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Determination of the Chemical Stability of Various Formulations of Tobramycin Eye-Drops by HPLC Method and Data Analysis by R-GUI Stability Software

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ABSTRACT

Tobramycin, is a water soluble aminoglycoside antibiotic produced by the fungus *Streptomyces tenebrarius* and used in a variety of pharmaceutical applications including ophthalmic solutions, suspensions and ointments; inhalation solutions and intravenous administration. There are commercially available eye drops formulations in the Argentinian market that have different conservation conditions. We formulated six eye drops solutions, studied their stability at 2-8°C, 25°C, and 40°C, 75% RH and quantified using USP method. Only half of the formulations studied were found to be stable for two years at ambient temperature which is their expected expiry date and only one for three years in the same condition. One system was unstable in the three conservation conditions studied, including appearance and pH.

INTRODUCTION

Tobramycin, D-streptamine, *O*-3-amino-3-deoxy-α-D-glucopyranosyl-(1→6)-*O*-[´2,6-diamino-2,3,6-trideoxy-α-D-*ribo*-hexopyranosyl-(1→4)]-2-deoxy-, is a water soluble aminoglycoside antibiotic produced by the fungus *Streptomyces tenebrarius* and used in a variety of pharmaceutical applications including ophthalmic solutions, suspensions and ointments; inhalation solutions and intravenous administration (Hanko and Rohrer, 2006; Manyanga *et al.*, 2013). It is active against a broad spectrum of gram-negative bacteria. There are commercially available eye drops formulations in the Argentinian market that have different conservation conditions (ambient temperature and refrigerator). Brandl and Gu studied the stability of Tobramycin in aqueous solution (Brandl and Gu, 1992). They concluded that although hydrolysis of tobramycin occurs at the pH extremes, it is not an important degradation pathway at neutral pH values. The major degradation pathway for tobramycin at neutral pH values where the drug is formulated (pH 5.8-7.4) is oxidation. Tobramycin oxidizes giving several products including nebramine, deoxystreptamine and deoxystreptamine-kanasaminide (Fig 1). According to these results we formulated six solutions and studied their stability at 2-8°C, 25°C, and 40°C, 75% RH. Direct analysis of tobramycin is not simple. This is due to the polar basic nature and the lack of UV absorbing chromophore in the molecule. Several methods have been described to determine tobramycin by adsorptive stripping voltammetric method (Sun *et al.*, 2005), electrophoresis capillary (El-Attug *et al.*, 2012; Ahmed and Ebeid, 2015) and ion pair LC methods (Hankoand Rohrer, 2006; Szúnyog *et al.*, 2000; Valentini *et al.*, 2008; Hanko *et al.*, 2008; Chopra *et al.*, 2010). A literature survey revealed some reversed phase liquid chromatographic methods for the quantitation of tobramycin employing mass detector (Keevil *et al.*, 2003; Guo *et al.*, 2006), ELSD (Megoulas *et al.*, 2005; Pfeifer *et al.*, 2015) and PED (Manyanga *et al.*, 2013). The literature showed also reversed phase HPLC methods with pre column derivatization with 1 Naphthyl isothiocyanate (Feng *et al.*, 2002), Fluorescein isothiocyanate (Mashat *et al.*, 2008), 2,4,6-Trinitrobenzenesulphonic acid (Dash and Suryanarayanan, 1991a; Dash and Suryanarayanan, 1991b), 4-Chloro-3,5-dinitrobenzotrifluoride (He *et al.*, 2011), 9-Fluorenylmethyl chloroformate (Zhang and Peng, 2012) and

