

A water-soluble prodrug of cyclosporine A for ocular application: A stability study

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Abstract

UNIL088 is a water-soluble prodrug of cyclosporine A (CsA) developed for topical eye delivery. Such a prodrug has to fulfil two paradoxical requirements as it must be rapidly hydrolysed under physiological conditions but also retain a long shelf-life in aqueous media. This study has been conducted to explore the stability of UNIL088 formulated as an eyedrop solution. The stability study of the prodrug was performed over a pH range of 5–7 at 20 °C and at various ionic strengths. The molecule was more stable at pH 5 than at pH 7 with conversion rate constant of 3.2×10^{-3} and 26.0×10^{-3} days⁻¹, respectively. The effect of temperature was studied at four different temperatures and activation energy was determined. Conversion of UNIL088 followed a pseudo-first-order kinetic with an activation energy of 79.4 kJ mol⁻¹. Due to its low solubility, CsA generated precipitated in the solution. The average size of CsA precipitates, determined by photon spectroscopy, was 0.22 and 1.08 μm at 7 and 14 days, respectively. The hydrolysis mechanism was partially elucidated by identification of the intermediate pSer-Sar-CsA. © 2005 Elsevier B.V. All rights reserved.

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1. Introduction

Cyclosporine A (CsA) is a powerful immunosuppressive drug, now routinely used in the prevention of graft rejection, the treatment of several autoimmune diseases as well as in the treatment of some parasitic diseases (Klyashchitsky and Owen, 1998). CsA is also used in ophthalmology for the prevention of corneal graft rejection and for the treatment of conditions with an immune component, such as categories of dry eye syndromes and uveitis. However, the poor solubility of CsA in water is a limiting factor for the formulation of solutions intended for ocular administration. Considerable efforts have been made to improve the availability and the tolerance of topically applied CsA, but to-date, none of the

delivery systems developed have been fully satisfactory (Lallemand et al., 2003). An emulsion of CsA, Restasis® (Allergan Inc., Irvine, CA), is now on the U.S. market (Sall et al., 2000). However, a drawback of topical emulsions is their poor ocular tolerance. To overcome the unfavourable properties of CsA, the concept of a water-soluble and enzyme activated prodrug was employed by synthesizing UNIL088, a double ester prodrug of CsA (formula shown in Fig. 7) (Wenger et al., 2002). The synthesis of ester prodrugs is a commonly used approach due to the frequent predominance of carboxylic and hydroxyl substitutes in drug molecules along with the availability of the enzymes in living organisms able to hydrolyse them. However, the major drawback of esters is their susceptibility to hydrolysis in aqueous solutions. Hence, an ideal ocular prodrug has to fulfil two paradoxical requirements, i.e., a rapid in vivo conversion into the parent drug (CsA) and a sufficient stability in the for-

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mulation. The aim of the present study was to determine the optimum storage and administration conditions of an eyedrop formulation of UNIL088. Since the prodrug was demonstrated to be very rapidly hydrolysed in the presence of tears (Lallemand et al., 2005), stability studies in phosphate buffer solutions (PBS) were performed as function of pH (range of 5–7), temperature (from 4 to 60 °C) and ionic strength over 14 days. Shelf-lives ($t_{90\%}$) and activation energy (E_A) of UNIL088 were determined. As CsA released from UNIL088 is poorly soluble in aqueous media, its precipitation process was followed by photon correlation spectroscopy. Finally, the chemical conversion mechanism was investigated by identifying an intermediate compound by high-performance liquid chromatography coupled with a mass spectrometer (HPLC–MS).

2. Materials and methods

2.1. Materials

UNIL088 was synthesized and characterised by the Institute of Chemical Sciences and Engineering (ISIC), Ecole Polytechnique Fédérale de Lausanne (EPFL), Switzerland according to a method described by Wenger et al. (2002). Phosphate buffer solutions (PBS) at 0.066 M used monopotassium phosphate (KH_2PO_4), disodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) and were made isotonic with sodium chloride (NaCl). These products, together with trifluoroacetic acid (TFA) were of analytical grade and were obtained from Fluka (Buchs, Switzerland). Water and acetonitrile (ACN) were of analytical grade (SDS, Peypin, France).

