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Research Article

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Forced degradation studies of a new antileishmanial molecule using a stability-indicating RP-HPLC method

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ABSTRACT

A stability indicating RP-HPLC method was developed and validated to study the forced stability of a quinoline-triclosan hybrid, a new agent (Quinoline-triclosan hybrid) for cutaneous Leishmaniasis treatment. The method was developed using a C18 column (250 mm x 4.6 mm, 5 µm) and acetonitrile-water mixture was used as mobile phase. System suitability parameters, linearity, precision and accuracy were determined in the method validation. Quinoline-triclosan hybrid was exposed to various levels of stress conditions including photolytic, oxidative and acid and basic hydrolytic stress. The percentage of degradation in each condition was determined by HPLC. The hybrid was stable at all tested stress conditions with the exception of exposure to light, which was the only factor capable of producing a significant degradation of the molecule. Therefore, it is necessary to protect this molecule from light during development of a dosage form.

Keywords: Cutaneous Leishmaniasis, validation, forced stability, HPLC method, quinoline-triclosan hybrid.

INTRODUCTION

More than 12 million people in the world are affected by Leishmaniasis, which are diseases caused by an intracellular protozoan parasite of the Leishmania gender [1]. Cutaneous leishmaniasis (CL) is the most prevalent clinical manifestation of leishmaniasis causing simple or multiple, localized or disseminated ulcers in the skin, but also in the mucous of mouth, nose and throat cavities. The most affected countries are Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica and Peru that account for at least 70% of global estimated incidence for CL [2].

Currently, the available drugs to leishmaniasis treat include pentavalent antimony compounds (meglumine antimoniate and sodium stibogluconate), miltefosine, pentamidine and amphotericin B. All these drugs, although still effective, are highly toxic and prone to generate resistance in the parasites. Moreover, treatment with these drugs result very expensive for governments, which makes them inefficient considering that major use would be in isolated areas of developing countries where leishmaniasis is extremely associated with poverty [3,4]. Therefore, is necessary to develop more effective and safer drugs.

In a previous study, a series of quinolone-triclosan hybrids were synthesized by Williamson reaction and their in vitro leishmanicidal activity and cytotoxicity were tested. Among these compounds, the hybrid *8-((3-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)propyl)oxy)-2-methylquinoline* or hybrid 19, showed significant activity against intracellular amastigotes of L. (V) panamensis and reduced cytotoxicity on human U937 macrophages [5]. In addition, treatment of golden hamsters (Mesocricetus auratus) experimentally infected with L. (V) braziliensis were

treated an oil-in-water cream formulation with 3% of the hybrid 19 per day during 15 days and produced cure in 6/8 hamsters and relapse in 1/8 hamsters (unpublished data). In order to start the development process of an adequate pharmaceutical formulation of this compound was mandatory to accomplish a forced or stress stability study, enabling the identification of factors that promote the chemical decomposition and the elucidation of degradation pathways [6]. This information becomes an important criterion for choice of excipients, manufacturing process and packaging materials [7].

The forced or stress stability study are performed at conditions more severe than accelerated stability. The ICH Q1A (R2) guide "Stability testing of new drug substances and products" [8] gives only general instructions on forced degradation studies. The selection of the conditions of degradation, such as drug concentration, solvent, temperature, pH, humidity, type and amount of reagent and duration of the study, play a pivotal role in this type of study. These must be higher than those used in studies of accelerated degradation but neither can be too extreme. In this sense, there is not a general consensus among the scientific community, giving rise to a wide range of conditions used in stress stability, which differ from investigator to investigator [9]. For these reasons, several researchers have focused their efforts in providing valid guidelines, looking that the results obtained in conditions of forced stability can be used to predict stability problems under natural conditions [10-12]. Other authors consider certain features of the molecule impede the harmonization of these studies, e.g. decomposition rate and solubility; and therefore is only possible consensus on some general rules as: to select conditions which produce at least 10% of degradation on the compound of interest [7].

To conduct a forced stability study is necessary to development and validate the analytical method to ensure that this method accomplish the requirements needed [12]. Considering that HPLC technique is the most used in stability studies with 65% prevalence [13], this work was aimed to develop and validate a stability-indicating method by HPLC and subsequently characterize the stability behavior of hybrid 19, applying a forced stability study under stress conditions proposed by Singh and Bakshi [11].