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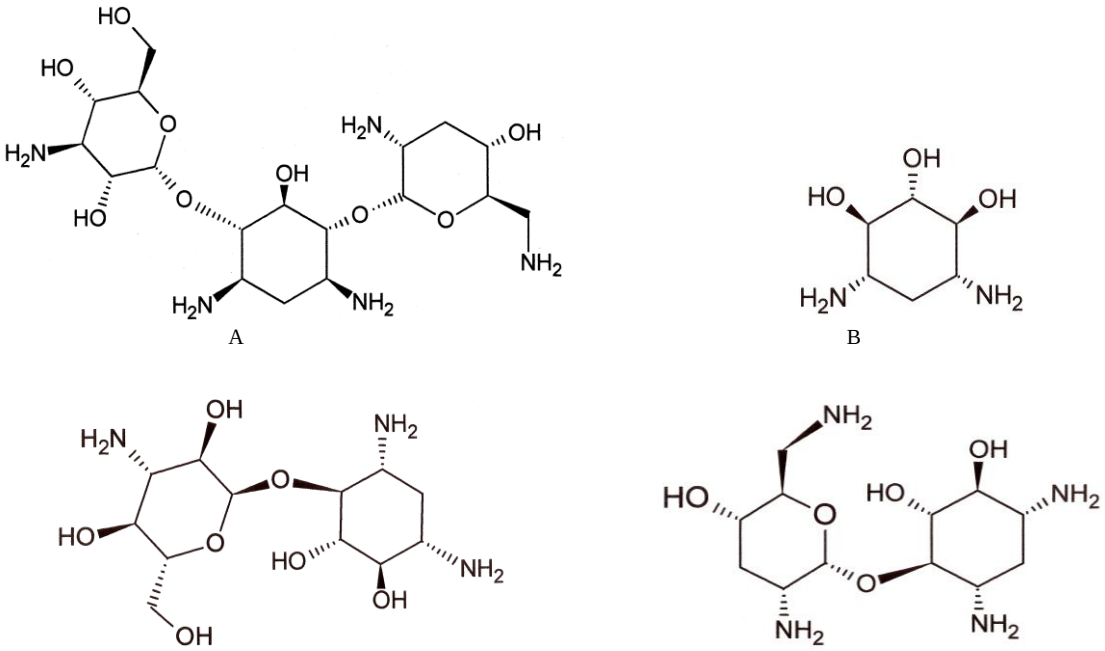

Fig. 1: A) Tobramycin, B) Deoxystreptamine, C) Deoxystreptamine-kanasaminide, D) Nebramine.

Table 1: Quantitative composition of formulations (g %)

Materials (g/100g)	System A	SystemB	System C	SystemD	SystemE	SystemF
Tobramicyn	0.3	0.3	0.3	0.3	0.3	0.3
Excipients:Benzalkonium chloride	1.0×10^{-2}					
Sodium chloride	1.0×10^{-2}	0.1	7.0×10^{-2}	2.0×10^{-2}		
Dibasic sodium phosphate	0.6					
Monobasic sodium phosphate	0.4					
Boric acid		1.24×10^{-2}		1.26×10^{-3}	1.9	1.9
Anhydrous sodium sulfate		1.52×10^{-3}		2.26×10^{-4}		
Tyloxapol		1.0×10^{-3}		0.1		
Edetate disodium			0.1			
Sodium metabisulfite			0.5			
Thimerosal			1.0×10^{-3}			
Sodium hyaluronate					0.3	0.3
Potassium sorbate						0.18
Water for injection csp	100	100	100	100	100	100

o-Phthalaldehyde pre column derivatization (Caturla *et al.*, 1992) and post column derivatization (He and Yang, 2010). This work describes the analysis of tobramycin in six formulations maintained in stability studies and quantitated using USP method (The United States Pharmacopeia, 2015). This method use 2,4-Dinitrofluorobenzene as pre- derivatizating agent. Russ and coworkers use a modification of this method for the quantitation of tobramicyn in an ophthalmic suspension (Russ *et al.*, 1998).

MATERIALS AND METHODS

Materials and reagents

Tobramicyn (955,9 µg/g calculated with reference to the dried substance) pharmaceutical grade was provided by Cross Chem (China), Benzalkonium chloride Merck (Germany), Sodium chloride Merck (USA), Dibasic sodium phosphate Anedra (Argentine), Monobasic sodium phosphate Anedra (Argentine), Boric acid J.T Baker (Mexico), Anhydrous sodium sulfate Anedra (Argentine), Tyloxapol Sigma-Aldrich (India), Edetate disodium Merck (Germany), Sodium hydroxide Anedra (Argentine), Sodium metabisulfite J.T. Baker (Mexico), Thimerosal Ningbo Hi-Tech (China), Sodium hyaluronate CPN SPOL SRO (Czech Republic), Potassium sorbate granular Merck (Germany) and distilled water.

All chemicals used were of analytical grade, sulphuric acid Merck (Germany), Buffer TRIS (Tris(Hidroxymethyl) aminomethane) Biopack (Argentine), 2,4-Dinitrofluorobenzene Sigma-Aldrich (USA), p-naphtholbenzein Sigma-Aldrich (USA). Acetonitrile and water were of HPLC grade. Solvents were filtered through a 0.45 µm membrane and degassed.

