

Formulation	Component	Quantity
	Transcutol®	3 %
	Liposomal solution	25 %
	Cholesterol	0.1 %
	Isopropile miristrate	10 %
	Propylene glycol	10 %
	Carbopol	2 %
	NaOH 10%	q.s. pH 7.5
	WFI	q.s. 20 g

Q.s.: a sufficient quantity

WFI: water for inyection

Table 2. Chemical stability results

Rapamycin quantity/weighed formulation quantity (Q, mg/g) ± SD

Time (days)	**0**
Ointment	7.21 ± 0.86
Emulsion	9.41 ± 0.28
Gel	8.40 ± 0.01
Liposomes	1.15 ± 0.11
Percentage content of remaining rapamycin (%CR) ± SD	**Percentage content of remaining rapamycin (%C**
Time (days)	**0**
Ointment	100.00 ± 0.00
Emulsion	100.00 ± 0.00
Gel	100.00 ± 0.00
Liposomes	100.00 ± 0.00

SD: standard desviation

Table 3. Physical and microbiological stability results.

Physical stability results: properties at t=0 days	**Ointment**	**Emulsion**	**Gel**	**L**
pH	7.0	6.0	6.0	7.
Uniformity	Level 2	Level 3	Level 3	L
Extensibility	Level 1	Level 2	Level 3	L
Absence of crystals	Level 3	Level 3	Level 1	L
Absence of phase separations	Level 3	Level 1	Level 3	L
Mean particle size, PdI	NA	NA	NA	1
Zeta potential (mV) ± SD	NA	NA	NA	-4
EE% ± SD	NA	NA	NA	8
Microbiological stability results: culture samples at t=28 and 56 days	Negatives	Negatives	Negatives	N

NA: not applicable

PdI: polidispersity index

mV: millivolts

SD: standard desviation

EE: encapsulation efficiency

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