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Analysis of acyclovir in vitreous humor by a validated HPLC method

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An HPLC-UV method was developed and validated for the determination of acyclovir in vitreous humor. The method was carried out in isocratic mode using 0.02 mol/L acetic acid/methanol (95:5) as mobile phase, a C18 column at 25 °C and UV detection at 254 nm. The method was linear ($r^2 > 0.99$) over the range of 35–700 μg/mL, precise (RSD < 5%), accurate (recovery ranged from 98.18 to 99.64%), robust, selective regarding of the vitreous humor, and robust remaining unaffected by deliberate variations in relevant parameters. The validated HPLC-UV method can be successfully applied to determine acyclovir directly injected into the vitreous cavity of rabbits' eye.

1. Introduction

Acyclovir [9-(2-hydroxyethoxylmethyl) guanine] (Fig. 1) is a guanine derivative nucleoside analog with strong antiviral activity against herpes simplex and varicella zoster viruses (Ormrod and Goa 2000). Currently, acyclovir has been used to treat acute retinal necrosis, a disease caused by herpetic viruses with devastating consequences for the eye (Tam et al. 2010).

The use of intravitreal injections for the treatment of acute retinal necrosis has become increasingly common and the effectiveness of this local treatment has been described as it allows immediate drug delivery at therapeutic levels directly into the target site (Meghpara et al. 2010). Intravitreal injection of acyclovir may be a potential alternative in acute retinal necrosis therapy as it may contribute to a better control of this aggressive retinitis mainly during the first 48 hours, while systemic acyclovir has still not reached therapeutic level in the retina (Schulman et al. 1986).

Analysis of acyclovir in biological matrices has been made possible by a number of high performance liquid chromatographic (HPLC) methods employing different modes of detection such as fluorescence (Peh and Yuen 1997; Svensson et al. 1997; Maes et al. 2009), HPLC with mass detection (Manish et al. 2009) and high-performance capillary electrophoresis (HPCE) (Vo et al. 2002). Sample preparation for these HPLC methods includes mainly a deproteinization step (using perchloric acid or organic solvents such as acetonitrile and methanol) and/or a solid-phase extraction step (using a hydrophobic column) as well as other techniques such as ultrafiltration (Nebinger and Koel 1993). There have been reported assays for acyclovir analysis in biological fluids using high-performance capillary electrophoresis (Zhang et al. 1996; Cabarcos et al. 2010) that employed UV and amperometric modes of detection, with limits of detection in the high hundreds of the μg/ml range (Vo et al. 2002). Therefore, the mentioned HPLC methods require detection devices, not generally available in pharmaceutical laboratories, and complex processes of sample preparation, which involve a long analysis time for estimating the drug, limiting its application in routine quality control.

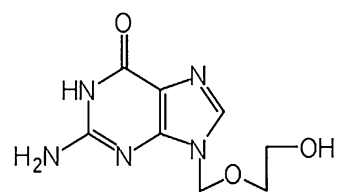

Fig. 1: Chemical structure of the acyclovir

In this study, a HPLC method with ultraviolet detection (HPLC-UV) was optimized and validated for determination of acyclovir in the vitreous humor of rabbits' eye. The proposed HPLC-UV offers many advantages in comparison to the mentioned analytical methods: (1) it requires an ordinary detection device for quantitation of acyclovir incorporated into the vitreous humor; (2) it is extremely simple and rapid, since it involves a single step for preparation of the biological samples and direct injection of them; (3) small sample volume requirements for detection and quantitation of the acyclovir. Therefore, it is hypothesized that this validated HPLC-UV method could be applied to determine acyclovir levels in pharmacokinetic studies in pre-clinical investigations involving rabbits as experimental animal models of acute retinal necrosis; and/or in toxicological analysis.