2.2. Solubility assessment

Aqueous solubility was evaluated at room temperature by adding UNIL088 until saturation to isotonic PBS (pH 5–7). The solubility of CsA was similarly determined in PBS pH 7. The resulting suspensions were filtered through 0.22 μm membranes (Millex-GV; 13 mm; 0.22 μm ; low protein retention, Millipore, Cork, Ireland). Filtrates were collected and the solubilised molecules were quantified spectrophotometrically at 210 nm (Spectrophotometer HP 8452 Hewlett-Packard GmbH, Waldbronn, Germany).

2.3. Chemical stability

All solutions of UNIL088 were prepared at a concentration equivalent to 0.2% (w/v) in CsA. The influence of pH on the transformation of UNIL088 was investigated in isotonic PBS (0.066 M) at pH 5–7 at 20 °C over 2 weeks.

The influence of ionic strength (I) was studied by incubating UNIL088 in NaCl solutions at concentrations of 0.4, 0.9 and 1.4% (w/v) with an I of 0.068, 0.154 and 0.240 M, respectively, without pH adjustment (pH \sim 5). To investigate the effect of temperature on stability, UNIL088 was incu-

bated at 4, 20, 40 and 60 °C in PBS at pH 7. Aliquots of 2 μl were sampled from each of these solutions at determined time intervals and diluted in 20 μl of ACN plus 55 μl of water. Each solution was then assayed for prodrug content by HPLC.

2.4. Physical stability

2.4.1. Determination of particle size distribution

The mean size \pm S.D. and polydispersity index (P.I.) of precipitates were measured by photon correlation spectroscopy using a Zetasizer[®] 3000HS (Malvern instruments Ltd., Worcestershire, UK). The P.I. values can range from 0 to 1; a higher value in this range indicates a less homogeneous particle size distribution. Measurements were made in triplicate every 2 days during 2 weeks.

2.5. HPLC assay

CsA and the prodrug were analysed and quantified by HPLC. The method was developed specifically to quantify in the same run hydrophobic CsA and hydrophilic prodrug. Analytical separations were conducted using a C4 column (300 Å, 5 μm , 4.6 mm i.d. \times 250 mm, type 214TP54, Vydac, Hesperia, California). The mobile phase contained ACN as organic modifier and acidified water (0.09%, v/v TFA). An organic gradient (60–100% ACN) over 15 min using volumetric mixing by the HPLC pump (W600 controller and multisolvent delivery pump, Waters, MA, USA) was used to separate components. The flow rate was set at 0.8 ml/min and the column oven at 40 °C. Seventy microliters of sample were injected via an automatic injector (W717 plus Autosampler Waters, MA, USA). The absorbance was measured at 210 nm (W2487 Dual λ Absorbance Detector, Waters, MA, USA). Millennium[®] 32 chromatography manager software (Version 3.2) was used for peak integration. The analyte peak was compared to the total peak area and was expressed as a percentage. The limit of quantification was estimated using the signal to noise ratio approach ($S/N = 10$) and confirmed by injections of an independent standard sample at a concentration of 2 $\mu\text{g ml}^{-1}$. Under these conditions, UNIL088 and CsA were separated with retention times (RT) of 10.50 and 12.50 min, respectively.

2.6. Characterisation of compounds by HPLC–MS

The HPLC method, described above, was coupled with a quadrupole mass spectrometer SSQ 7000 Finnigan MAT (Thermo Electron Corporation, TX, USA). Electrospray ionisation mode (ESI) was used at a capillary temperature of 200 °C in positive ion mode. Sheath gas (N_2) pressure was at 3.5 bar and spray voltage at 4.5 kV. Skimmer pump pressure was 950 mTorr, capillary voltage of approximately +5 V. Data acquisition was performed between $m/z = 30$ and 2500 at a scanning speed of 2 s.

2.7. Data analysis

The data obtained were plotted as natural logarithm (\ln) of the percentage of the prodrug remaining versus time. The observed conversion rate constants (k_{obs}) of UNIL088 were calculated from the slope of the linear fits of the experimental data. The shelf-life ($t_{90\%}$, i.e., the time at which 90% of the original amount of UNIL088 is still present) was obtained from the k_{obs} values. The activation energy (E_A , i.e., the minimum amount of energy required to ensure that a reaction occurs) was obtained by the calculation of k_{obs} at 4, 20, 40 and 60 °C, with further fitting to the Arrhenius equation:

$$\ln(k_{\text{obs}}) = \ln Z - \frac{E_A}{RT} \quad (1)$$

where E_A is the activation energy (kJ mol^{-1}), T the absolute temperature (K), R the gas constant and Z is a constant. $-E_A/RT$ is the slope of the linear plot from which E_A is determined. $\ln Z$ is the intercept with the y -axis. The majority of chemical reactions have an E_A between 40 and 130 kJ mol^{-1} ; an E_A below 80 kJ mol^{-1} indicates that the system will react at room temperature.

