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Title: Determination of triamcinolone acetonide in silicone oil and aqueous humor of vitrectomized rabbits' eyes: Application for a pharmacokinetic study with intravitreal triamcinolone acetonide injections (Kenalog® 40)

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PII: S0731-7085(13)00484-6
DOI: http://dx.doi.org/doi:10.1016/j.jpba.2013.10.025
Reference: PBA 9306

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received date: 3-6-2013
Revised date: 11-10-2013
Accepted date: 15-10-2013

Please cite this article as: G.M. Fernandes-Cunha, J.B. Saliba, R.C. Siqueira, R. Jorge, A. Silva-Cunha, Determination of triamcinolone acetonide in silicone oil and aqueous humor of vitrectomized rabbits' eyes: Application for a pharmacokinetic study with intravitreal triamcinolone acetonide injections (Kenalog*regd* 40)., *Journal of Pharmaceutical and Biomedical Analysis* (2013), http://dx.doi.org/10.1016/j.jpba.2013.10.025

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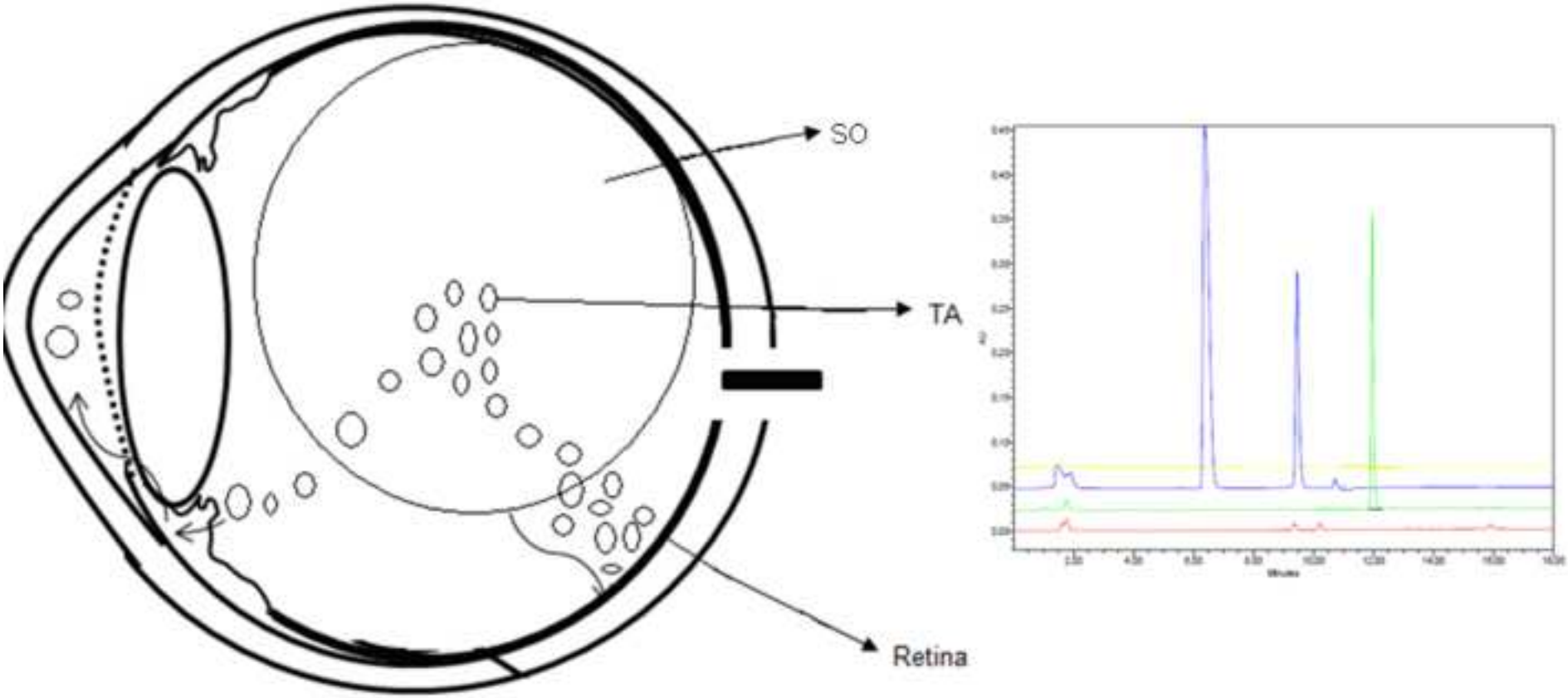
***Graphical Abstract**



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- High-performance liquid chromatography method was developed and validated.
- Administration of TA injections in rabbits' eyes.
- Method was successfully applied to quantify the drug in SO and aqueous humor.
- TA remained in SO and aqueous humor of rabbits' eyes for 4 weeks.
- SO may play an important role in the elimination of lipophilic drugs.