2. Investigations, results and discussion

In this study, an HPLC-UV method was optimized and validated for determination of acyclovir in the vitreous humor after direct injection of the drug into the vitreous cavity of rabbits' eye. Different analyses of drugs in the vitreous humor were reported previously, for amphetamines, benzodiazepines (Clauwaert et al. 2000; De Letter et al. 2000), cocaine and its metabolites (Mackey-Bojack et al. 2000; Fernandez et al. 2006) and opiates (Antonides et al. 2007), following a complex process

Table 1: System suitability of the proposed HPLC-UV method

	Resolution (R)*	Theoretical plates (N)	Tailing factor (T)	Retention time and RSD
Proposed method	2.47	3026	1.50	7.427 min RSD = 0.31
Required limits[1]	R < 2	–	T < 2	RSD < 2.0%

*Resolution between the chromatographic peak of the acyclovir and the nearest peak of the vitreous humor.
[1]Required limits for system suitability previously described (The United States Pharmacopeia 2008).

of drug extraction. However, the proposed HPLC-UV method offers the advantage of being simple, since it involves a single step for preparation of the biological samples, without any process of extraction, and direct injection in the chromatograph. Additionally, to date there are no descriptions of a HPLC-UV method for assaying acyclovir in the vitreous humor under the selected chromatographic conditions.

The official compendia recommend an HPLC-UV method for assaying acyclovir raw material using a mobile phase constituted of glacial acetic acid and water (1:1000) (The United States Pharmacopeia 2008). However, it was verified that this chromatographic condition was not suitable for the determination of the acyclovir added in the vitreous humor matrix due to the high retention time of the drug of approximately 20 min and the formation of an asymmetric peak of the acyclovir. For the purpose of decreasing the retention time of drug, in this study, a mobile phase composed on 0.02 mol/L acetic acid and methanol (95:5) was applied. The pH of the eluent was 3.23, providing ionization of the drug molecule, and consequently, reducing its affinity to the stationary phase and decreasing the retention time of the acyclovir. This is consistent with the pK_a value for guanine ($pK_a = 3.18$) of which acyclovir is an analogue (Smith and Walker 1985). An increase in the percentage of methanol in the mobile phase further decreases the retention of acyclovir, but it promotes the overlapping between chromatographic peaks of the drug and vitreous humor. Then, a minimal proportion of this organic solvent (5%) was selected to compose the mobile phase in order to provide resolution between peaks. Finally, the adjustment of the proportion of the components of the mobile phase contributed to the attainment of a favorable retention time of acyclovir (7.4 min) and a symmetric chromatographic peak of the drug. Additionally, the wavelength of 254 nm was selected for analysis, since it provided maximum chromatographic response of the acyclovir, and consequently, high sensitivity of the HPLC-UV method.

System suitability was carried out to confirm that the equipment was adequate for determination of acyclovir in vitreous humor. For this test, six replicate injections of the standard solution of acyclovir at 700 μg/mL were analyzed considering resolution, theoretical plates, tailing factor and retention time of the drug. Table 1 shows the obtained results of system suitability in comparison with the required limits (The United States Pharmacopeia 2008). Accordingly, the proposed method fulfills the requirements within the accepted limits.

The specificity of the method was investigated by evaluating the lack of interference of the simulated and collected vitreous humor on the chromatographic peak of the acyclovir. The components of the vitreous humor eluted at approximately 8.2 min and at retention times lower than 4 min, and the acyclovir eluted in 7.4 min (Fig. 2A, 2B and 2C). The chromatographic peaks were completely resolved and any substance presented the same retention time of the active principle, allowing the unequivocal determination of the drug. Additionally, peak purity higher than 99.0% was obtained for the drug in the chromatograms of acyclovir spiked in the vitreous humor from rabbits' eye, indicating that other components did not coelute with the drug peak. According to the obtained results, the method demonstrated specificity for acyclovir in direct contact with the components of the collected vitreous humor.

The linearity of the method was tested by plotting a calibration curve over the range of 35 to 700 μg/mL of acyclovir, which was subjected to regression analysis by the least square method. The representative linear equation was $y = 213557169.88x + 896259.92$, where y and x were area and concentration (μg/mL), respectively. The significance of the intercept obtained in the calibration curve was tested and this parameter was not statistically significant ($p > 0.05$), consequently, it can be considered that the curve passes through the origin (Saliba et al. 2011). The correlation coefficient (r) was higher than 0.99, showing highly significant correlation between concentration and peak area (Brazil 2003). The coefficient of determination (r^2) of the calibration curve was 0.9999, implying that 99.99% of total variance of the peak areas was explained by the varying acyclovir concentration. Finally, the linear model proved to be adequate since the residues fol-

