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Short communication

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 1 μg/mL). The triamcinolone acetonide remained in silicone oil and aqueous humor of vitrectomized rabbits' eyes for four weeks after the intravitreal injections.

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.

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, 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

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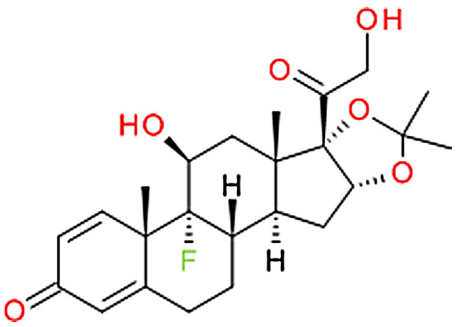

Fig. 1. Chemical structure of triamcinolone acetonide.

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 mm × 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 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 min. Then, an aliquot (1000 μL) of TA working solution was transferred to the plastic tube and mixed in a vortex for 5 min. After that, the final solution was evaporated during 48 h. 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 min and centrifuged at 300 × *g* for 5 min. 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 min and centrifuged at 300 × *g* for 5 min 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

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 R.S.D. 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 h 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 24 h 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 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.

To date, the exact TA amount present in the vitreous cavity after vitrectomy surgery is not known. In this study we presented a