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Preparation of the eye-drops

The six systems were prepared with the same drug substance of Tobramycin. Formulations are described in Table 1. In a glass flask containing 350 mL of distilled water at 25 °C, the salts and other excipients were dissolved once each and stirring

vigorously. A nitrogen flow was placed before the Tobramycin was added. Then, the pH was corrected around 7.5 with NaOH 1N or HCl 3N. The solutions were diluted with distilled water to 500 mL. The solutions were passed through a 0.22 µm nylon membrane filter before injection (25 mm disposable filter; Cat. N° R04SP02500 Osmonics Inc., Minnesota, USA). Approximately 5 mL of Tobramycin solutions were transfer in plastic bottles of HDPE/LDPE and placed in stability conditions: 2-8 ºC, 25 ºC and 40 ºC, 75% HR.

Instrumentation

The HPLC system consisted of a dual piston reciprocating Thermo Finnigan pump (Waltham, Massachusetts, United States, Model P2000), a Rheodyne injector (Model 7125), a UV-Vis KONIK detector (Barcelona, Spain, Model KNK-027-757) with operating software WinPCC Chrom XY (Buenos Aires, Argentine) was used during the study.

HPLC conditions

The experiment was performed on a reversed phase C18 column (Phenomenex, Torrance, CA, USA) 300 x 4.6mm, 10µm. The separation was carried out under isocratic elution with acetonitrile:buffer TRIS. For the preparation of the mobile phase, 2 g of buffer TRIS were dissolved in 800 ml of water. To this solution, 20 ml of Sulfuric acid was added and diluted to 2000 ml with acetonitrile. The flow rate was 1.1 mL/min. The wavelength was monitored at 365 nm, and the injection volume was 20 µL. The HPLC was operated at ambient temperature. In these conditions the retention time (t_R) was roughly 6 minutes.

Preparation of standard solution

An accurately weighed quantity of 33 mg of tobramycin was transferred to a 50 mL volumetric flask, added 1 mL of 1 N sulfuric acid and dissolved in 20 ml of water and then taken to volume with water. Then, 10.0 mL were withdraw in a 50 mL volumetric flask, diluted with water to volume, and mixed. The solutions were passed through a 0.45 µm nylon membrane filter before injection (25 mm disposable filter; Cat. N° Y02025WPH µicroclar, Buenos Aires, Argentina).

Sample Preparation

Approximately 1 mL of eye drops tobramycin solution 0.3% (3 mg/mL) were exactly weighed, placed into a 25 mL volumetric flask and taken to volume with water**.**

The solutions were passed through a 0.45 µm nylon membrane filter before injection (25 mm disposable filter; Cat. N° Y02025WPH µicroclar, Buenos Aires, Argentina).

Derivatization

A solution of 2,4-Dinitrofluorobenzene containing 10 mg per mL in alcohol was prepared and maintained during 5 days in refrigerator. A stock solution of TRIS was prepared in water containing 15 mg per mL. 40 mL were transferred to a 200 mL volumetric flask and diluted to volume with dimethyl sulfoxide. This reagent was used within 4 hs. To separate 50 mL volumetric flask, 4.0 mL of standard preparation, 4.0 mL of sample preparation and 4.0 mL of water were transferred and added 10 mL of 2,4-Dinitrofluorobenzene solution and 10 mL of TRIS solution each. The volumetric flask were kept in a water bath at 60 ± 2°C during 50 ± 5 minutes. The flask were removed from the bath, and allowed to stand for 10 minutes. Added acetonitrile to about 2 mL below the 50 mL mark, allowed to cool to room temperature, then diluted with acetonitrile to volume, and mixed.

The resolution solution was prepared transferring 2 mL of a fresh solution of p-naphtholbenzein in acetonitrile (containing 0.24 mg per mL) to a 10 mL volumetric flask and diluted with derivatized standard preparation for volume and used promptly.

It was injected a blank of mobile phase, another with the derivatization solution diluted as the standard solution and the resolution solution. The relative retention times were about 0.6 for p-naphtholbenzein and 1.0 for tobramycin, and the resolution, R, between the two peaks was not less 4.0.The derivatized standard preparation was chromatographed, recorded the responses until the relative standard deviation for replicate was not more than 2.0 %. Separately were injected equal volumes of the derivatized standard preparation and the derivatized assay preparation into the chromatograph, recorded the chromatograms and measured the area responses for the major peaks and calculated the quantity.

pH Determinations

The pH data for all the systems were obtained with model Altronix TPX I (Saen S.R.L., Buenos Aires, Argentina). The pH was measured as directed in USP 38 <791>, using an indicator glass electrode. The buffer solutions for standardization were from Merck (Darmstadt, Germany) at pH 4.00 and 7.01.

