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Influence of temperature and shaking on stability of insulin preparations: degradation kinetics

Alexis Oliva, José B. Fariña*, Matías Llabrés

Dpto. Ingeniería Química y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de La Laguna, 38200 La laguna, Tenerife, Spain

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Abstract

Two commercial human insulin preparations were stored at different conditions in order to study the influence of temperature and shaking on their stability. Deamidation was the predominant degradation process in the case of the suspension, while dimerization prevailed for the solution, mainly when shaking was applied. The formation process of desamido B3 insulin follows first order kinetics; the dimer formation fit better the Prout-Thompkins nucleation model. Results obtained for suspension confirm the suitability of Arrhenius plot for both storage conditions. However, in the case of solution, the Arrhenius relationship was only valid when it was stored without shaking, since with shaking no linearity was observed in the temperature range studied.

Keywords: Insulin; Degradation; Arrhenius; Stability; Kinetics; HPLC

1. Introduction

Several authors have studied the suitability of the Arrhenius relationship in different chemical pathways of peptides and protein degradation, such as deamidation (Patel and Borchardt, 1990a), hydrolysis (Helm and Müller, 1991; Motto et al., 1991), racemization (Friedman and Masters, 1982) and polymerization (Brange, 1992). However, one of the main problems found was to determine the suitable temperature range for stability studies, where the Arrhenius plot would be valid.

The stability predictions based on the Arrhenius relationship are inappropriate for protein preparations which exhibit complex degradation mechanisms and, in some cases, the degradation mechanisms of this type of drug could change as a function of temperature (Gu et al., 1991). Extrapolation of stability data obtained with protein drugs should be limited to the temperature range

* Corresponding author. Tel.: + 34 922 603622; fax: + 34 22 630095; e-mail: jfarina.@ull.es

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where the degradation mechanisms are the same and not susceptible to unfolding. Temperatures have been proposed as valid below 40°C (Pearlman and Nguyen, 1992). However, other authors have used temperatures over 40°C, for example, Brange et al. (1992a) used the interval from 4 to 45°C for insulin stability studies and Yoshioka et al. (1994) used temperatures between 40 and 70°C for α-chymotrypsin.

Many proteins follow a first-order degradation kinetics, i.e interleukin 1β (Gu et al., 1991), LH/RH (Motto et al., 1991), secretin (Tsuda et al., 1990), histrelin (Oyler et al., 1991), α-chymotrypsin (Yoshioka et al., 1994) and insulin (Brange et al., 1992a); others follow a pseudo first-order degradation kinetics, for example, the degradation of ACTH (Motto et al., 1991). Insulin, followed a first order degradation kinetics (Brange et al., 1992a), being deamidation and polymerization the main degradation pathways (Brange et al., 1992b; Darrington and Anderson, 1995). Brange et al. (1992b) carried out an accelerated stability assay in the temperature range of 4–45°C with the objective of obtaining information on the Arrhenius behaviour using several types of insulin pharmaceutical preparations.

Arrhenius plot for deamidation and polymerization of insulin in commercial preparations was only valid at certain temperatures. At temperatures above 30°C, the rate of deamidation increases more than expected from the Arrhenius equation, because the formation of reactive intermediate is facilitated. Morever, the temperature-dependence of the dimerization and polymerization reactions depends on the type of preparation and the strength and species of insulin (Brange et al., 1992b).

Brange (1992) obtained from the Arrhenius plot, a value of activation energy (E_a) for the deamidation reaction of 19.2 kcal/mol in neutral insulin solutions containing NaCl and methylparaben at temperature intervals of 4–25°C, and 25.3 kcal/mol in neutral insulin solutions with glycerol and phenol at a temperature interval of 25–45°C. These values are in good agreement with similar data from the literature (Geiger and Clarke, 1987; Patel and Borchardt, 1990b). On the other hand, for the covalent di and polymerization of insulin in neutral solution, they obtained, a temperature range of 25–45°C, E_a value of 28.4 kcal/mol. In other pharmaceutical preparations, E_a cannot be estimated because of a lack of linearity in Arrhenius plots at the temperature range studied (Brange, 1992).

The aim of our study was to investigate the kinetics of insulin transformation products in solutions and suspensions during storage of these pharmaceutical preparations at temperatures from 20 to 60°C to determine the influence of shaking in the degradation process and on the other hand, to verify the validity of Arrhenius plot to estimate the expiration date of studied preparations.