EXPERIMENTAL SECTION

Reagents and materials

The hybrid 19 was synthesized via Williamson reaction of *O-triclosan alkyl bromide* and *8-hydroxyquinaldine* [5]. Acetonitrile HPLC LiChrosolv® (Merck Millipore), ortho-phosphoric acid Suprapur® (EMD Millipore), hydrochloric acid 37% Ensure® (Merck), hydrogen peroxide 30% Ensure® (Merck), sodium hydroxide Ensure® (Merck), ethanol LiChrosolv® (Merck), HPLC grade water was obtained from a Synergy® Millipore system.

HPLC instrument

The cromatograph used was a Shimazdu Prominence UFCL with LC-20AD series binary pump systems, SIL-20A HT auto sampler, SPD-M20A diodes array detector and DGU-20A5 degasser and LC Solutions software was used to acquire and process the data. The column used was ZORBAX Eclipse Plus C18 (250mm×4.6mm, 5.0µ).

Chromatographic conditions

The mobile phase consisted of water pH 3.0 (ortho-phosphoric acid 85%) (A) and acetonitrile (B). A gradient method was used varying the solvent B percentage as follows: the initial percentage was 10% and it was maintained during 3.5 min, changed to 70% (3.5-3.55 min), increased to 85% (3.55-12.5 min), and held for 2.5 min (12.5-15 min); finally it decreased to 10% within 2 min (15-17 min) and it kept to 8 min for re-equilibration of the column (17-25 min). Flow rate was 1.0 mL/min. Run time was set to 25 minutes and the detection was carried out at 285 nm, the volume injection of the sample was 20 µL.

Chromatographic method development

Water, acetonitrile, methanol and mixtures of them were used as possible mobile phases or solvents to dissolve the sample. Because of the structural features of the hybrid 19, stationary phases C8, C18 and cyano were tested. Finally, the gradient was adjusted to achieve the best chromatographic parameters.

Validation sample preparation

Samples were prepared from a stock solution of 1.0 mg/mL in acetonitrile and sonicated for 5 minutes until complete dissolution. Appropriate dilutions were performed in the range 20-180 µg/mL using water: acetonitrile 50:50. The samples were filtered through 0.45 µm membrane and dispensed into 1 mL HPLC vials. Chromatographic parameters and system suitability were determined using a degraded sample by photolysis. The concentration sample was 0.1 mg/mL in water: acetonitrile 50:50 and it was irradiated in a solar simulation chamber 1500e ERICHSEN Solarbox, at 700 W/m2 for 6 hours.

The method was validated according to the following parameters: linearity, precision, accuracy, system suitability and stability of the solution. For linearity assay, seven solutions of concentrations between 20.0 and 180.0 µg/mL were prepared in water: acetonitrile 50:50. Two calibration curves per day were done during three consecutive days. All statistical analyzes were performed using the software STATGRAPHIS® Centurion XVI; Grubb's test was applied to determine the existence of outliers and Levene's test to evaluate the variance homogeneity at each level of concentration; the R^2 coefficient was determined and used to identify the best curve fit.

The inter-day precision was determined in five assays with samples sets at three different concentrations (20.0, 100.0 and 190.0 µg/mL) of the compound. This process was reproducing for three consecutive days; the variation degree was determined by the Pearson's Coefficient of Variation. The accuracy was also determined in these sets of samples. The error percentage in each measurement was determined and expressed in relation to 100 %.

To evaluate the sample stability, samples freshly prepared at 100.0 µg/mL in water: acetonitrile 50:50 were injected (t_0); in addition, these samples were dispensed in vials for chromatography and stored during 24 hours and then injected (t_f); the reduction in the chromatogram area was determined and expressed as a percentage.

Forced degradation study

Working samples at 0.3 mg/mL in water: ethanol 2: 3, were prepared from a stock solution at 1.0 mg/mL in ethanol. The samples were subjected to the conditions proposed by Singh and Bakshi and summarized in Table 1 [11]. Photolytic degradation was performed in a solar simulator (Solarbox1500e), equipped with xenon lamp set at 600W/m^2 for 5 hours, following the ICH guide: "Photostability testing of new drug substances and products" [14]. Then, samples subjected to acid and basic hydrolytic degradation were neutralized at pH near to 7.0 to quench the reaction. Acetonitrile was added to samples to achieve a concentration of 120.0 µg/mL and to avoid a possible precipitation. Finally, samples were filtered throughout 0.22 µm membrane filter before injected. The analysis was done in triplicate for each condition of degradation.