Software

To evaluate the shelf life of Tobramycin eye drops, an R-package "stab" software version 3.0.1 was used (Lee *et al.*, 2010; Hassan *et al.*, 2015). The software meets ICH Q1E specifications (Guidance for the industry: ICH 2003).

RESULTS AND DISCUSSION

In the present study, the stability of different eye drops formulations containing Tobramycin were studied. The eye drops produced in this work are similar to those it can be found in the Argentinian market. The trouble is that the products have different conservation conditions.

The stability studies are executed to estimate the mechanism of the active pharmaceutical ingredients´ (APIs) degradation in crude and in dosage forms.

The preparations were analyzed by HPLC with a previous derivatization reaction and UV detection. The stability results were analysed with R-package "stab" software version 3.0.1., having a single-factor analysis, for single-batch based on ICH Q1E specifications (Guidance for the industry: ICH 2003). The assay results are mentioned in Table 2. With reference to pH for tobramycin eye-drops solutions, USP 38 (The United States

Pharmacopeia, 2015) sets a range from 7 to 8. The appearance and pH results are indicated in Table 3, in bold pH data that are out of specification. Only system C does not meet this requirement in any of the storage conditions. First order analysis was made at one sided lower control analysis at 90% confidence interval (Fig. 2-4). The results are presented in Table 4. In refrigerator (2-8 °C) systems A, B, D and E shown a shelf life of 5 years or more while system F present 4.83 years and system C 1.58 years. At ambient temperature (25 °C), systems B was the most stable with 4.58 years and systems D and E greater than 24 months. At the accelerated condition (40 °C and 75% RH), only system B was stable at 1.75 years. System B was found most stable whereas system C was the least stable. With regard to appearance, System C seems to turn to yellowish at 6 months at ambient temperature. The same occur to System F since 9 months and System A since 18 months.

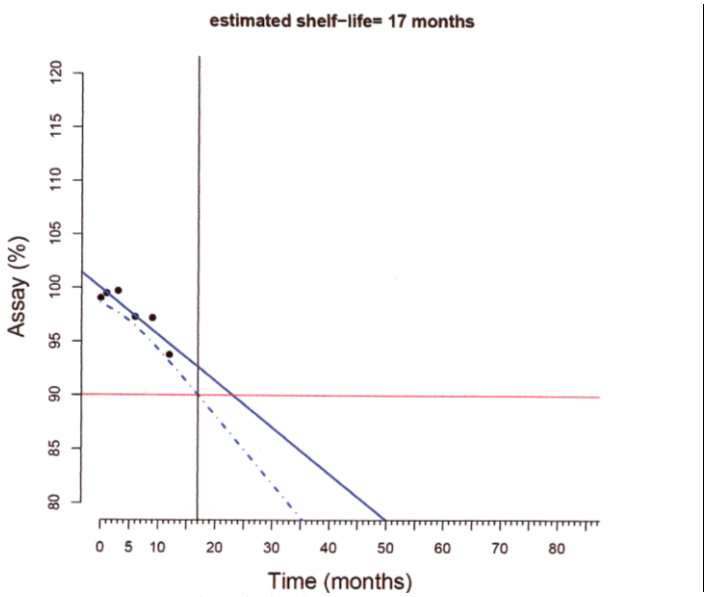

Fig. 2: Estimation of shelf life of Tobramycin System D al 40 °C, 75% RH

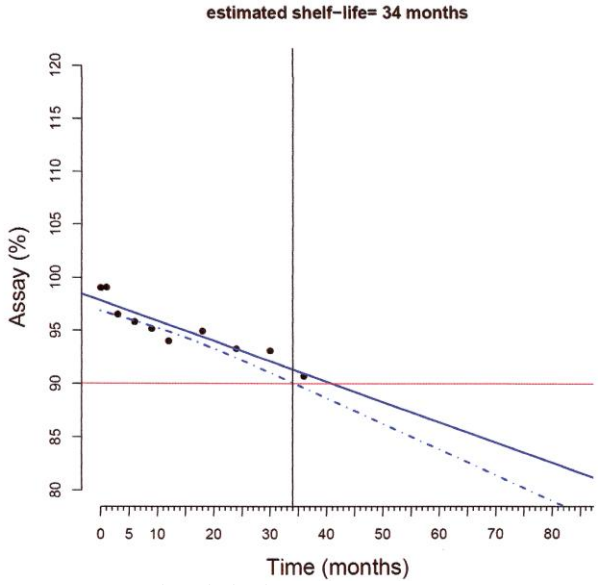

Fig. 3: Estimation of shelf life of Tobramycin System D al 25 °C.