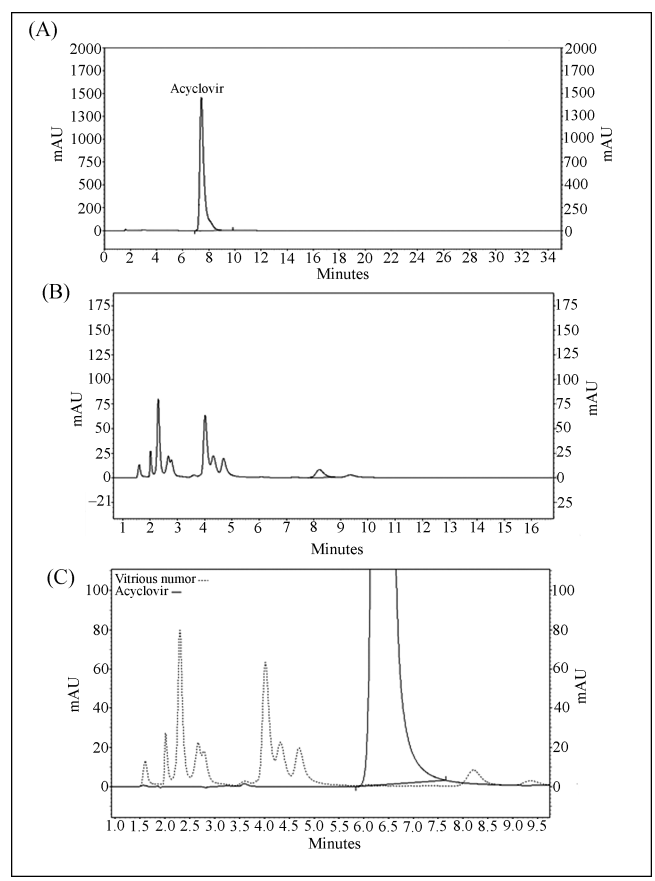

Fig. 2: (A). Chromatogram of the acyclovir at 700 μg/mL in simulated vitreous humor solution. (B) Chromatogram of the vitreous humor collected from rabbits' eye. (C) Overlay of the chromatograms 2A and 2B. Chromatographic conditions: C_{18} column 250 mm × 4.6 mm at 25 °C; 0.02 mol/L acetic acid and methanol (95:5, v/v); 3 mL/min of flow rate; wavelength of 254 nm

Table 2: Mean content of acyclovir in the intra-day and inter-day precision

Acyclovir concentration (μg/mL)	Intra-day precision		Inter-day precision	
	Mean content	RSD	Mean content	RSD
35	98.26	4.67	99.64	4.31
70	98.33	3.05	98.18	2.30
700	99.55	3.15	99.57	3.34

Table 3: Percent recovery of acyclovir spiked in the vitreous humor of rabbits' eye

Acyclovir concentration (μg/mL)	Percent recovery	RSD
35	99.64	4.31
70	98.18	2.26
700	99.57	3.32

lowed a normal distribution pattern and were independent, the homoscedasticity could be observed and the lack of fit was not significant. The LOQ and LOD were calculated as 0.16 μg/mL and 0.048 μg/mL, respectively.

The repeatability (intra-day) and intermediated precision (inter-day) were expressed as the relative standard deviation (RSD) of a series of measures of different concentrations of acyclovir incorporated into simulated vitreous humor solutions. The data obtained for precision were summarized in Table 2. The RSD values were lower than 5% for all levels of concentrations tested, thus indicating appropriate intra and inter-assay precision (Brazil 2003).

The accuracy of the HPLC-UV method was expressed as the percent of recovery of acyclovir spiked in the vitreous humor of rabbits' eye. Recovery ranged over 98.18% and 99.64%, as demonstrating in Table 3. All the values were between 98.0 and 102.0% of the theoretical concentration, confirming the accuracy of the proposed method.

The results obtained on the robustness test are shown in Table 4. The HPLC-UV method appears to be robust regarding all the variables analyzed, as the difference between results obtained under nominal and modified conditions were lower than the critical value for all analytical parameters studied. During the assays, the retention time of acyclovir was not significantly changed and peak symmetry was maintained. However, it could be noted that the wavelength and filter unit were the factors that had more influence on method performance. Thus, these analytical parameters should be carefully controlled.