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Determination of triamcinolone acetonide in silicone oil and aqueous humor of vitrectomized rabbits' eyes: Application for a pharmacokinetic study with intravitreal triamcinolone acetonide injections (Kenalog® 40).

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ABSTRACT

A simple and accurate method including liquid-liquid extraction and protein precipitation procedures from silicone oil and aqueous humor samples followed by high-performance liquid chromatography (HPLC-UV) was developed and validated to determine the pharmacokinetic profile of triamcinolone acetonide in silicone oil and aqueous humor of rabbits' eyes submitted to the *pars plana* vitrectomy surgery. The method was successfully applied to quantify the drug remaining in silicone oil and aqueous humor (LOQ range of 1µg/mL). The triamcinolone acetonide remained in

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silicone oil and aqueous humor of vitrectomized rabbits' eyes for four weeks after the intravitreal injections.

Keywords: Silicone oil, Triamcinolone acetonide, Intravitreal injections, Vitrectomy, HPLC-UV.

1. Introduction

The *Pars Plana* vitrectomy surgery, in which the humor vitreous is replaced with an internal tamponade, the silicone oil (SO), is performed in order to repair the retinal detachment caused by proliferative vitreoretinal diseases [1,2]. The intravitreal injections of triamcinolone acetonide (TA) (Fig. 1), a synthetic lipophilic corticosteroid with low solubility in aqueous solution, are applied to overcome surgical complications [3-5]. However, the quantity of the drug present in the SO after vitrectomy surgery is unknown, possibly leading to inadequate therapy.

INSERT FIGURE 1

Some methods have been reported for determining TA in rabbit and human's eyes. Oliveira et al determined TA concentration in humor vitreous of rabbits' eyes by HPLC-UV using a C-18 column and ACN/H_2O (60:40 v/v) as mobile phase [6]. However, this method was applied to quantify TA only in humor vitreous therefore modifications need to be done to quantify the drug in different matrices. In other study, Beer et al applied a HPLC-MS method to determinate TA in aqueous humor of human's eyes [7]. Regardless the high sensitivity of HPLC-MS method, the major limitation of this kind of analysis is the matrix effects in which the matrix coextracted with the analyte can alter the signal response. Furthermore, the methods using HPLC-MS are expensive and not readily available in all laboratories [8]. In this study, we chose to develop a HPLC-UV method to quantify TA in SO and aqueous humor of rabbit's eyes,

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since it is simple to perform, it is cost and time-effective and have low limits of detection.

Thus, in the present work an easy, manageable and rapid HPLC-UV method combined with SO drug extraction and aqueous humor protein precipitation for quantifying TA in vitrectomized rabbits' eyes was developed and validated. The method was applied for *in vivo* study in which rabbits groups received injections of TA.

2. Experimental

2.1 Chemical and reagents

TA reference standard was purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). SO (5000 cTs) was obtained from Ophthalmos (São Paulo, SP, Brazil). TA aqueous suspension was purchased from (Bristol-Myers, NJ, USA). Ultra-pure water was obtained from a Millipore system (Bedford, MA, USA). Acetonitrile and methanol (HPLC grade) were purchased from Tedia (Fairfield, OH, USA) and ethyl acetate (HPLC grade) was obtained from Vetec (Rio de Janeiro, RJ, Brazil).

2.2 Instrumentation and analytical conditions

The reversed-phase HPLC system was a Waters apparatus (Massachusetts, USA) equipped with a 717 plus autosampler model, consisting of a 515 pump and a 486 ultraviolet detector. Data collection and integration were achieved using Enpower (version 6.2) software. The analytical column used was a Chromolith® Merck C_{18} (5 µm particle size; 100 x 4.6 mm i.d). The mobile phase used for TA quantification consisted of HPLC water (A) and acetonitrile (B). Separation was carried out applying a gradient elution at ambient temperature using a flow rate 1.0 mL/min. The mobile phase combinations were: 0 min, 90% A, 10% B; 10 min, 90→50% A, 10→50% B; 5 min of isocratic elution; 50% A, 50% B; 3 min, 50→90% A, 50→10% B. A re-equilibration

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interval of 15 min in the initial conditions was introduced between subsequent analyses. Detection was achieved at 239 nm.