3. Results

The solubility of UNIL088 in isotonic PBS pH 7 is approximately 25,000 times higher than that of CsA, being respectively, $128.28 \pm 0.07 \text{ mg ml}^{-1}$ and $0.0052 \text{ mg ml}^{-1}$. At pH 6 and 5, solubilities in PBS were, respectively, 115.00 ± 0.02 and $92.33 \pm 0.01 \text{ mg ml}^{-1}$ indicating that the solubility of the prodrug increases with increasing pH.

In PBS, UNIL088 underwent a pH sensitive hydrolysis leading to a quantitative generation of CsA. The conversion of UNIL088 as a function of time is represented in Figs. 1 and 2. At pH 7, the observed conversion rate constant, k_{obs} , is $26.0 \times 10^{-3} \text{ days}^{-1}$ while at pH 5 it is approximately eight times lower ($3.2 \times 10^{-3} \text{ days}^{-1}$) (Table 1). Likewise, the hydrolysis process is affected by temperature, as the conversion rate increased with higher temperatures, with $k_{\text{obs}} = 5.9 \times 10^{-3}$ and $978.1 \times 10^{-3} \text{ days}^{-1}$, respectively, at 4 and 60 °C (Table 1). At 20 °C and pH 7, the shelf-life of

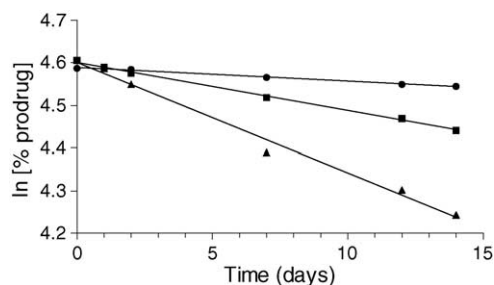


Fig. 1. Semi-logarithmic representation of percentage of UNIL088 vs. time (days) when incubated in PBS pH 5 (●), 6 (■) and 7 (▲) at 20 °C.

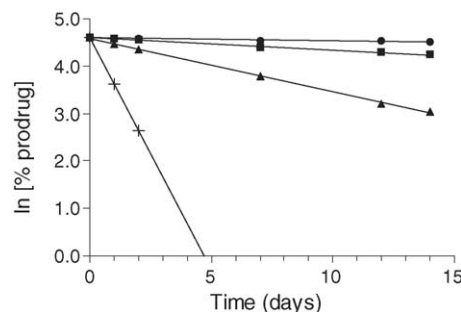


Fig. 2. Semi-logarithmic representation of percentage of UNIL088 vs. time (days) when incubated in PBS pH 7 at 4 (●), 20 (■), 40 (▲), 60 °C (+).

Table 1

Conversion rate constants k_{obs} and shelf-lives $t_{90\%}$ of UNIL088 in solution at various pH and temperature conditions

Temperature (°C)	pH of PBS	k_{obs} ($\times 10^{-3} \text{ days}^{-1}$)	$t_{90\%}$ (days)
4	7.0	5.9	17.90
20	5.0	3.2	32.93
	6.0	11.3	9.32
	7.0	26.0	4.05
40	7.0	112.7	0.93
	7.0	978.1	0.11

UNIL088 ($t_{90\%}$) is 4 days and is extended up to 18 days at 4 °C. The activation energy determined from the Arrhenius relationship (Fig. 3) is 79.4 kJ mol^{-1} at pH 7 ($R^2 = 0.9962$). Ionic strength had no significant effect on the rate of conversion (data not shown).

When the pH 5 solution (20 °C) was analysed by HPLC, the chromatogram showed a peak at 7.20 min (Fig. 4) which was not detected at pH 6 and 7. This compound was apparent from the beginning of the incubation of UNIL088 and its concentration increased with time. It is more hydrophilic than UNIL088 since it was less retained by the C4 column. Its molecular mass m/z was 1441 (Fig. 5). This mass corresponds to the Serine-Sarcosine-CsA phosphate intermediate (pSer-Sar-CsA, see Fig. 7).