Acyclovir directly injected into the vitreous cavity of rabbits' eye was quantified by applying the validated HPLC-UV method. The concentration of acyclovir in the collected vitreous humor, after 2 days of injection, was equal to 0.28 μg/mL (n = 3). The relative standard deviation for replicates was 2.36%.

In conclusion, an HPLC method for determination of acyclovir in vitreous humor of rabbits' eye was developed using ultraviolet detection, a common detection device. This chromatographic method was considered simple and rapid, since the preparation of the samples did not involve complex and prolonged processes of extraction. Furthermore, the HPLC-UV method was validated in terms of selectivity, linearity, limits of quantitation and detection, precision, accuracy and robustness. Finally, it provided unequivocal determination of acyclovir in direct contact with the components of the vitreous humor extracted from the posterior segment of rabbits' eye, and may be applied to determine acyclovir levels in pharmacokinetic studies in pre-clinical investigations involving rabbits as experimental animal models of acute retinal necrosis; and/or in toxicological analysis.

3. Experimental

3.1. Materials and reagents

Acyclovir reference standard was purchased from Sigma Aldrich (99% of purity). Ultrapure water was produced by a Milli-Q® purification system (Millipore, USA). Methanol HPLC grade was purchased from Merck® (Brazil). The other solvents and reagents used were of analytical grade.

3.2. Instrumentation and chromatographic conditions

The HPLC analyses were carried out on a Merck Hitachi LaChrom Elite (Germany) which included a quaternary pump, autosampler and diode array detector (DAD). The Ace C18 column (250 × 4.6 mm i.d.; 5 μm particle size) from Advanced Chromatography Technologies (Scotland) was used and maintained at 25 °C. The mobile phase comprised 0.02 mol/L acetic acid and methanol (95:5), at a flow rate of 3 mL/min. The injection volume was 20 μL and detection was performed at 254 nm.

3.3. Preparation of solutions

3.3.1. Simulated vitreous humor solution

Approximately 6.4 g of sodium chloride, 750 mg of potassium chloride, 480 mg of calcium chloride, 300 mg of magnesium chloride, 3.9 g of sodium acetate and 1.7 g of sodium citrate were accurately weighed, transferred to a 1000 mL volumetric flask and dissolved in ultrapure water. The simulated humor vitreous solution was filtered through a 0.45 μm filter (Sartorius, Germany).

Table 4: Effects of the analytical parameters in percent recovery of acyclovir of the HPLC-UV method

Analytical parameter		Recovery of acyclovir (%)	Effect X – x (absolute value)*
Washing volume of the syringe of injection (μL)	A	98.88	0.06
	a	98.82	
Wavelength (nm)	B	98.44	–0.83
	b	99.27	
Mobile phase flow rate (mL/min)	C	98.83	–0.05
	c	98.87	
Proportion of the mobile phase (0.02 mol/L acetic acid and methanol)	D	98.73	–0.25
	d	98.98	
Injection volume (μL)	E	99.03	0.37
	e	98.67	
Temperature of sample compartment (°C)	F	98.90	0.10
	f	98.80	
Filter unit	G	100.87	4.04
	g	96.83	

* Difference between average of the values obtained at nominal conditions and average of the values obtained at altered conditions.

3.3.2. Stock solution of acyclovir

Stock solution of acyclovir of 1.75 mg/mL was prepared by accurately weighing 43.75 mg of acyclovir reference standard into a 25 mL volumetric flask and dissolving it in 0.1 mol/L sodium hydroxide.

3.3.3. Standard solution of acyclovir

Aliquots of the stock solution of acyclovir were diluted with simulated humor vitreous solution to obtain solutions with different concentrations of acyclovir. The concentration of acyclovir in each standard solution was defined in the description of the validation parameters. Each standard solution of acyclovir was filtered through a 0.45 μm filter (Sartorius, Germany).

3.3.4. Vitreous humor from rabbits' eye

Rabbits were sacrificed using a lethal dose of intraperitoneal injection of pentobarbital 70 mg/kg (Euthanyle; Brouwer, Buenos Ayres, Argentina) (n = 5). Vitreous humor samples of rabbits' eye were collected by inserting a needle into the lateral canthus of each eye. All the vitreous humor in each eye was aspirated, and care was taken to avoid damage to any loose tissue fragments around the vitreous chamber. The obtained samples were placed into polystyrene conical tubes and kept at –18 °C without any preservatives until analysis (Cabarcos et al. 2010). The collected vitreous humor was filtered through a 0.45 μm filter (Sartorius, Germany).
The collection of the humor vitreous from rabbits' eye was performed in accordance with stipulations set forth by Ethics Committee in Animal Experimentation of the Fundação Ezequiel Dias, which gave approval to the protocol.

