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Measuring triamcinolone acetonide in aqueous humor by gas chromatography-electron-capture negative-ion mass spectrometry

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Abstract

Intravitreal triamcinolone acetonide (IVTA) injection has been used in the treatment of various posterior segment diseases. One of the side effects of IVTA is raised intraocular pressure, which may be secondary to triamcinolone acetonide (TAA)'s effects on the trabecular meshwork that affects aqueous outflow. In order to study the biological effects of TAA on the trabecular meshwork, we firstly need to reliably and accurately detect the concentration of TAA in tissue cells or fluids. In this study we have described a technique of using gas chromatography-electron-capture-negative-ion mass spectrometry (GC–NCI–MS) to develop a simple, sensitive, selective and validated method to detect TAA in aqueous humor (AH) of rabbits following IVTA and subconjunctival TAA injections. We derivatized TAA from extracted aqueous sample by acetic anhydride and BSTFA, respectively, and analyzed by GC–NCI–MS. The detection limit was 0.3 ng/ml, linearity over 0.995 from 0 to 300 ng/ml. The reproducibility ranged from 10.4 to 3.9 for concentrations from 3 to 300 ng/ml, and recovery was over 95% for the concentrations 10, 60, and 200 ng/ml. No interference was found from 159 aqueous samples. There was no TAA residue carried to the next injection from previously high concentration injection, 10,000 ng/ml. We have provided an alternative, rapid, and robust method other than LC–MS–MS for TAA detection in AH.

Keywords: Triamcinolone acetonide; Aqueous humor; GC–NCI–MS

1. Introduction

Intravitreal triamcinolone acetonide (IVTA) has been used in the treatment of various ophthalmic conditions including uvetitis [1,2], proliferative diabetic retinopathy [3], intraocular fibrovascular proliferation [3], choroidal neovascularization [4], and diabetic macular edema [5]. Some studies have also suggested that IVTA could be helpful in anterior ocular diseases such as iris neovascularization and proliferative ischaemic retinopathies [6]. However, pseudo-endophthalmitis with triamcinolone acetonide (TAA) crystals has been found in the anterior chamber following intravitreal TAA injection [3]. In order to study the feasibility of TAA treatment in anterior ocular diseases and any side effects such as crystallization in the anterior chamber and an increase in intraocular pressure [7], a rabbit animal model was used for this study. We investigated the level of TAA in the aqueous humor (AH) following IVTA and subconjunctival TAA injections. However in order to do this, a sensitive, specific, and validated TAA detection method was required.

Many published methods for bioanalysis of TAA, as other steroids, use triple quadrapole mass spectrometry [8–14], which, however, is expensive and not commonly available in routine laboratories. The methods were based on published procedures for corticosteroid analysis in urine or serum but not established specifically for TAA in AH [10,11]. Only a few published methods employ a more common routinely available laboratory equipment, gas chromatography mass spectrometry with electron capture negative ion (GC–NCI–MS), for TAA bioanalysis in urine [15] or bronchoalveolar lavage fluids [16]. GC–NCI–MS has the advantages of lower capital cost when compared with triple quadrapole MS, low background noise, high sensitivity, and fewer fragmentations for molecular ion determination. The disadvantages of these published methods are long processing

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time and that they are labor intensive. They are not designed for AH TAA analysis. We have aimed to develop a rapid, sensitive, accurate, and validated method for TAA determination in AH by GC/MS.

2. Experimental Methods

2.1. Standard and reagents

TAA, BSTFA were purchased from Sigma (St. Louis, MI). Heptadeuterated TAA ($^2H_7$-TAA) was brought from C/D/N Isotopes Inc. (Pointe Claire, Quebec, Canada). The isotopic purity of the deuterium atoms in the 16α, 17α-isopropylidenedioxy substituent was more than 99%, while the isotopic purity of the deuterium atom at C6 was 74%. Acetic anhydride and ethylacetate were obtained from BDH (Poole, UK). BSTFA-TMCS mixture and Power Sil Prep were purchased from Alltech (Deerfield, IL). Anhydrous sodium sulphate was purchase from Fluka (GMbH, Buchs). Pyridine came from RDH (Seelze, Germany). All other reagents were purchased as highest grade.