After 1 week, the solution at pH 7 started to change colour and to become turbid, due to the precipitation of CsA (concentration of CsA $\sim 0.4 \text{ mg ml}^{-1}$), with large visual precipitates observed at 2 weeks. Other solutions at 20 °C remained clear

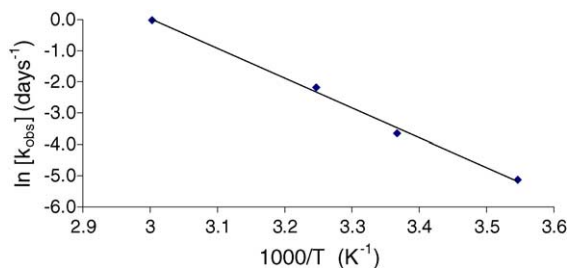


Fig. 3. Arrhenius plots of logarithm of k_{obs} as determined at 4, 20, 40 and 60 °C.

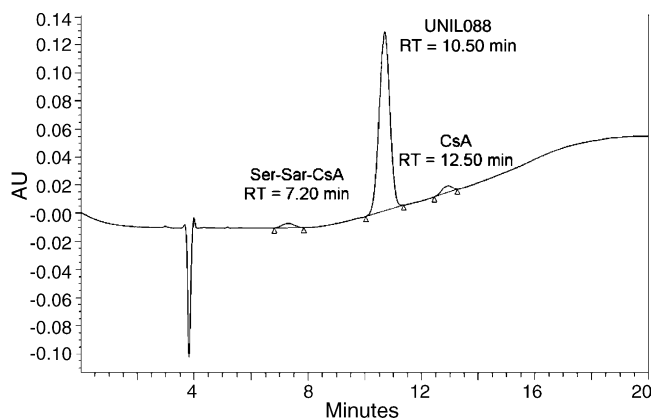


Fig. 4. HPLC chromatogram of UNIL088 solution incubated at 20 °C in PBS pH 5.

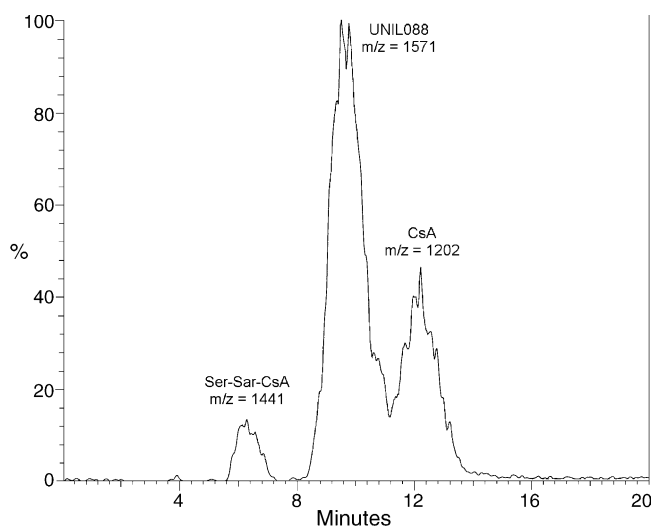


Fig. 5. Mass spectrometer chromatogram of UNIL088 solution incubated at 20 °C in PBS pH 5.

over the 2 weeks. Photon microscopy measures of the precipitated solution revealed a three-phase process resulting in the growth of CsA precipitates. Small precipitates of an average size of 49.2 ± 2.7 nm appeared 1 day after preparation (2.5% of UNIL088 hydrolysed). The precipitates grew slowly during a first phase of 7 days (Fig. 6). At days 7–8, the particles underwent a dramatic size increase to reach a plateau at day

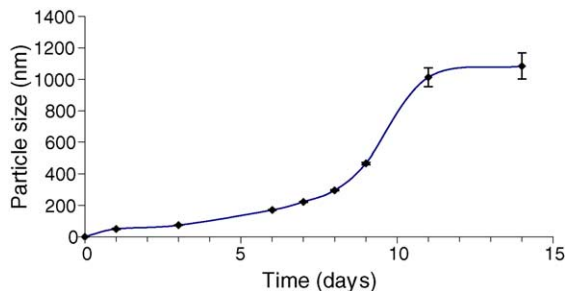


Fig. 6. Mean size \pm S.D. of CsA particles as a function of time in a PBS pH 7 solution of UNIL088 at 20 °C.

11 of a mean size of 1012.8 ± 59 nm with a P.I. of 0.44 and 21% of particles above 2 μ m. This sudden increase appeared when 20% of the prodrug is converted. The higher values of P.I. and the higher standard deviations observed from day 8 indicate a less homogeneous distribution of the particle sizes.