2. Materials and methods

2.1. Chromatography system

The HPLC system was a Water apparatus consisting of a pump, model 600E Multisolvent Delivery System, a UV-Vis detector, a model 490E Programmable Multiwavelength Detector and data aqusition sofware, Maxima 820. Deionized water prepared with a MilliQ apparatus (Millipore Waters) was used throughout; all other chemicals and reagents were HPLC grade. All solvents were filtered with 0.45 μm (pore size) filters (Millipore).

2.1.1. RP-HPLC

A reversed-phase C-18 column (Delta-Pack, 300 Å, Waters) and a mix 74:26 of 0.2 M sodium sulphate buffer (pH 2.3) and acetonitrile as the eluent at a flow rate of 1.0 ml/min at room temperature and detection at 214 nm was used (Farid et al., 1989, modified).

To validate the analytical method, seven standard solutions were prepared using human insulin (Batch: H01003, Novo biolabs) at concentrations of 2–8 μg/ml. Each sample was replicated four times. The analysis of variance (ANOVA) of linear regression confirms the linearity of the method used through the rejection of the null hypothesis of deviation of the linearity for a significative level of 0.05 ($\alpha = 0.05$); the coefficient of variation of predicted concentrations was equal to 2.6% (Hunter and Lamboy, 1981).

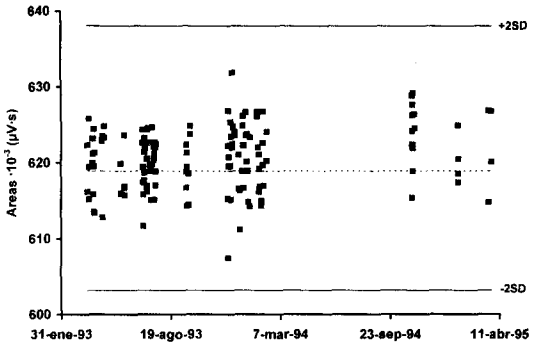


Fig. 1. Control chart corresponding to the RP-HPLC system.

To calibrate the RP-HPLC system, we analyzed an insulin solution sample daily, as standard. Insulin solution, used as standard, was stored at between 2 and 8°C for 2 years. Insulin solution (100 μl) was diluted with 0.05 M HCl to obtain a final concentration of 7.27 μg/ml Each work day, the standard was analyzed to determine the peak area. The estimated area value for standard concentration was 618 901 and the historical C.V was equal to 1.41%. The upper and lower limits for the control chart were established at ± 2 S.D. of this value, taking as standard deviation the value obtained from variance of analytical method. Fig. 1 shows the control chart corresponding to the RP-HPLC system.

In the case of pharmaceutical preparations, 1 ml homogeneous samples were withdrawn from vials and samples were stored at 0°C until analysis. Insulin suspension samples were isolated by centrifugation, 4000 r.p.m. for 20 min (Econospin, Dorvall Instruments Du Pont). Supernatants were removed and the dry residues were diluted with 0.05 M HCl. Samples containing insulin in solution were diluted with 0.05 M HCl to obtain concentrations values within the calibration range.

2.1.2. SE-HPLC

SE-HPLC method proposed by Brange et al. (1992b) was used. A Protein Pack C-18 column (Waters) and using an eluent with 2.5 M acetic acid, 4 mM L-arginine, and 4% (v/v) acetonitrile at a flow rate of 1.0 ml/min and detection was performed at 280 nm. In this case, 200 μl homogeneous samples were diluted with the mobile phase.

SE-HPLC was only used to detect aggregates products of insulin, specially, the covalent insulin dimer as well as other degradation products which could be formed under different storage conditions.

2.2. Stability studies

In this study, we used two human insulin pharmaceutical preparations, a regular rapid acting solution and a long-acting insulin zinc suspension, crystalline. The type of preparation and their composition are include in Table 1. Both preparations were stored at differents temperatures 40, 50 and 60°C with variations less than ± 1°C, on a continuous motion system at 100 r.p.m (Ika-vibrax Schot-Ibérica) and without shaking. At the time, both preparations were stored protected from light at room temperature, thermostatically controlled over 2 years. The mean temperature value was 20.7 ± 1.6°C, while the mean kinetics temperature (MKT) obtained using the formula of Haynes (1971) was 22.7°C to the solution and 22.5°C to the suspension, respectively.