2.2. Animal sampling

New Zealand albino rabbits (Charles River Canada, St-Constant, Quebec, Canada) aged 6 months, mean initial weight of 3.6 kg (range from 3.1 to 4.0 kg), were acclimatized under a 12-h light–12-h dark cycle with standard chow and water provided ad libitum for 1 week. The animals were divided into two groups. Group A (5 rabbits) received 12 mg TAA intravitreally, group B (5 rabbits) received 12 mg TAA subconjunctivally. TAA injections and AH sampling were performed on the right eye of each rabbit. The left eye served as control. The AH was collected on days 0 (before injection), 1, and 10. All intravitreal injections were performed under general anesthesia by intramuscular injection of xylazine (5 mg/kg, TranquiVed; Vedco Inc., St. Joseph, MO) and ketamine (35 mg/kg, Ketaset; Fort Dodge Animal Health, Fort Dodge, IA). On day 0, 12 mg of 0.1 ml TAA (Kenalog-40, Bristol-Myers Squibb, Princeton NJ) was injected intravitreally. Each injection was performed via a 30-gauge needle. The injection site was 3 mm behind the limbus superotemporally and the needle was directed towards the centre of the globe. The needle was withdrawn 30 seconds after injection to prevent any reflux. TAA was visualized in the vitreous cavity immediately after the injection. All subconjunctival TAA injections were performed under general anesthesia by intramuscular injections of xylazine (5 mg/kg, TranquiVed; Vedco Inc., St. Joseph, MO) and ketamine (35 mg/kg, Ketaset; Fort Dodge Animal Health, Fort Dodge, IA). Injection site was 1 mm behind the limbus superotemporally. Also, on day 0, group B rabbits were injected with 12 mg in 0.3 ml TAA subconjunctivally. All AH samplings were performed under general anesthesia. A 30-gauge insulin needle and syringe entered the corneal limbus superonasally and about 0.1 ml AH was taken. Fifty microlitres of the sample was aliquoted in two 500 μl tubes, wrapped with foil and snapped frozen in nitrogen. Ocular chloramphenicol ointment (Alcon, Fort Worth, TX) was given once immediately after each aqueous sampling or TA injection. Guidelines as set by The Chinese University of Hong Kong were followed for the use and care of laboratory animals.

2.3. Chromatography and mass spectrometry

TAA was chromatographed with a 30 m × 0.25 mm × 0.25 μm DB-5MS capillary column on an Agilent 6890 plus series gas chromatograph system, equipped with an Agilent 7683 autosampler and interfaced to an Agilent 5973 N mass spectrometer in negative chemical ionization (NCI) mode (Agilent, CA). The reagent gas used was methane at a flow rate of 2 ml/min, and the carrier gas was helium at a flow rate of 1 ml/min. Data were acquired and analyzed by an Enhance Chemstation, version C.00.00. Injection mode was splitless with inlet temperature 280 °C for 1 min and split at 1:50 afterward. Injection volume was 1 μl. The GC oven temperature program was initially set at 160 °C for 1 min, then increased to 220 °C at 40 °C/min, then to 310 °C at 10 °C/min, and maintained at this temperature for 10 min. The transfer line temperature was kept at 310 °C. Ion source temperature and quadrupole temperature were both set at 150 °C. Selected Ion Monitoring mode was used to detect TAA and $^2H_7$-TAA at 548.2 and 555.2 amu, respectively. Electronic multiplier offset was 400 mV. The dwell time was 70 ms for each mass.

2.4. Standard and sample preparation

Ten milligram internal standard, $^7H_2$-TAA, was dissolved in 5 ml ethylacetate and stored at −80 °C as stock solution. It was diluted to 200 ng/ml as working standard with ethylacetate. Standard TAA was prepared as 20 μg/ml in ethylacetate. It was further diluted to 200 ng/ml and 2 ng/ml as working standard. The standard curve was constructed by adding 10 μl of 200 ng/ml internal standard and appropriate volumes of working standards into 2 ml ethylacetate/water mixture (1:1) so that the final concentration of standard TAA in the GC vial were 0, 0.3, 3, 10, 30, 60, 100, and 300 ng/ml with internal standard as 100 ng/ml. The standards were processed as in sample preparation.