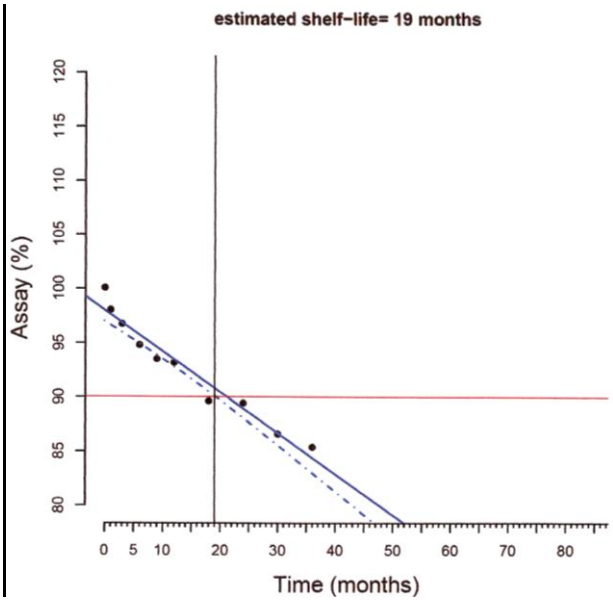

Fig. 4: Estimation of shelf life of Tobramycin System C al 2-8 °C.

Table 2: The remaining percentage content of the active ingredient tobramycin in eye-drops solutions.

	Time (months)									
System A	**0**	**1**	**3**	**6**	**9**	**12**	**18**	**24**	**30**	**36**
40 ºC, 75% HR	97.65	93.08	88.19	88.32	74.52	62.92				
25 °C, 60% HR	97.65	99.92	96.24	95.11	95.84	94.77	91.50	91.45	85.95	85.30
2-8ºC	97.65	98.48	96.84	96.71	97.50	97.24	97.25	98.47	98.24	99.54
SystemB										
40 ºC, 75% HR	99.42	97.09	98.87	95.90	96.70	96.47				
25 °C, 60% HR	99.42	100.94	97.56	95.67	97.63	98.08	97.38	99.12	95.29	94.51
2-8ºC	99.42	97.41	96.72	96.66	97.73	98.15	98.09	98.48	99.86	98.99
SystemC										
40 ºC, 75% HR	100.09	82.42	80.11	75.40	73.82	68.78				
25 °C, 60% HR	100.09	94.32	84.70	80.53	80.82	79.56	77.70	75.87	76.06	69.97
2-8ºC	100.09	98.08	96.78	94.84	93.55	93.18	89.65	89.48	86.62	85.43
SystemD										
40 ºC, 75% HR	99.04	99.48	99.71	97.30	97.19	93.78				
25 °C, 60% HR	99.04	99.09	96.52	95.84	95.16	94.00	94.95	93.27	93.12	90.72
2-8ºC	99.04	97.41	97.12	95.77	96.73	97.17	97.53	97.46	97.53	96.56
SystemE										
40 ºC, 75% HR	99.24	97.20	97.54	88.70	85.77	81.49				
25 °C, 60% HR	99.24	100.77	96.29	96.31	96.24	93.47	95.56	95.88	90.03	87.55
2-8ºC	99.24	97.58	96.75	96.34	96.53	96.52	97.27	99.04	97.87	100.74
SystemF										
40 ºC, 75% HR	98.6	92.18	93.61	86.68	81.30	69.08				
25 °C, 60% HR	98.6	98.70	94.62	92.79	93.24	91.88	90.48	84.73	85.73	83.82
2-8ºC	98.6	96.04	96.07	95.30	95.80	94.99	95.45	94.80	93.12	95.52

Table 3: Appearance and pH of the formulations.

