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Evaluation of different preparation methods for a preservative free triamcinolone acetonide preparation for intravitreal administration: a validated stability indicating HPLC-method

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Intravitreally applied triamcinolone acetonide (TA) is used to treat a variety of macular diseases. Commercially available products of TA are mainly intended for *intramuscular* application and contain benzyl alcohol (BA) as a bacteriostatic preservative. Since this agent damages ocular tissues, different methods such as filtration techniques and centrifugation are usually used to eliminate BA from commercial products (40 mg/mL TA, 9.9 mg/mL BA). In this study, we evaluated these methods in regard to their ability to eliminate benzyl alcohol and to guarantee standard doses of triamcinolone acetonide. A new formulation without BA (TA 40 mg/mL) was developped according to the following criteria: autoclavability, stability, and suitability for intravitreal use. For QA/QC evaluation a new rapid and simple HPLC procedure (C18 RP column, mobile phase consisting of methanol-water, 48:52, v/v) to quantify the respective compounds was developed and validated according to ICH guidelines. The HPLC method was proven to be selective, linear, precise and accurate. Analysis of preparations based on commercial products undergoing different filtration techniques showed variable results: TA concentrations of 22-80% of the declared amount were found, and BA content was not reduced to safe levels (up to 39% of initial content remained). Centrifugation methods decreased the concentration of the preservative adequately, however agglomerated TA crystals were observed, leading to irreproducible and deviating particle sizes that are potentially harmful with ocular use. The newly developed preservative free formulation (TA 40 mg/mL) delivered uniform doses of TA, revealed no drug loss during forced light exposure and was proven to be stable, sterile and bacterial endotoxin free after autoclaving and after storage for three months,. The new formulation may offer an alternative for the in-house production of intravitreally applicable TA preparations in hospital pharmacies and should enhance medication safety.

1. Introduction

Intravitreal administration of triamcinolone acetonide (TA) is used for the treatment of eye diseases, such as proliferative diabetic retinopathy and uveitis as well as for a variety of macular diseases, including refractory cystoid macular edema, diabetic macular edema, macular edema associated with central retinal vein occlusion and exudative macular degeneration. TA is used during post-pars plana vitrectomy as well as during anterior vitrectomy. Furthermore, it can be used during cataract eye surgery with iris neovascularisation (Jermak et al. 2007; Martidis et al. 2002; Jonas et al. 2001; 2003a, b, 2004, 2006b; Larsson et al. 2009; Roesel et al. 2009). A recent study about peribulbar injection of TA combined with laser grid photocoagulation has demonstrated that it is a safe and effective therapy for diabetic macular edema (Liu et al. 2009). The therapeutically relevant dose is usually 4 mg/0.1 mL (Jermak et al. 2007; Martidis et al. 2002; Jonas et al. 2006a, b).

TA is available as an injectable suspension. Commercial preparations available in Europe are e.g. Kenalog®, Kenacort® A or Volon® A (40 mg/mL), which were developed and marketed for i.m. and intraarticular use. Intravitreal use should be avoided since all these preparations contain benzyl alcohol (BA) as a bacteriostatic preservative which is harmful to the eye. Thus steps to remove the preservative by different cleaning procedures are necessary. Nevertheless, complications such as infectious and sterile endophthalmitis and pseudoendophthalmitis have been revealed to be a result of intravitreal administration of suspensions where authors have claimed that BA was filtered out (Jonas et al. 2006a,b; Lorenzo Carrero et al. 2008). It is still unclear whether these adverse reactions are due to remaining amounts of BA or to the drug TA. It has been reported that pure TA is safe (McCuen et al. 1981) whereas several studies have proven the intravitreal toxicity of BA (Chang et al. 2007; Morrison et al. 2006; Chang et al. 2008; Macky et al. 2007). In regard to these studies, it is generally recommended to decrease the BA concentration at least to the level of 10% of its initial content.

Various techniques, such as the filtration method with or without a three-way cock, and centrifugation are described to eliminate BA (Jonas et al. 2001, 2003b, 2004; García-Arumí et al. 2005;

Kube et al. 2006; Nishimura et al. 2003; Hernaez-Ortega and Soto-Pedre 2004, 2006; Spandau et al. 2005; Bitter et al. 2008). The main objective is to maximally reduce BA at the lowest possible loss of TA.Applying different filtration methods, highly variable TA and BA amounts were found, ranging from 45-97% of the initial concentration of TA and from 0.3-10% of the initial concentration of BA (García-Arumí et al. 2005; Kube et al. 2006; Spandau et al. 2005; Rodriguez-Coleman et al. 2004; Nishimura et al. 2003). After centrifugation, TA as well as BA were quantified showing concentrations being between 93-112% for TA and 0.06-16.9% for BA (Bitter et al. 2008; Hernaez-Ortega and Soto-Pedre 2006).