Table 1. Drug classification system according to its stability[a]

Category of drug	Acid and basic hydrolysis condition (HCL & NaOH)[b]	Oxidation condition (H2O2)[b]
Practically stable	5N/48 hours/refluxing	30%/48 hours/R.T.
Very stable	2N/24 hours/refluxing	10%/24 hours/R.T.
Stable	1N/12 hours/refluxing	3%/24 hours/R.T.
Labile	0.1N/8 hours/refluxing	3%/6 hours/R.T.
Very labile	0.01N/8 hours/40 °C	1%/3 hours/R.T.
Extremely labile	0.01N/2 hours/25 °C	1%/30 minutes/R.T.

[a] Adapted with permission from S. Singh; M Bakshi [11]

[b] Concentration, time and temperature respectively related for each degradation condition.

R.T. room temperature

RESULTS AND DISCUSSION

Method development

The UV absorption spectra of hybrid 19 in methanol and acetonitrile exhibited an absorbance maximum at 285 nm and a shoulder between 310-330 nm (Figure 1). Thus, HPLC detector was set at 285 nm. The molecule under study is composed by triclosan and 8-hydroxyquinoline, bonded them through an ether linkage, and since triclosan exhibits a characteristic peak at 290 nm [15] and 8-hydroxyquinoline has an absorbance band at 310 nm [16-18], the absorptions observed could correspond to these nucleuses separately.

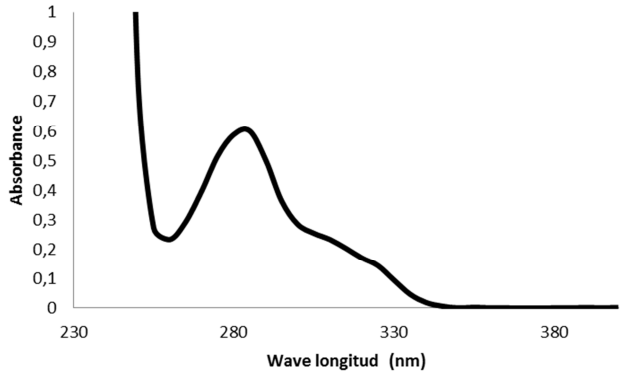

Figure 1: Hybrid 19 UV spectrum in acetonitrile

The effect of stationary phase and mobile phase was evaluated. According to the non-polar nature of the hybrid 19, three types of stationary phase were assayed and results were summarized in Table 2. The C8 column generated short retention time with acetonitrile concentrations upper than 60%, however decreasing proportions of organic solvent, the retention time increased greatly; all peaks showed symmetry problems and was not possible to guarantee the integration of the target peak.

Table 2. Effect of the stationary phase and organic solvent in the mobile phase for the hybrid 19 retention and symmetry in HPLC analysis[a]

Column	Methanol (%)[b]	Acetonitrile (%)[b]	tR	Observations
	80		10	Left asymmetry
	70		27	Left asymmetry
C8		80	3.5	Left asymmetry
		60	6.4	Left asymmetry
		40	45	Left asymmetry
		60	3.4	Symmetric
Cyano		50	6.8	Symmetric
		40	7.3	Symmetric
	90		11	Slight asymmetry
C18	85		11.7	Slight asymmetry
		80	4.6	Left asymmetry
		60	8.9	Left asymmetry
		50	20.5	Left asymmetry

[a] *Concentration at 100.0 µg/mL, flux 1.0 mL/ min, injection volume 20 µL, detection at 285 nm.*
[b] *% of organic solvent in aqueous-organic mobile phase.*

The cyano column resulted in short retention times, even using low amounts of organic solvent and producing overlaping between signals of hybrid 19 and solvent, for this reason this stationary phase was discarded.

The best symmetry factor of target peak was observed with C18 stationary phase and methanol as organic solvent in the mobile phase. The mobile phases: 80% acetonitrile and 85% methanol, used with the C18 column showed similarly strength elution (Snyder's polarity index, P': 6.68 and 6.46 respectively), nevertheless acetonitrile caused smaller retention times indicating higher affinity with the hybrid 19 and therefore requiring small amounts of the solvent to reduce the duration of the method.