		System A			**System B**			**System C**			**SystemD**			**SystemE**			**SystemF**		
Time (month)		**2-8 °C**	**25°C**	**40ºC**	**2-8 °C**	**25°C**	**40ºC**	**2-8 °C**	**25°C**	**40ºC**	**2-8°C**	**25°C**	**40ºC**	**2-8°C**	**25 °C**	**40ºC**	**2-8°C**	**25°C**	**40ºC**
0	Appearance	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	pH	7.52	7.52	7.52	7.50	7.50	7.50	7.55	7.55	7.55	7.40	7.40	7.40	7.42	7.42	7.42	7.40	7.40	7.40
1	Appearance	a	a	a	a	a	a	a	a	c	a	a	a	a	a	a	a	a	c
	pH	7.69	7.69	7.68	7.50	7.50	7.49	7.48	7.23	**6.98**	7.37	7.33	7.37	7.47	7.44	7.50	7.40	7.41	7.41
3	Appearance	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	pH	7.69	7.68	7.68	7.51	7.53	7.52	7.40	7.17	**6.97**	7.33	7.34	7.34	7.49	7.49	7.50	7.43	7.42	7.40
6	Appearance	a	a	a	a	a	a	a	c	d	a	a	a	a	a	a	a	a	c
	pH	7.70	7.70	7.69	7.52	7.56	7.55	7.37	**6.99**	**6.92**	7.26	7.37	7.36	7.50	7.55	7.50	7.42	7.43	7.42
9	Appearance	a	a	c	a	a	a	a	d	d	a	a	a	a	a	a	a	c	c
	pH	7.69	7.67	7.59	7.44	7.42	7.45	7.33	**6.90**	**6.78**	7.16	7.19	7.23	7.52	7.45	7.47	7.44	7.39	7.42
12	Appearance	a	a	c	a	a	a	a	d	d	a	a	c	a	a	c	a	c	c
	pH	7.63	7.65	7.49	7.30	7.39	7.40	7.19	**6.85**	**6.62**	7.11	7.17	7.15	7.47	7.47	7.44	7.40	7.41	7.34
18	Appearance	a	b	-	a	a	-	a	d	-	a	a	-	a	a	-	a	c	-
	pH	7.67	7.65	-	7.51	7.41	-	7.15	**6.85**	-	7.24	7.17	-	7.57	7.44	-	7.48	7.34	-
24	Appearance	a	c	-	b	a	-	a	d	-	a	a	-	a	a	-	a	c	-
	pH	7.67	7.65	-	7.45	7.52	-	7.03	**6.96**	-	7.14	7.27	-	7.53	7.50	-	7.42	7.41	-
30	Appearance	a	c	-	c	a	-	a	d	-	a	a	-	a	a	-	a	c	-
	pH	7.66	7.63	-	7.36	7.47	-	**6.84**	**6.86**	-	7.05	7.24	-	7.44	7.48	-	7.38	7.41	-
36	Appearance	a	c	-	c	a	-	b	d	-	a	a	-	a	a	-	a	c	-
	pH	7.65	7.61	-	7.32	7.43	-	**6.77**	**6.75**	-	7.08	7.19	-	7.51	7.48	-	7.48	7.42	-

a: limpid solution, colorless., b: limpid solution, slightly yellowish., c: limpid solution, yellowish., d: limpid solution, yellow

Table 4: Software originated results for apparent First-Order Rate Constant (k_{obs}) for the degradation of tobramycin eye-drops.

System	$K_{obs.}$ month^{-1}	Correlationcoefficient	t_{90} years (months)
A40 ºC, 75% RH	2.655	0.9648	0.08 (1 month)
25 °C	0.368	0.9679	1.67 (20 months)
2-8ºC	0.045	0.6497	> 5
B 40 ºC, 75% RH	0.204	0.6814	1.75 (21 months)
25 °C	0.101	0.6401	4.58 (55 months)
2-8ºC	0.049	0.5786	> 5
C40 ºC, 75% RH	1.970	0.8503	0.08 (<1 month)
25 °C	0.598	0.8380	0.08 (<1 month)
2-8ºC	0.378	0.9757	1.58 (19 months)
D40 ºC, 75% RH	0.431	0.9116	1.42 (17 months)
25 °C	0.190	0.9197	2.83 (34 months)
2-8ºC	0.013	0.1944	>5
E40 ºC, 75% RH	1.530	0.9807	0.42 (5 months)
25 °C	0.275	0.8811	2.17 (26 months)
2-8ºC	0.063	0.5495	>5
F40 ºC, 75% RH	2.169	0.9661	0.17 (2 months)
25 °C	0.404	0.9574	1.25 (15 months)
2-8ºC	0.070	0.6477	4.83 (58 months)

CONCLUSIONS

Only half of the formulations studied were found to be stable for two years at ambient temperature which is their expected expiry date and only one for three years in the same condition. In refrigerator, five formulations were found to be stable for four years or more. One system was unstable in the three conservation conditions studied, including appearance and pH. These results indicate the need for exhaustive stability studies carried out in the pharmaceutical industry.

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