2.3 Preparation of standard solution

Stock solution of TA was prepared by dissolving the accurately weighed reference substance in methanol. The working solution of TA was prepared immediately before the use by diluting the stock solution to a final concentration of 40 µg/mL.

2.4 Extraction of TA from SO

First, 300 µL of ethyl acetate were added in a plastic tube containing 100 µL of SO. This solution was mixed in a vortex for 5 minutes. Then, an aliquot (1000 µL) of TA working solution was transferred to the plastic tube and mixed in a vortex for 5 minutes. After that, the final solution was evaporated during 48 hours. The extraction consisted of the addition of different solvents such as acetonitrile, methanol and water to 100 µL of evaporated SO. Different extraction times were analyzed (5, 10, 15 min) as well as different quantity of solvents (500 µL two times, and 1000 µL). The mixture was stirred for 5 minutes and centrifuged at 300 x g for 5 minutes. The supernatant was then collected, filtered and transferred to a vial. A 20 µL aliquot was injected into the chromatographic system.

2.5 Samples preparation

A 500 µL aliquot of acetonitrile was added to the TA SO and aqueous humor samples. The samples were vortex mixed for 5 minutes and centrifuged at 300 x g for 5 minutes at ambient temperature. The supernatant was collected, filtered and lyophilized. The residue from lyophilization was resuspended in 100 µL of acetonitrile. A 20 µL aliquot was injected into the HPLC system to determinate the amount of TA.

2.6 TA method validation

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The validation process was carried out as described in the literature [9]. TA method selectivity was assayed by injection of SO and aqueous humor blank extracted samples and by the injection of TA blank injectable suspension. TA method linearity was assessed by six and five-point calibration curves in methanol in triplicate in three consecutive days. The concentration range evaluated was 1.0-120.0 µg/mL. The curves were evaluated by residuals and fitted by weighted linear regression. The LOQ was established analyzing the means of six replicates. The LOD was defined as the concentration giving a sign-to-noise ratio (S/N) of 3. The intra-day precision was evaluated by RSD values of sample solutions analyzed on the same day (n=6), at the middle concentration of the calibration curves, whereas inter-day precision was accessed by analyzing sample solutions prepared on two different days, by two different analysts (n=12). The extraction recovery of the method was determined by comparing the peak areas obtained from the SO samples with those of direct injected standards, at the same concentration. The evaluation was done by analyzing five replicates containing 1, 40, 120 µg/mL of TA. The stability of the analyte in SO was evaluated using the working solution in six replicates. The drug was left in ambient temperature for 72 hours in SO. The stability of the drug during the run-time in the HPLC auto-injector was investigated of one concentration levels 40 µg/mL. Samples were prepared and kept in the sample rack of the auto-injector and injected into the HPLC system 24h after preparation. Then the stability was investigated by analyzing the concentrations found.

2.7 Application to a pharmacokinetic study

The validated method was used to determine the concentration of TA in rabbits' vitrectomized eyes after administration of the intravitreal injection of TA. Nine New Zealand albino adult male rabbits weighing 2.0-2.5 Kg were used for *in vivo* studies. All experimental procedures involving animals were performed in agreement with the

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Ethics Committee of Universidade de São Paulo (USP-Ribeirão Preto) and according to the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research. They were placed in two groups; group I (n=3) underwent standard *pars plana* vitrectomy with the injection of 1000 µL of SO and served as experiment control; group II (n=6) underwent standard *pars plana* vitrectomy with the injection of 1000 µl of SO and 100 µl of TA (Kenalog®40; 40 mg/mL) were injected intravitreally just after the surgery was over. In group I and II SO and aqueous humor were collected from vitreous cavity and anterior cavity at 1 and 4 weeks after the administration of the drug by intravitreally injection. The samples were immediately placed in eppendorf tubes and stored at -20°C until HPLC-UV analysis.

3. Results and discussion

3.1 Conditions for HPLC-UV

The selectivity aspect was accessed by analyzing whether blank solutions interfere at TA retention time. No interfering peaks were detected in the analyte peak region (Fig. 2). The retention time of TA was approximately 12.3.