3.3.5. Vitreous humor from rabbits' eye spiked with stock solution of acyclovir

An aliquot of 10 mL of the stock solution of acyclovir was transferred to a 25 mL volumetric flask and dissolved in humor vitreous solution collected from rabbits' eye. The spiked solution of 700 μg/mL of acyclovir was filtered through a 0.45 μm filter (Sartorius, Germany).

3.4. Method validation

The method was validated for specificity, linearity, precision (repeatability and intermediated precision), limits of quantitation and detection, accuracy and robustness in accordance with standard procedure (ICH 2005).

3.4.1. System suitability

System suitability was carried out by making six replicate injections of a standard solution containing 700 μg/mL of acyclovir prior to sample analyses. The acceptance criterion was a resolution greater than 2 between the chromatographic peaks of acyclovir and humor vitreous (The United States Pharmacopeia 2008). Additionally, the limits for system suitability were set for the theoretical plates, tailing factor and retention time of acyclovir.

3.4.2. Specificity

The chromatographic peaks of following solutions were recorded: (1) standard solution of acyclovir at 700 μg/mL; (2) simulated vitreous humor solution; (3) collected vitreous humor from rabbits' eye; (4) vitreous humor from rabbits' eye spiked with stock solution of acyclovir at 700 μg/mL. To achieve the specificity of the method, no peak, with the same retention time of acyclovir, was allowed. Additionally, spectral purity of the chromatographic peak of acyclovir was evaluated using the UV spectra recorded by a diode array detector (DAD).

3.4.3. Linearity

The calibration curve was obtained using five standard solutions in different concentrations of acyclovir (35, 105, 175, 350 and 700 μg/mL) in 3 independent replicates run in random order. These assays were performed on 2 different days. The calibration curves constructed were assessed using residue analysis (homoscedascity, normality, and independence of residues) and linear regression analysis was done by the ordinal least squares method (Souza and Junqueira 2005).

3.4.4. Sensitivity

The sensitivity of the method was evaluated by determining the lower limit of quantification (LOQ) and the detection limit (LOD). The LOQ was set as the lowest acyclovir concentration that could be determined with adequate precision and accuracy, whereas the LOD was the lowest acyclovir concentration that could be detected but not quantified under the stated experimental conditions (Causon 1997; Santana 2004). The LOQ and LOD were calculated by using the standard deviation and the slope of the calibration curve and the Eqs. (1) and (2), respectively:

$$\mathrm{LOQ} = 10\,\sigma/b \tag{1}$$

$$\mathrm{LOD} = 3\,\sigma/b \tag{2}$$

where σ is the standard deviation of the response and b is the slope of the calibration curve.

3.4.5. Precision

The precision of the method was determined based on repeatability (intra-day) and intermediate precision (inter-day). Repeatability was assessed through the assay of standard solutions of acyclovir at concentrations of 35, 70, and 700 μg/mL on the same day (n = 6 for each concentration). Intermediate precision was verified by evaluating the results on 2 different days (n = 6 for each concentration). The precision was expressed as relative standard deviation (RSD) amongst responses.

3.4.6. Accuracy

Standard solutions of acyclovir at 35, 70 and 700 μg/mL were prepared and analyzed. Moreover, humor vitreous from rabbits' eye spiked with known amount of acyclovir performing 35, 70 and 700 μg/mL of the drug were also prepared and analyzed. Solutions were prepared in triplicate with 5 injections of each solution (n = 15). The percent recovery of added acyclovir was calculated comparing peak areas of the resultant solutions with standard solutions of acyclovir at the same concentration.