2.5. Sample preparation

Twenty microliter of AH sample and 10 μl of 200 ng/ml internal standard were spiked into 2 ml ethylacetate/water mixture (1:1). After vortexing for 1 min and allowing the mixture to settle down, the glass tube was briefly centrifuged at 1100 × *g* for phase separation. After transferring the ethylacetate layer to the other glass tube, the aqueous phase was re-extracted twice by 1 ml of ethylacetate. These ethylacetate extracts were pooled and dried by anhydrous sodium sulphate. The dried extracts were evaporated under nitrogen. The residue was derivatized by 10 μl acetic anhydride and 60 μl pyridine at 60 °C for 20 min. After evaporating by nitrogen, the residue was dissolved in 40 μl acetonitrile and further derivatized by 20 μl BSTFA at 70 °C for 20 min. After drying by nitrogen, the residue was redissolved in 20 μl dodencane. One microliter was injected into GC–MS.

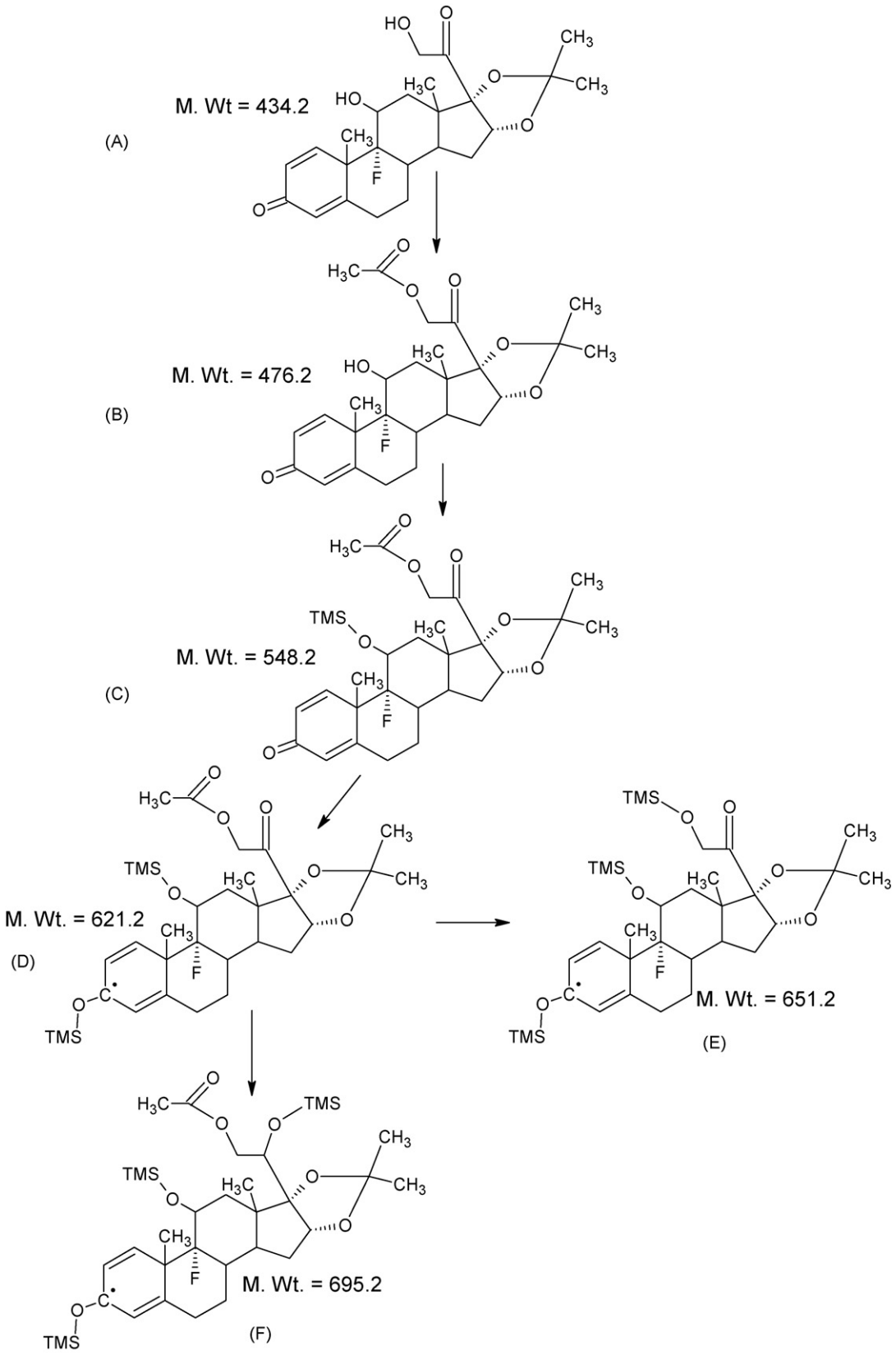

Fig. 1. Diagram showed TAA (A) derivatized by various silylating reagents under different conditions to form different derivatives.

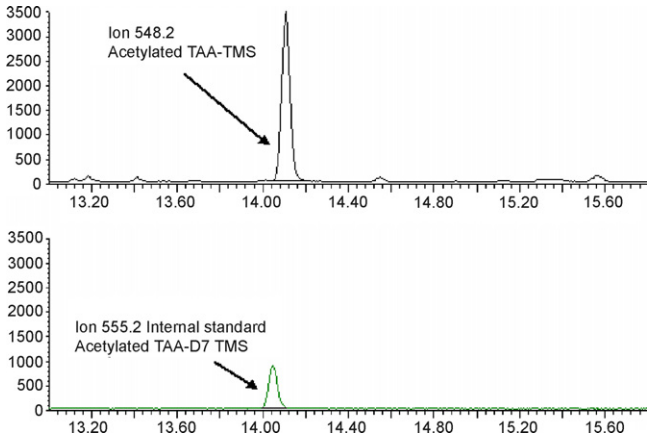

Fig. 2. Chromatogram of acetylated TAA–TMS and acetylated $^{2}H_{7}$-TAA–TMS in aqueous humor indicated the peaks are symmetric without apparent interference by matrix from aqueous humor.

2.6. Validation

Linearity was assessed by adding various amounts of standard TAA and 10 μl of internal standard, $^{2}H_{7}$-TAA, into blank AH to produce concentration ranges of 0, 0.3, 3, 10, 30, 60, 100, and 300 ng/ml TAA with 100 ng/ml $^{2}H_{7}$-TAA. They were extracted into ethylacetate/water mixture as described in sample processing. Reproducibility was assessed by repeat processing as above for five times. Recovery was obtained by adding 10, 60, and 200 ng/ml of TAA into 20 μl of blank AH samples and adding to the ethylacetate/water mixture. After the extractions and addition of 10 μl internal standard, the samples were processed as sample preparation. Standard curve was constructed by using 0, 0.3, 3, 10, 30, 60, 100, and 300 ng/ml with 100 ng/ml internal standard and directly evaporated, derivatized, and analyzed. The three concentrations were calculated according to the calibration curve. The percentage of calculated concentration compared with adding concentration was the recoveries in different concentration. Detection limit of the method was defined as the concentration that gives a signal-to-noise ratio 3 after sample preparation. The limit of quantitation (LOQ) was defined as the concentration that gives a signal-to-noise ratio 10 after sample treatment.