INSERT FIGURE 2

To date, the exact TA amount present in the vitreous cavity after vitrectomy surgery is not known. In this study we presented a combination of a HPLC-UV method with SO extraction which provides an exact amount of the drug in the vitreous cavity.

3.2 Development of the procedure for sample extraction

We have chosen the ethyl acetate as the ideal solvent once it was able to solubilize both constituents in order to obtain a dispersed system. The extraction studies were conducted by testing three solvents (acetonitrile, methanol and water). The solvent that provided highly efficient separations, a good symmetric peak and recovery rates higher than 90% was acetonitrile. The best recovery was achieved with

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two aliquots of 500 µL, which can be explained based on the greater contact surface between the drug and the solvent. No significant differences were found between the different times therefore the 5 min time was chosen because it was the shortest time. The recovery method here developed is simple, robust and efficient, resulting in a fast and easily-handled analysis.

3.3 Method validation

No significant interference was detected in the retention times of the analyte in the chromatograms (Fig. 2). Calibration curves were shown to be linear over the range 1.0-120.0 µg/mL. Typical standard curve was $y=4.05 \times 10^{-4}x + 4.33 \times 10^{-4}$, with a weighted factor 1/*x*. Regression coefficient was 0.9992, showing an excellent correlation. Linearity data are presented in Table 1. The obtained LOQ and LOD were respectively 1.0 µg/mL and 0.2 µg/mL. The obtained data for intra-run and inter-run precision and accuracy are shown in Table 2. The mean R.S.D. values in the intra-run precision was 2.17% and inter-run precision was 1.22% and. The mean accuracy value in the intra-run assay was 92.0% and in the inter-run assay was 85.5%. The mean recovery rates of TA (n=18), determined at three concentrations, was 101% (Table 3). The results of stability experiments showed that there were no significant degradation of TA in SO samples following 72 hours after extraction and no significance decomposition was observed after the reconstituted samples of TA had been stored in auto injector at room temperature for 24 h. The measure concentration after 24h and 72h were all > 100% of the initial values for the drug at the concentration of 40 µg/mL.

INSERT TABLE 1

INSERT TABLE 2

INSERT TABLE 3

3.4 Application to a pharmacokinetic study

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The HPLC method was successfully applied to determine the TA in a pharmacokinetic study in rabbits' vitrectomized eyes. Fig. 3 shows the concentrations of intravitreal TA in SO and aqueous humor (group II). The mean concentration of intravitreal TA that remained in SO was of 60.76 ± 1.6 µg/mL (0.15% of initial injection concentration) and 28.20 ± 8.7 µg/mL (0.07%), and in aqueous humor it was of 3.64 ± 1.6 µg/mL and 7.35 ± 0.9 µg/mL on weeks 1 and 4 respectively. In this study, we showed the remaining TA concentration, both in aqueous humor and in SO in vitreous cavity, which can provide additional information to develop a more reliable TA injections intervals and initial dosage. Additionally, we obtained a TA half-life values of 3.5 days in the vitreous cavity of vitrectomized rabbits' eyes whereas in other study it was found a higher TA half-life in non-vitrectomized rabbits' eyes (8 ± 2,8 days) after an initial dosage of 4mg/mL [6]. Our results have lead us to affirm that the TA is cleared 2.3 times faster than in non-vitrectomized eye cavity, suggesting that the SO plays an important role in the TA clearance kinetic profile.

INSERT FIGURE 3

4. Conclusion

To our knowledge, this is the first method for quantifying TA in SO using HPLC-UV. The method was validated and showed to be robust and reproducible allowing elucidating the *in vivo* pharmakocinetic of TA in vitrectomized eyes. We successfully showed that the SO may play an important role in the elimination of lipophilic drugs which is additional information when planning intravitreal injection of TA in vitrectomized eyes. In this work, we suggest that the SO potentially can interfere in the elimination of lipophilic drug administrated in vitreous cavity.

Acknowledgements

This publication was made possible by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil), Fundação de Amparo à

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Pesquisa de Minas Gerais (FAPEMIG/Brazil) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Competing Interests

The authors declare that they have no competing interests.

References

[1] W.F. Smiddy, M.W. Flynn, Vitrectomy in the management of diabetic retinopathy, Survey of Ophthalmology. 43 (1999) 491-501.