A heat of activation of 16 kcal/mol was used to the suspension and 25.5 kcal/mol to the solution, values calculated according to the Arrhenius relationship using data from accelerated testing excluding room temperature data.

Table 1
Composition of commercial human insulin preparations used in this study

Type of preparation	Concentration	Preservative	Isotonic agents	Other additives
Solution	40 IU/ml	m-cresol	Glycerol	—
Insulin-zinc suspension	40 IU/ml	Methylparaben	NaCl	Zn^{+2} Na-acetate

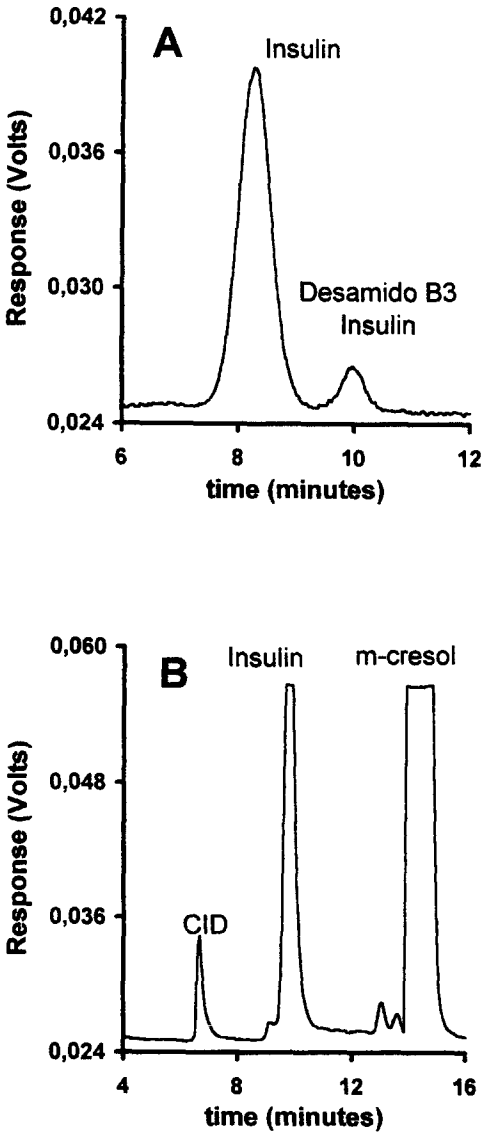


Fig. 2. RP-HPLC chromatogram corresponding to a suspension sample (A) and SE-HPLC chromatogram corresponding to a solution sample (B) stored at 40°C without shaking (CID, Covalent insulin dimer).

3. Results

In the case of suspension, the only degradation product detected by RP-HPLC was the desamido B3 insulin (see Fig. 2A). However, insulin aggregation products such as covalent dimer insulin by SE-HPLC were not detected at any time.

The quantification of insulin degradation was carried out from data obtained by RP-HPLC. The suspension degradation, at all temperatures and storage conditions, follows a first order process as can be seen from Fig. 3A. The formation