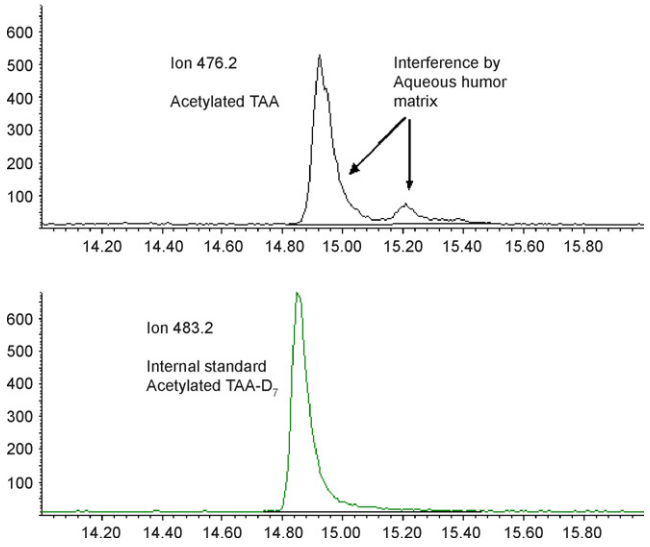

Fig. 3. Chromatogram of acetylated TAA and $^{2}H_{7}$-TAA in aqueous humor showed the peak has long tailing and interfered by matrix from aqueous humor.

Table 1
Reproducibility of various concentrations of spiked triamcinolone acetonide obtained from five samples

Spiked concentration (ng/ml)	Intraday variation (%) CV (%) ($n=5$)	Interday variation (%) CV (%) ($n=5$)
0.3	26.4	18.3
3	10.4	4.5
10	5.5	2.3
30	6.2	1.7
60	5.6	3.2
100	4.3	1.2
300	4.3	2.6

CV: coefficient of variation. Intraday variations were obtained by the CV of five batches of samples on the same day while interday variations were obtained by calculating CV of the mean of the five batches in each day for 5 days.

Table 2
Recoveries of spiked triamcinolone acetonide at final concentration of 10, 60, and 200 ng/ml by processing five samples at each concentration

Spiked concentration (ng/ml)	Recovery (%) ($n=5$)
10	96.5 ± 3.5
60	97.3 ± 3.2
200	98.7 ± 2.7

3. Results

Our derivatization procedure successfully produced silylated derivative from the TAA molecule (Fig. 1). The gas chromatogram was clear with little tailing for the TAA–TMS peak (Fig. 2). In contrast, the acetylated TA peak entailed a long tailing (Fig. 3).

The linearity, r^2, from 0 to 300 ng/ml was above 0.995 ($y = 0.0121x + 0.0035$). The reproducibility of the method from 3 to 300 ng/ml was from 10.4 to 3.9% (Table 1). The abso-

Table 3
Concentrations (ng/ml) of TAA in rabbit aqueous humor at day 0, 1, and 10 after intravitreal and subconjunctival injections

Treatment	Subjects	Day		
		0	1	10
		Concentration (ng/ml)		
Intravitreal	A1	0.0	0.0	13.6
Intravitreal	A2	0.0	165.3	22.0
Intravitreal	A3	0.0	9.4	0.0
Intravitreal	A4	0.0	13.4	33.9
Intravitreal	A5	0.0	25.7	25.4
Subconjunctival	B1	0.0	7.9	0.0
Subconjunctival	B2	0.0	0.0	0.0
Subconjunctival	B3	0.0	0.0	Sample lost
Subconjunctival	B4	0.0	0.0	0.5*
Subconjunctival	B5	0.0	46.7	13.5

* Below quantitation limit.

lute recoveries at 10, 60, and 200 ng/ml were more than 95% (Table 2). The detection limit was about 0.3 ng/ml. The quantitation limit was about 3 ng/ml. No interference peaks were found after assaying 150 trial blank samples and 9 intravitreal samples.

After 12 mg IVTA, the TAA concentration in AH of five rabbits ranged from 0 to 165.3 ng/ml at day 1 and 0 to 33.9 ng/ml at day 10. For those who received 12 mg subconjunctival injection, the TAA concentration ranged from 0 to 46.7 ng/ml at day 1 and 0 to 13.4 ng/ml at day 10 (Table 3). Large concentration variation was found among subjects.