The gradient effect was explored using water pH 3.0: acetonitrile as mobile phase and C18 column. 85% phosphoric acid was used to achieve pH 3.0 in mobile phase and thus improve the efficiency of the column. Two samples were used: one freshly prepared solution of hybrid 19 at 100.0 µg/mL, dissolved in water:acetonitrilo 50:50, and onother solution prepared as above described, and subject to irradiation in the solar simulator for 6 hours at 700 W/m^2. Finally, a good resolution of hybrid 19 peak from its degradation products with a shorter time of analysis was achieved applying the gradient method described in the experimental section as chromatographic conditions.

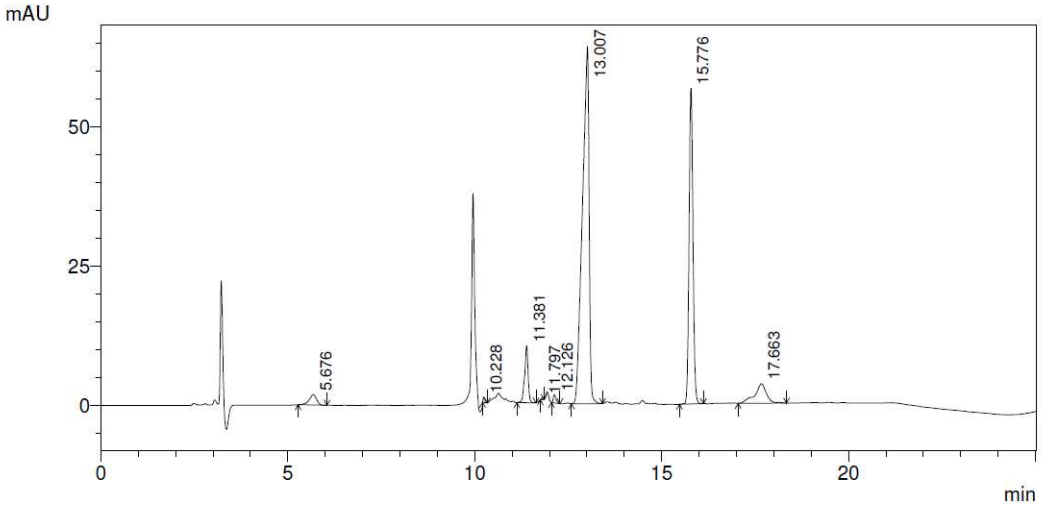

Figure 2: Chromatographic profile of photodegraded solution of hybrid 19. C18 column and water pH 3.0: acetonitrile as mobile phase under gradient

Retention time average of the hybrid 19 was 13.43 ± 0.21 minutes, the capacity factor was 3.196, and the peak area varied less than 1.2% in system proficiency testing, tailing factor was 0.66 and theoretical plates for the molecule

was 12,616, the hybrid 19 peak resolution value was 3.843. The above calculations were performed using the chromatographic analysis software with dead time value of 3.1 min and 250 mm as column length.

Figure 2 shows the chromatographic profile of photo-degraded sample, where seven degradation products are observed at retention times of 5.6, 10.2, 11.4, 11.8, 12.1, 15.8 and 17.7 min. Degradation compounds did not interfere with the identification and quantification of the hybrid 19, suggesting that the method is indicative of stability. The peak at 9.7 minutes was generated by the gradient method and it was considered as a base line perturbation.

The UV spectra suggested the formation of a degradation compound containing both triclosan and 8-hydroxyquinoline nucleus (t_R15.8 min), other are formed by ether bond cleavage of hybrid 19. Peaks at 10.2, 11.4, and 12.1 min showed absorbance spectra attributed to the triclosan nucleous, while peaks at 5.6 and 17.7 min presented the absorbance attributed to 8-hydroxyquinoline.

Method validation

The absence of outliers in the linearity of the method determined by Grubb's test at significance level of 5% allowed working with 42 data from six regressions. The verification of the homogeneity variance between seven concentration levels was performed using Levene's test (95% of confidence). Results showed that the variance in the analyzed levels was heteroscedastic; due to this result, a weighted linear regression was done to reduce the error in the estimated concentration; the model: Area=3465.4+11538.3*concentration, presented a R^2 = 99.4%. This was the best fit found.