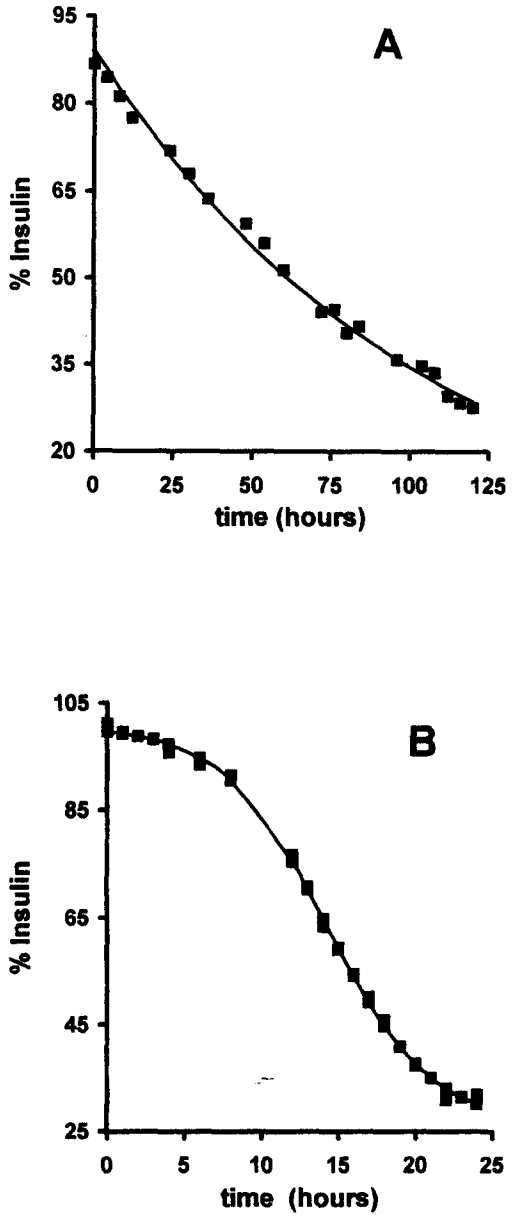


Fig. 3. Degradation kinetics of insulin corresponding to suspension (A) and solution (B) samples stored at 60°C with shaking.

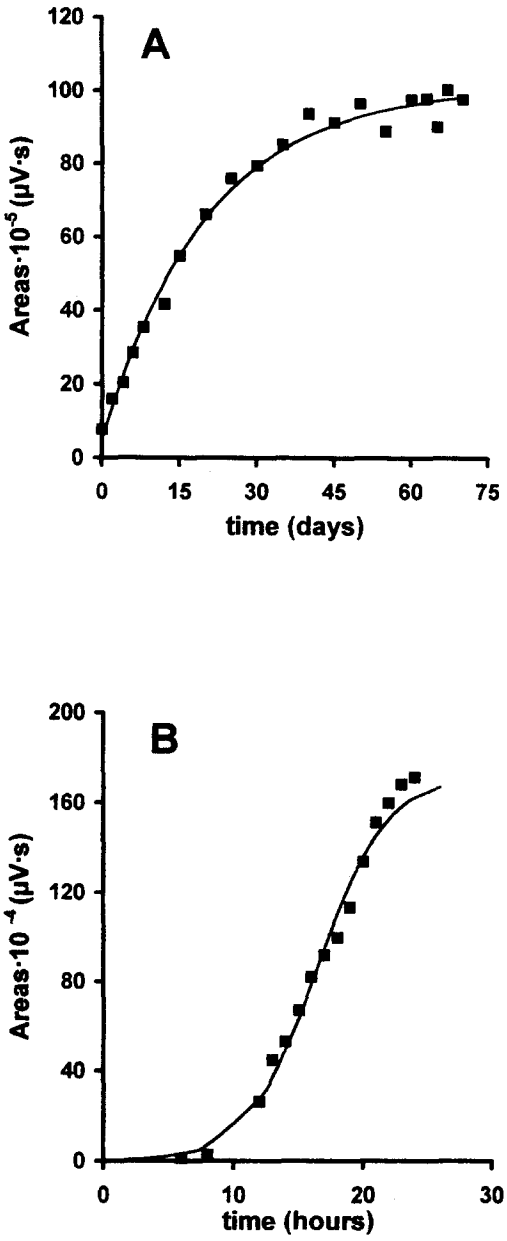


Fig. 4. Formation kinetics corresponding to desamido B3 insulin (A) and 10 covalent insulin dimer (B).

process of desamido B3 insulin also follows a first order kinetics (see Fig. 4A).

On the other hand, for solution, the main degradation product detected by RP-HPLC was the desamido B3 insulin, although in the assays with shaking it can also be the desamido A21 insulin. The aggregation products such as covalent insulin dimer were separated by SE-HPLC (Fig. 2B), although this product could be only detected in samples stored at temperatures higher than 40°C major with and without shaking, even the samples stored at 60°C with shaking high molecular weight transformation products by SE-HPLC were also detected.

For solution, a first order degradation kinetic domain in all cases except data obtained at 60°C with shaking, which fit better the Prout-Thompkins nucleation model (see Fig. 3B). In this case, the NonLinearFit function of Mathematica 2.1 (Wolfram Research) programm was used. The remaning fraction of insulin 'β' at time 't' was calculated throughout the Eq. (1):

$$\beta = \beta_\infty + \frac{\beta_0 - \beta_\infty}{e^{k(t-t_0)}} \quad (1)$$

where β is the remaining fraction at time t; β_∞ is the remaining fraction at time infinite; β_0 is the the remaining fraction at time zero; k is the degradation rate constant, t is the time and t_0 is the time where the fraction remaining is 50% of the initial one. The covalent insulin dimer formated, expressed as transformated fraction, 'α', at time 't' was calculated from Eq. (2).

$$\alpha = \frac{e^{k(t-t_0)}}{1 + e^{k(t-t_0)}} \quad (2)$$

where k is the dimer formation rate constant and t_0 is the time where 50% of product has formed.