4. Discussion

We noted two important papers that described the use of GC–NCI–MS for determination of TAA in biological samples. One reported vigorous reaction conditions to hydrolyze and oxidize TAA to 1,4-dien-3-one system in ring A and combine with the 11-keto group [15]. However, this method required long processing time and the response to TAA was about 10 times lower than to dexamethasone. The other study analyzed TAA in bronchoalveolar lavage fluid by the virtue of the intrinsic electron capture properties of TAA after acetylation [16]. Although no detection limit was reported, the authors claimed that "reliable result" was obtained as 6 pg/ml after extraction of 2 ml of the fluid. TAA analysis by LC–MS–MS could only detect 5 ng/ml after 5 ml urine extraction [12]. The performance of GC–NCI–MS was therefore superior. When considering 20 μl samples would be available for AH, the detection limit was 600 pg/ml when the GC–NCI–MS method was applied. However, when we adopted this method in our analysis system, we found the 0.7 min long TAA peak tail (Fig. 3). This tailing is due to the 11β-hydroxyl moiety that interacts with the silanols in the column. It rendered integration difficult, and the sensitivity was lowered. Although mass spectrometer was a highly selective detection method, we found some matrix interfering peak overlapping on the tailing in some samples. We also found residue TAA in the injection port being carried over to next few injections after a previous higher concentration injection (>100 ng/ml). This is most likely to be due to the presence of involatile acetyl TAA derivatives that contained the hydroxyl group. Such cross-over effects have to be avoided. Any residue left from high concentration sample will seriously affect the results of subsequent sample analysis.

In this study, we silylated and acetylated TAA. Although it has been claimed that steric hindrance exists in the hydroxyl moiety of the TAA molecule [17], which limits reagent access for acetylation and silylation, we have successfully silylated the 11β-hydroxyl quantitatively. This silylation does not only improve the chromatographic properties but also enhances the selectivity by adding another derivatization group without affecting the electron capture ability (Fig. 2). Better chromatographic properties give a sharper peak that improves the sensitivity that is essential when handling small sample volume of AH, which is usually not more than 50 μl. In our analysis, no residue TAA was carried over to the next injection even after 10000 ng/ml TAA was injected.

We have tried different strong silylating reagent including BSTFA, BSTFA/TMCS (1%) mixture and Power Sil Prep. BSTFA/TMCS (1%) mixture at 70 °C was too strong to give a single TMS derivative, instead double and triple TMS derivative structures D, E and F were shown (Fig. 1). Since the derivatization did not give quantitative single form derivative and the electron stablizing conjugating moiety was disrupted, the responses of both compounds were low. Power Sil Prep (TMSIM:BSA:TMCS; 3:3:2) was so powerful that it could derivatize TAA at 50 °C. It also produced different structural forms (structures C, D and E), as well as left involatile residue after evaporation, which rendered the final dissolution difficult. Further extraction was needed to remove these residues. BSTFA and pyridine were tried at 70–80 °C for 10 min, but they also produced di-TMS and tri-TMS derivatives (structures D and E). Pyridine might have acted as catalyst to enhance the derivatization [18]. BSTFA in acetonitrile (1:2) gave a quantitative single TMS derivative (structure C) without involatile residue after evaporation. It was also essential to dry the ethylacetate extract before evaporation because proton/deuterium exchange was found between water and internal standard. Standard TAA peak was found even only when $^{2}H_{7}$-TAA internal standard was analyzed.

We made an attempt to investigate the transfer of TAA from vitreous humor and subconjunctival compartment to AH. We took the AH samples from 3 days as day 0, 1, and 10 that represented as negative control, the immediate TAA and possibly stabilized TAA levels, respectively. Although large variation of concentration was found within each group, comparatively higher dose was found in the intravitreal injection group in both the first and the tenth day. Only low concentration was maintained 10 days after intravitreal injection and almost no TAA was detected 10 days after subconjunctival injection. Large variation was found due to biological variation in absorption lag time in individual rabbits. This was found in our later study in which the pharmacokinetic profiles of TAA in the aqueous humor of each rabbit were similar (unpublished results). The variation of absorption lag time may be because the dissolved tramcinolone had to be absorbed and transferred via a few tissue layers from the sclera or vitreal chamber to the anterior aqueous humor. While the optimal TAA therapeutic dosing in the treatment various ocular conditions remains to be determined, the crystallization of TAA in the anterior chamber is unlikely with the dosage of 12 mg under normal circumstances because according to our study, the solubility of TAA at room temperature was about 11 μg/ml at room temperature.

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