4. Discussion

The increased solubility of the prodrug UNIL088 in aqueous media is mainly due to the phosphate group of the pro-moiety. In neutral solution this group is fully ionised, increasing both polarity and aqueous solubility of the molecule. Depending on pH, the phosphate monoester exists either as the monoanion or the dianion (except at very low pH values where the neutral species exists). The phosphate group was chosen because it is ubiquitous in living organisms and presents a good biocompatibility.

An ideal ocular prodrug must release the active parent molecule very rapidly after topical administration as 90% of the instilled dose is usually removed from ocular surface within 4 min (Lee, 1993). In addition, the prodrug must have adequate chemical and physical stability, especially in ready-to-use eyedrop solutions. The present study was conducted in order to estimate the shelf-life, conditions and limitations of use of UNIL088 in aqueous solution.

Biocompatible conditions ranging from pH 5 to 7 have been investigated. The conversion profile of UNIL088 is consistent, in the pH range investigated, with other degradation studies of esters. It is well established that the degradation of esters follows a V-shaped pattern when represented as $\ln(k) = f(\text{pH})$ (Patel et al., 1968; Gogate et al., 1987). At low pH ($\text{pH} \sim 1$), $\ln(k)$ is higher due to acid ester hydrolysis, while $\ln(k)$ is lowest at pH between 4 and 5 due to increased stability. At alkaline pH, $\ln(k)$ increases dramatically as esters undergo alkaline hydrolysis. UNIL088 seems to follow such behaviour, as it is more stable at pH 5 than at pH 7. Since the hydrolysis is pH dependent, several chemical catalysers, such as water, hydrogen ion, hydroxyl ion or phosphate ion can contribute to the observed hydrolysis. It is supposed that with the presence of a buffer maintaining constant concentrations of these ions, their contributions to hydrolysis remain constant and are combined together to yield k . At $\text{pH} \geq 5$ the ester hydrolysis is mainly hydroxide ion-catalysed. The speed of conversion can be expressed as:

$$\frac{dC}{dt} = k[\text{OH}^-][\text{UNIL088}] \quad (2)$$

where k is the conversion rate constant and $[\text{UNIL088}]$ is the molar concentration of UNIL088. As pH is constant, $[\text{OH}^-]$ is assumed to be constant; such hydrolysis is considered to be of pseudo-first-order and Eq. (2) can be rewritten:

$$\frac{dC}{dt} = k_{\text{obs}}[\text{UNIL088}] \quad (3)$$

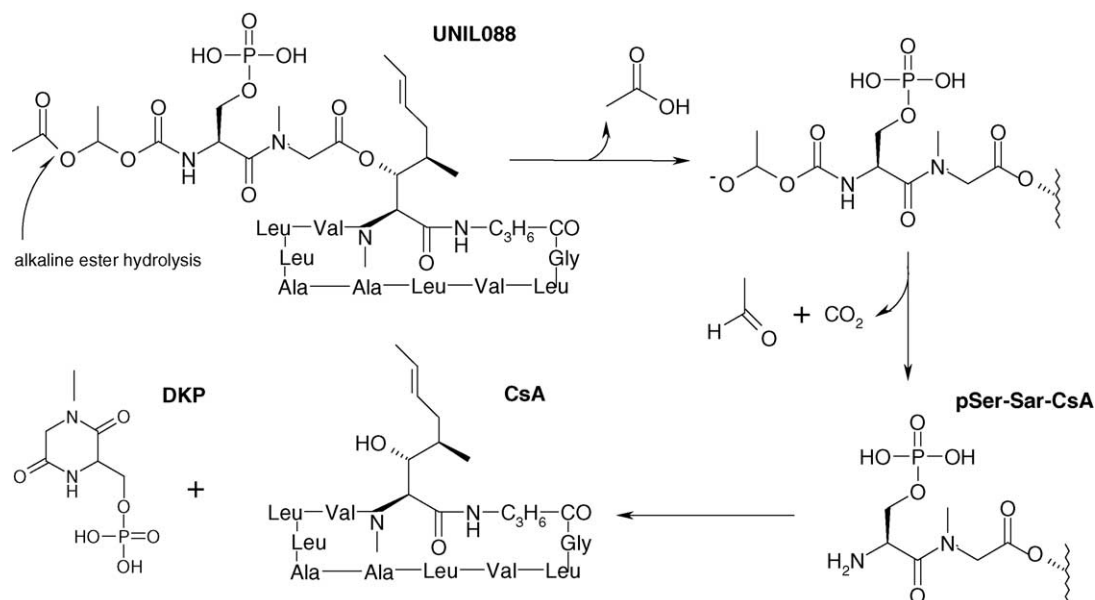


Fig. 7. General structure of UNIL088 and the proposed mechanism leading to the release of CsA under chemical hydrolytic activation.

where $k_{\text{obs}} = k[\text{OH}^-]$. The reaction order is confirmed by the linear profiles of the plotted $\ln[\text{UNIL088}]$ as a function of time (Figs. 1 and 2).