Table 2 shows the storage conditions used in accelerated stability testing as well as time of storage and the degradation rate constant values obtained for both formulations at different storage conditions.

Fig. 5 shows Arrhenius plot for both formulations at different storage conditions. By extrapolation from Arrhenius plot, rates constant at 5 and 20°C could be obtained and so the expiration time ($t_{10\%}$) at these temperatures (Table 3). Fig. 6 shows Arrhenius plot for assays carried out at 40, 50, 60°C and at room temperature without shaking for both preparations.

Table 2
Degradation rates constant values obtained for both formulations at different storage conditions

T (°C)	No shaking (suspension)			Shaking			
	$k(d^{-1})$[a]	r[b]	t[c]	$k(d^{-1})$	r	n[d]	t
40	0.00801	0.997	120	0.0446	0.994	60	25
50	0.0173	0.997	60	0.106	0.995	60	10
60	0.0173 (0.0413)	0.997 (0.995)	60 (25)	0.106 (0.227)	0.995 (0.994)	60	10 (5)
Solution							
40	0.00413	0.997	270	0.0506	0.998	60	24
50	0.0147	0.997	70	0.149	0.996	60	7
60	0.481 (0.0481)	0.995	25	7.08[e]	0.994	60	1

[a]Experimental rate constant per day.
[b]Coefficient of correlation.
[c]Time of storage in days.
[d]Number data.
[e]Insulin degradation kinetic fitted a Prout-Thompkins model

4. Discussion

Deamidation was the predominant degradation process in case of suspension while dimerization prevailed for solutions, mainly when shaking was applied. As was mentioned above, the desamido B3 insulin formation follows a first order kinetics, being the dimer formation better fitted by the Prout-Thompkins nucleation model (see Fig. 4).

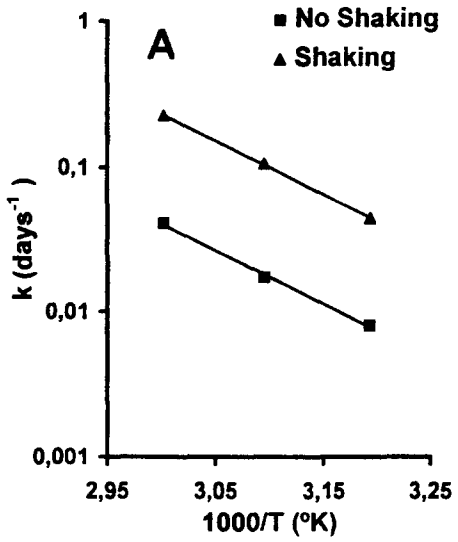
The activation energy (E_a) values obtained from assays with and without shaking for suspension at experimental temperatures were quite close, 17.0 and 16.9 kcal/mol, respectively (see Fig. 5A). However, the pre-exponential factor (A) change in function of storage conditions, being higher with shaking ($2.6 \cdot 10^{10}$ per day) than without ($5.62 \cdot 10^{9}$ per day). This fact seems to be logical since the shaking must facilitate the collision between molecules.

The estimated values for k at ambient temperature (20.7°C) and MKT (22.5°C) were higher than values experimentally obtained at 20.7°C. For this reason expiration time experimentally obtained was 140 days, being 81 and 67 days for the above estimates values of k (Table 3). If we include the 20.7°C assay in Arrhenius plot, the E_a value was 19.8 kcal/mol, value similar which obtained by Brange (1992). Expiration time estimated for 5°C, mean value corresponding to interval recomended by manufacturer (2–8°C), was 2.36 years, higher than expiration time date indicated for the manufacturer (2 years)

The results obtained for suspension assays at 40, 50 and 60°C with and without shaking allowed us to confirm the suitability of Arrhenius plot for insulin stability studies including relatively high temperatures like 60°C contrary to other authors affirmations (Gu et al., 1991; Brange et al., 1992a). However, it will be necessary to include in the studies temperatures close to those recomended by manufacturers to store their products. In our case we would need some more points between 10 and 20°C because the obtained values for $t_{10\%}$ at 5°C can change from 2.36 to 1.15 years if we include or not the k value at 20°C in the Arrhenius plot.