[2] G.W. Abrams, S.P. Azen, B.W. McCuen, H.W. Flynn, M.Y. Lai, S.I. Ryan, Vitrectomy with silicone oil or long-acting gas in eyes with severe proliferative vitreoretinopathy: results of additional and long-term follow-up. Silicone Study report 11, Arch Ophthalmol. 115 (1997) 407-408.

[3] H. Lewis, T.M. Aaberg, Causes of failure after repeat vitreoretinal surgery for recurrent proliferative vitreoretinopathy, Am J Ophthalmol. 111 (1991) 15-19.

[4] J.B. Jonas, Intravitreal injection of crystalline cortisone as adjunctive treatment of proliferative vitreoretinopathy, Br J Ophthalmol. 87 (2000) 1064-1071.

[5] W.M. Munir, J.S. Pulido, M.C. Sharma, B.M. Buerk, Intravitreal triamcinolone for treatment of complicated proliferative diabetic retinopathy and proliferative vitreoretinopathy, Canadian Journal of Ophthalmology. 40 (2005) 598-604.

[6] R.C. Oliveira, R.C. Siqueira, M.A. Bonini-Filho, A. Haddad, F.M. Damico, A. Maia Filho, P.T.B.Crispim, J.B. Saliba, J.A.S. Ribeiro, I.U. Scott, A. Silva-Cunha, R. Jorge, Vitreous pharmacokinetics and retinal safety of intravitreal preserved versus non-preserved triamcinolone acetonide in Rabbit Eyes, Current Eye Research. 37 (2012) 55-61.

[7] P.M. Beer, S.J. Bakri, R.J. Singh, W. Liu, G.B. Peters, M. Miller, Intraocular concentration and pharmacokinetics of triamcinolone acetonide after a single intravitreal injection, Ophthalmology. 110 (2003) 681-686.

ACCEPTED MANUSCRIPT

231 [8] V. Michael, S. Christoph, A decade of HPLC–MS/MS in the routine clinical
232 laboratory — Goals for further developments, Clinical Biochemistry. 41 (2008) 649-662.
233 [9] Food and Drug Administration. FDA guidance for Industry: Analytical Procedures
234 and Methods Validation, U.S. Department of Health and Human Services, Food and
235 Drug Administration, Center for Drug Evaluation and Research, Rockville, 2000.

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Table 1
Precision and accuracy data of back calculated concentrations of calibrations samples for triamcinolone acetonide in organic solvent.

Analyte	Nominal concentrations(µg/mL)	Observed concentration (µg/mL)	Precision (%R.S.D.)	Accuracy (%)
Triamcinolone acetonide	1 10 20 40 80 120	1.1 10.1 20.5 41.8 78.3 120.4	2.6 4.0 2.3 4.1 1.6 3.9	109.0 101.1 102.3 104.6 97.9 100.3

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Table 2

Precision and accuracy data for triamcinolone acetonide in organic solvent by HPLC-UV.

Analyte	Assay	Nominal concentration (µg/mL)	Observed concentration (µg/mL, mean±S.D.)	Precision (%R.S.D.)	Accuracy (%)
Triamcinolone acetonide	Intra-run (n=6)	40.0	36.8 ± 0.8	2.8	92.0
	Inter-run (n=12)	40.0	34.2 ± 0.7	2.2	85.5

S.D., standard deviation; R.S.D., relative standard deviation

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Table 3

Recovery data for triamcinolone acetonide from silicone oil by HPLC-UV.

Analyte	Nominal concentration (µg/mL)	Recovery (%)	%R.S.D.	Mean recovery (%)
Triamcinolone acetomide *(n*=9*)*	1.0 20 80	97.9 105.1 99.9	2.1 1.8 3.8	101.0

R.S.D., relative standard deviation.