Sample stability was also tested in the assay conditions. It was found a reduction in integration area was less than 1% in samples prepared and stored 24 hours prior to HPLC analysis. These results suggested that it is possible to make injections with samples prepared up to 24 hours in advance.

Results obtained for precision and accuracy of the method were summarized in Table 3. The variation coefficient for intra- day assay varied between 1.25% and 1.42 %, while the variation coefficient in the inter-day assay had an average value of 2.49%. These values are within the ranges permitted for analysis methods of pharmaceutical products and raw materials [19]. The accuracy evaluated at three concentration levels was 102.5 %, indicating that the method tends to deliver 2.5% more values than the real value of the molecule concentration.

Table 3. Precision and accuracy of chromatographic method

	Intra-day precision		**Inter-day precision**		**Accuracy**
Concentration (µg/mL)	Mean* ± SD	Coefficient of variation	Mean* ± SD	Coefficient of variation	% EE+100
20.16	20.26 ± 0.29	1.42	20.06 ± 0.9	4.15	99.5
100.8	104.62 ± 1.31	1.25	104.29 ± 1.25	0.95	103.5
191.52	195.99 ± 2.66	1.36	200.14 ± 8.39	2.36	104.5

** n= 5*

Forced degradation study

Although is recommend carry out stress stability test with 1.0 mg/mL solution of the active ingredient [8,13]; due to the low aqueous solubility of the hybrid 19, a solution with a lower concentration was prepared and it was necessary a binary mixture of water:ethanol 2:3 as solvent, being the maximum amount of water allows to dissolve 0.3 mg/mL of the hybrid 19, whereby it prevented that the active ingredient and the reagents precipitated in the reaction medium. Because approximately 70% of active ingredients discovered in last years have low solubility in water [20], the concentration and the dissolution medium are current problems in forced stability studies. One possibility to overcome this difficulty is working with samples as suspensions [7], however in this approach the solution was used because solid state stability is greater. In preliminary tests, it was found that acetonitrile as solvent promoted degradation reactions of hybrid 19, therefore this was replaced by ethanol.

The degradation percentages for each stress conditions evaluated were summarized in Table 4. In this study, a sufficient degradation percentage is more than 5% [6]. In acid condition aln sufficient degradation was achieved with the following conditions: 2 N HCl / 24 hours / reflux. These conditions corresponds to a very stable molecule. The low solubility of sodium hydroxide in ethanol was found as complication in basic degradation. A sufficient degradation was accomplished with 1 N NaOH / 12 hours / reflux. All together these results suggest that the hybrid 19 may be classified as stable in basic conditions. On the other hand, when the hybrid 19 was exposed to 10% H_2O_2 during 24 hours at room temperature, a sufficient degradation was reached, indicating that the hybrid 19 was very stable against oxidizing agents.

As is showed in Table 4, a sufficient degradation was found under photolytic conditions. In agreement with the ICH Q1B guideline "Stability testing: Photostability testing of new Drug Substances and products" [12] the hybrid 19 is classified as photolabile.

Table 4. Hybrid 19 degradation against stress conditions[a]

Type of degradation	Conditions	Degradation percentage[b]
Acid degradation	HCl 0.1N/Reflux/8h	2.45 ± 0.37
	HCl 1N/Reflux/12h	3.03 ± 0.46
	HCl 2N/Reflux/24h	22.15 ± 0.45
Basic degradation	NaOH 0.1N/Reflux/8h	3.17 ± 0.40
	NaOH 1N/Reflux/12h	8.18 ± 0.76
Oxidative degradation	H_2O_2 3%/R.T./6h	1.21 ± 1.09
	H_2O_2 3%/R.T./24h	3.39 ± 0.55
	H_2O_2 10%/R.T. /24h	15.03 ± 1.51
Photolytic degradation	Exposition> 200 W-h/m2	8.97 ± 0.67

[a] concentration of sample subject to decomposition: 0.3 mg/mL
[b] mean ±sd, n=3
R.T. room temperature

CONCLUSION

The *8-((3-(5-chloro-2-(2,4-dichlorophenoxy)phenoxy)propyl)oxy)-2-methylquinoline* can be exposed to condition such as hydrolysis and oxidation but should be protected from light, because this can produce a considerable degradation of the active ingredient. Therefore, is necessary to implement strategies to prevent photolysis during the process of formulation and storage.

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