3.4.7. Robustness

The method proposed by Youden and Steiner (1975) was carried out to evaluate the robustness. Seven analytical parameters were selected and investigated at two levels as indicated by capital letters (nominal values) and lowercase letters (conditions with small variation in nominal values) in Table 5. Eight runs were performed following the experimental design of Youden and Steiner in order to determine the influence of each parameter in the final result.
The standard solution of acyclovir at 700 μg/mL and the vitreous humor from rabbits' eye spiked with stock solution of acyclovir at 700 μg/mL were injected three times for each combination. In each combination, the recovery of the spiked acyclovir was analyzed.
The results of each experiment were represented by letters ranging from s to z (Table 5). To estimate the effect of each variable in the final result, the difference between the mean of the four values corresponding to the capital letters (nominal conditions) and the mean of the four values corresponding to the lowercase letters (altered conditions) was calculated (Youden and Steiner 1975). Thus, to evaluate the influence, for example, of wavelength in the final result of the analyses, Eq. (3) was used as given below:

$$\text{Effect G/g} = \frac{(\mathrm{s} + \mathrm{v} + \mathrm{x} + \mathrm{y})}{4} - \frac{(t + u + w + z)}{4} \tag{3}$$

The effect of the analytical parameter was considered to be significant if the value of the difference was greater than $\left(S\sqrt{2}\right)$, where, S is the standard deviation of the eight results (Bedregal et al. 2008).

3.5. Application of the validated HPLC-UV method

Acyclovir directly injected into the vitreous cavity of rabbits' eye was quantified by applying the validated HPLC-UV method. The percent of acyclovir in the collected vitreous humor was calculated comparing peak areas of the resultant solutions with standard solutions of acyclovir at 700 μg/mL.
Briefly, rabbits were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketamina; Agener, São Paulo, Brazil) and 6.7 mg/kg xylazine hydrochloride (Calmiun; Agener, São Paulo, Brazil). Pupils were dilated with topical 0.5% tropicamide (Mydriacyl; Alcon, São Paulo, Brazil) and the eye anesthetized with 0.5% proxymetacaine hydrochloride (Anestalcon; Alcon, São Paulo, Brazil) (n = 3). Before the injection of acyclovir, anterior chamber paracentesis (0.1 mL of aqueous humor) was performed with a 27-gauge needle to avoid increase in intraocular pressure and to minimize drug reflux. Intravitreal injection was performed using a 30-gauge needle attached to a 1- mL tuberculin syringe inserted approximately 3 mm posterior to the limbus, and 0.1 mL of acyclovir (10 mg/mL) was slowly injected directly into the vitreous. The right eye of each rabbit was injected with the acyclovir solution and the left eye with saline as control.
After 2 days of injection, rabbits were sacrificed using a lethal dose of intraperitoneal injection of pentobarbital 70 mg/kg (Euthanyle; Brouwer,

Table 5: Parameters, variation and factorial combination for robustness test studies

Analytical parameter	Value (X/x)		Factorial combination							
			1	2	3	4	5	6	7	8
	A	a								
Washing volume of the syringe of injection (μL)	300	500	A	A	A	A	a	a	a	a
	B	b								
Wavelength (nm)	256	252	B	B	b	b	B	B	b	b
	C	c								
Mobile phase flow rate (mL/min)	1.30	1.10	C	c	C	c	C	c	C	c
	D	d								
Proportion of the mobile phase (0.02 mol/L acetic acid and methanol)	95.5:4.5	94.5:5.5	D	D	d	d	d	d	D	D
	E	e								
Injection volume (μL)	22	18	E	e	E	e	e	E	e	E
	F	f								
Temperature of sample compartment (°C)	26	24	F	f	f	F	F	f	f	F
	G	g*								
Filter unit	cellulose	PVDF	G	g	g	G	g	G	G	g
Results			s	t	u	v	w	x	y	z

*PVDF – Polyvinylidene fluoride

Buenos Ayres, Argentina). Vitreous humor samples of rabbits' eye were collected by inserting a needle into the lateral canthus of each eye. All the vitreous humor in each eye was aspirated, and care was taken to avoid damage to any loose tissue fragments around the vitreous chamber. The obtained samples were placed into polystyrene conical tubes and kept at –18 °C without any preservatives until analysis (Cabarcos et al. 2010). The collected vitreous humor was filtered through a 0.45 μm filter (Sartorius, Germany).

The collection of the humor vitreous from rabbits' eye containing acyclovir was performed in accordance with stipulations set forth by Ethics Committee in Animal Experimentation of the Fundação Ezequiel Dias, which gave approval to the protocol.

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