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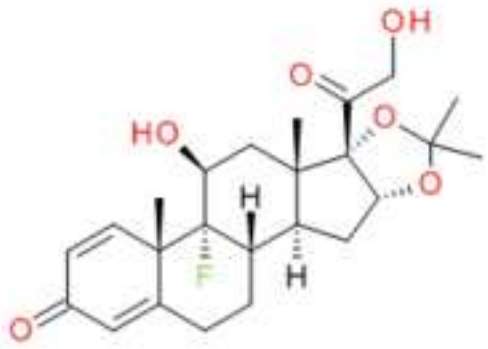
Figure(s)



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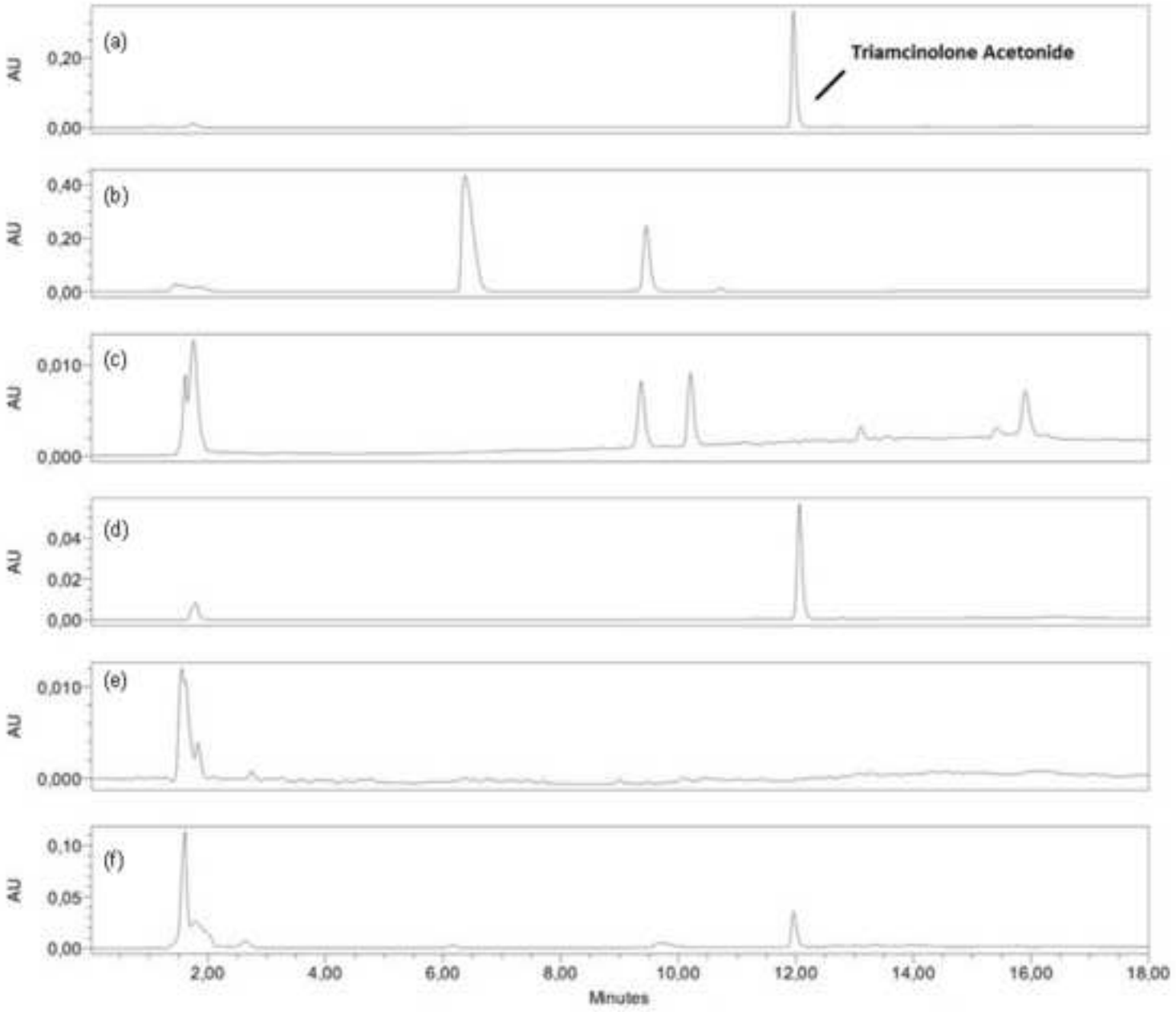
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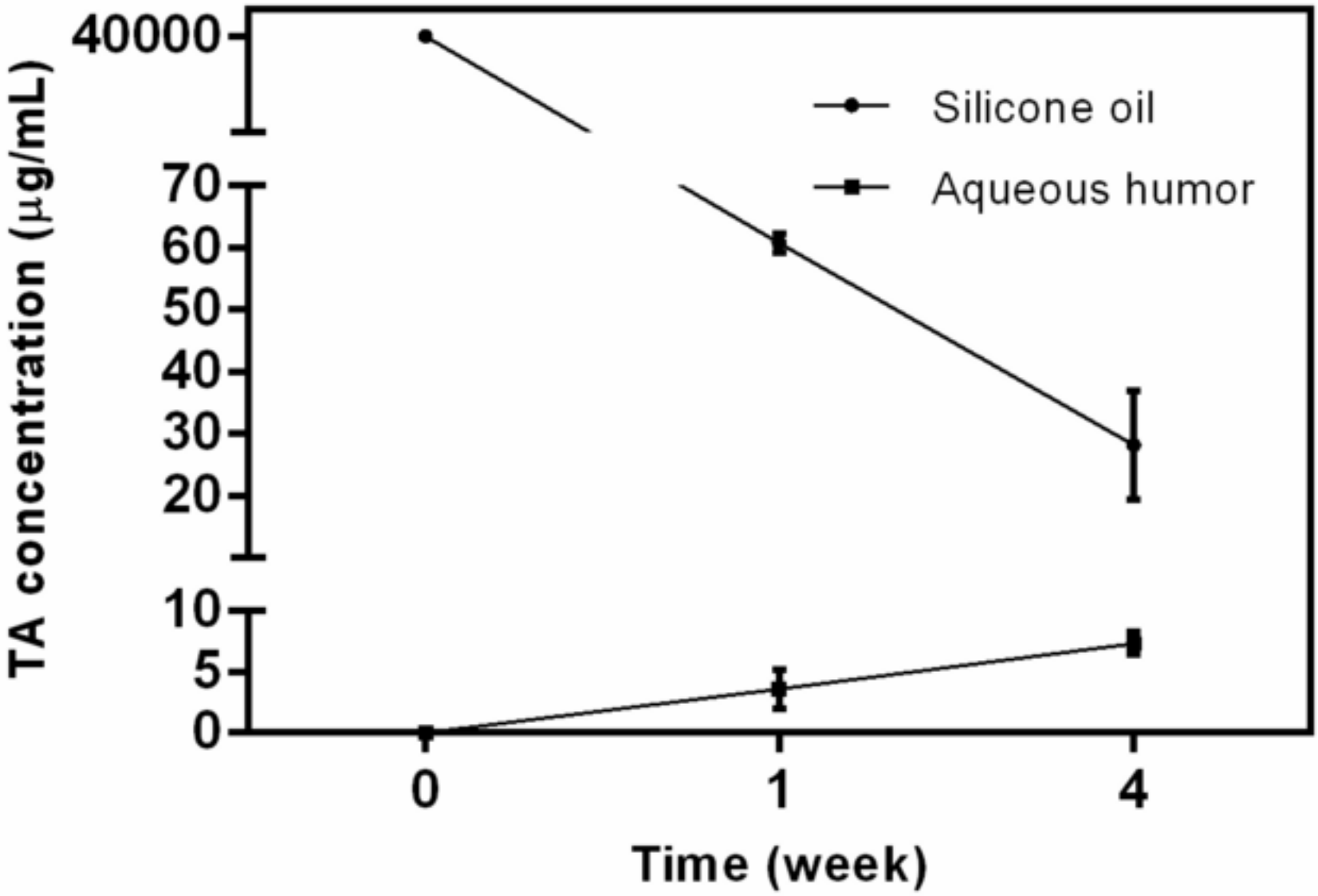
Figure(s)


40000
70
60
50
40
30
20
10
5
0
TA concentration (μg/mL)
Silicone oil
Aqueous humor
0
1
4
Time (week)

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FIGURES CAPTION

Fig. 1. Chemical structure of triamcinolone acetonide.

Fig. 2. Representative chromatogram of: (a) triamcinolone acetonide standard (b) triamcinolone acetonide blank formulation (c) extraction of silicone oil blank sample (d) extraction of triamcinolone acetonide from silicone oil sample (e) aqueous humor blank sample (f) triamcinolone acetonide from aqueous humor sample. The chromatograms demonstrated no interfering peaks co-eluted with the compound of interest.

Fig. 3. Triamcinolone acetonide profile elimination after an intravitreal injection of 40mg/mL during the follow-up 4 weeks. TA concentrations (µg/mL) are shown in silicone oil and aqueous humor. The values are shown as mean ± standard deviation, n = 3.

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