Since CsA has a very low solubility in PBS pH 7 ($5.2 \mu\text{g ml}^{-1}$), it will reach its maximal solubility and start precipitating when 0.2% of the initial UNIL088 content (2.6 mg ml^{-1}) undergoes conversion. Hence, the precipitation of CsA is expected to occur before the calculated $t_{90\%}$ of UNIL088 is reached (4 days). Using the k_{obs} determined at pH 7 and 20°C , one can calculate that the time for CsA to start precipitating is 1 h 50 min. However, no precipitate or changes in the colour of the solution were observed before 7 days. Photon microscopy measures showed very small particles 1 day after preparation of the solution indicating that the precipitation commences very early as predicted by calculation. Precipitation is a progressive nucleation and crystal growth phenomenon, confirmed by the increasing size of the particles with time. The dramatic increase observed at days 7–8 is probably due to an agglomeration of small crystals in large agglomerates as confirmed by the low homogeneity displayed by the P.I. value. The European Pharmacopoeia (fourth edition) allows the presence of particles in collyria (in a sample corresponding to $10 \mu\text{g}$ of solid phase, 20 particles can be larger than $25 \mu\text{m}$, only 2 larger than $50 \mu\text{m}$ and none above $90 \mu\text{m}$). As the average size at 4 days ($t_{90\%}$) is around 100 nm , the solution fulfils the Pharmacopoeia requirements in term of particles size. $t_{90\%}$ can, therefore, be safely used as an indicator of expiry date. The low E_A of UNIL088 (below 80 kJ mol^{-1}) indicates that it is labile in aqueous solution at room temperature and hence that storage at 4°C would be preferable.

The mass spectrometry investigation in PBS pH 5 clearly identified the intermediate pSer-Sar-CsA, confirming part of the supposed transformation mechanism presented in

Fig. 7. In presence of hydroxide ion, the (acyloxy)alkyl-oxy-carbonyl group, at the end of the solubilising moiety, underwent a nucleophilic substitution of the carbonyl to release acetic acid and an unstable carbamic acid derivative. This derivative loses CO_2 and releases the dipeptide phosphate CsA: pSer-Sar-CsA. The next step is not confirmed yet and remains hypothetical. The terminal amine of the pSer-Sar-CsA may attack the ester connecting the promoiety to CsA via an addition–elimination reaction. The released compounds would then be CsA and diketopiperazine phosphate (DKP) (diketopiperazine phosphate has not been isolated but the mechanism has been described by Hamel et al. (2004)). In acidic pH, the terminal amine of the intermediate pSer-Sar-CsA should be partly or completely protonated. The addition–elimination reaction of the amine on the ester, necessary to release the pSer-Sar dipeptide in this condition is occurring very slowly and the intermediate pSer-Sar-CsA can be isolated and identified by HPLC–MS, explaining the accumulation this compound in acidic solution.

5. Conclusions

The stability of an eyedrop solution is of great importance for its efficacy. However, it should be noted that stability issues can be less critical for prodrugs compared to conventional drugs as the compound generated from the prodrug is the therapeutic molecule. Hence, CsA can be present in the solution as long as it does not alter the favourable properties of the prodrug solution. CsA is itself very stable due to its lipophilic nature and its cyclic structure (Kumar et al., 2001). It was shown to be stable over 7 days at room temperature and up to 28 days at 4°C when formulated at 1% in artificial tears (Fiscella et al., 1996). Although UNIL088 has a longer

shelf-life at pH 5 than at pH 6 and 7, a neutral pH must be preferred for the formulation because of its biocompatibility. As the hydrolysis mechanism is temperature-dependent the stability of the solution is prolonged at 4 °C. Besides chemical stability of the prodrug, physical stability must also be monitored carefully due to the risk of precipitation. Complete elucidation of the conversion mechanism is currently under investigation, as well as improvement of the stability by several approaches.

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