In case of solution, the Arrhenius relationship was also valid, but only for assays without shaking. We have only observed linearity in non-shaking assays in the temperature range studied, obtaining a value of activation energy of 25.5 kcal/mol. However, with shaking linearity was not observed in the experimental temperature intervals. Combination of shaking and high temperature (60°C) promote a different degradation kinetics with a k value which does not fit the Arrhenius plot (see Fig. 5B).

The $t_{10\%}$ at 20°C obtained using the k value from Arrhenius relationship was 378 days (Table 3), the same value as that obtained using the k value at 20.7°C. When the k value used was obtained from extrapolation at 22.7°C (MKT value), the $t_{10\%}$ value was 279 days, so we can say that 2 degrees variation on storage temperature could change the expiration date by a 100 days.



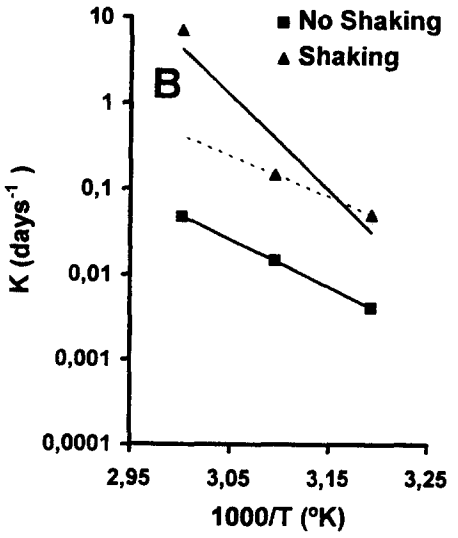


Fig. 5. Arrhenius plot corresponding to suspension (A) and solution (B) assays for both storage conditions.

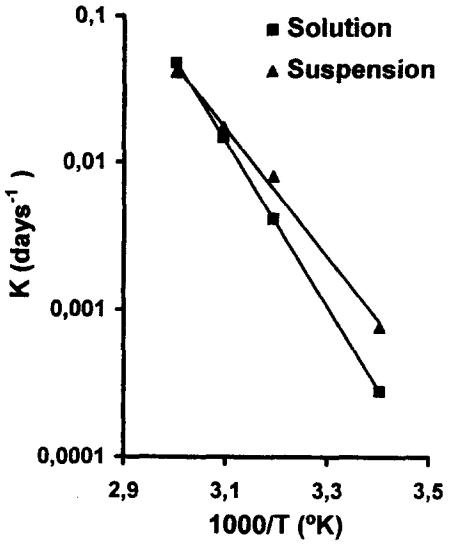


Fig. 6. Arrhenius plot corresponding to 40, 50 and 60°C and at room temperature assays for both preparations without shaking.

Using the k values obtained at 20.7, 40, 50 and 60°C, the Arrhenius plot is linear ($r = 0.999$) and the $t_{10\%}$ at 5°C was higher than 5 years (see Fig. 6).

Finally, from results it is easy to deduce the significance of shaking in degradation of insulin, specially when it is combined with temperature, in solution for example, at 60°C, a k value increase from 0.048 per day to 7.08 per day can be observed.

Table 3
Rate constants and expiration times ($t_{10\%}$) values estimated from Arrhenius extrapolation (k_{est}) and experimentally (k_{obs}) for two formulations

T (°C)	Suspension		Solution	
	k_{est} d^{-1}	$t_{10\%}$ (days)	k_{est} d^{-1}	$t_{10\%}$ (days)
5	$2.51 \cdot 10^{-4}$	419	$2.40 \cdot 10^{-5}$	4381
20.7	$1.30 \cdot 10^{-3}$	81	$2.82 \cdot 10^{-4}$	374
MKT[a]	$1.58 \cdot 10^{-3}$	67	$3.78 \cdot 10^{-4}$	279
	k_{obs} d^{-1}	$t_{10\%}$ (days)	k_{obs} d^{-1}	$t_{10\%}$ (days)
20.7	$7.60 \cdot 10^{-4}$	140	$2.79 \cdot 10^{-4}$	378

[a]Mean kinetic temperature.

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