Preparation of preservative free formulations have been described only in a few publications. A retrospective *in vivo* study was conducted to test a TA preservative free formulation (available in US, New England Compounding Center, Farmingham, MA) containing polysorbate 80, buffering agents, polyglycol and sodium chloride. The preparation had a good safety profile as no adverse events were detected (Bakri et al. 2005). Moreover, 0.2% polysorbate 80 has been shown to be innocuous to the eye (Younis et al. 2008). Polysorbate 80 is also contained in Triesence® suspension (TA for visualization during pars plana vitrectomy) recently approved by the FDA (Dyer et al. 2009). Another formulation is proposed based on suspending TA powder in normal saline and adding 0.5% hydroxypropylmethylcellulose (HPMC) (Kim et al. 2006). Although it has been shown that viscoelastic substances generally elevate the intraocular pressure (Liesegang 1990; Glasser et al. 1986; Fernandez-Vigo et al. 1989), the effect is transient. After the injection of 2% HPMC the intraocular pressure returns to preoperative levels within 24 h even without being washed out from the anterior chamber (Glasser et al. 1986; Fernandez-Vigo et al. 1989). When choosing HPMC as additive, it has to be considered that particles contained in the raw material have to be removed by filtration before use (Liesegang 1990; Sieber and Mühlebach 1982). The application of sodium hyaluronate and balanced salt solution is another option to prepare a preservative free formulation (Bitter et al. 2008). Unfortunately sodium hyaluronate cannot be autoclaved, as this would lead to depolymerization and thus to a change in its viscosity (Liesegang 1990).

As all clinical studies emphasize the utmost importance to avoid BA in products for intraocular use, in-house manufacturing procedures in hospital pharmacy departments so far were mainly based on commercial products of TA and subsequent steps to eliminate the BA, such as different filtration techniques or centrifugation. To compare results, these different types of procedures for removing BA should be evaluated and resulting preparations tested for TA and BA quantity. For this purpose a HPLC assay had to be developed which had to be selective for BA and TA, and stability indicating with respect to TA.

Photodegradation of TA and possible phototoxic effects have been investigated. Since TA was proven to undergo photodegradation (Matysová et al. 2003; Miolo et al. 2003), this aspect needs to be particularly considered in validation procedures (according to the current International Conference on Harmonization guideline: ICH Q2R1 2005) and stability assays of formulations.

Since most results published to date indicate that TA and BA amounts vary considerably after different purification procedures, a further aim was the development of a preservative free formulation for intravitreal administration, containing 40 mg/mL TA. The suspension should be stable, autoclavable, easily dispersable, packed in single dose containers fulfilling all requirements for ophthalmic and intravenous formulations according to Ph. Eur. / USP (European Pharmacopoeia 6th / United States Pharmacopeia 2010). The degree of standardization of the tested preparation methods in accordance with GMP quality requirements should be evaluated in order to find the most suitable preparation technique.

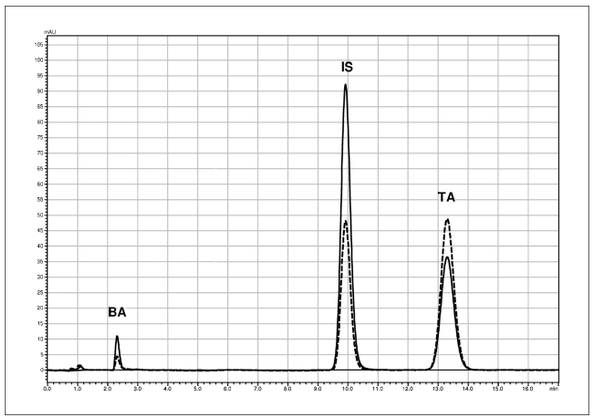

Fig. 1: Representative chromatogram, detection at λ = 254 nm (—) and λ = 240 nm (···); benzyl alcohol (BA) t_{ret} 2.31 min, internal standard (IS), propylparaben, t_{ret} 9.91 min, and triamcinolone acetonide (TA) t_{ret} 13.30 min, eluent methanol-water (48:52, v/v)

2. Investigations, results and discussion

2.1. Assay validation

A stability-indicating HPLC method, to quantify BA and TA simultaneously, was developed and validated according to current ICH guidelines (ICH Q2R1 2005). The validation included specificity, linearity, precision, LOD/LOQ and accuracy. The specificity of the method was tested by injecting an aliquot of the "filtration" medium (Ringer's solution) and the following: a standard solution containing TA, a standard solution containing BA, a standard solution containing propylparaben as internal standard (IS) and a diluted Volon® A sample. TA and BA raw material as well as TA and BA contained in Volon® A elute at identical retention times. Furthermore, the IS does not co-elute with either the component. For a typical chromatogram of a sample containing TA, BA and IS compare Fig. 1. Samples that were stressed with solar light and elevated temperatures showed the formation of several degradation products (Fig. 2). None of the peaks of the degradation products observed in stressed samples interfere with the peaks corresponding to BA or TA, peak purity indices were found to be >0.999 in all chromatograms. For the linearity assay the five levels of concentration within the range of 54.40-6.80 μg/mL TA and 88.48-5.53 μg/mL of BA, were

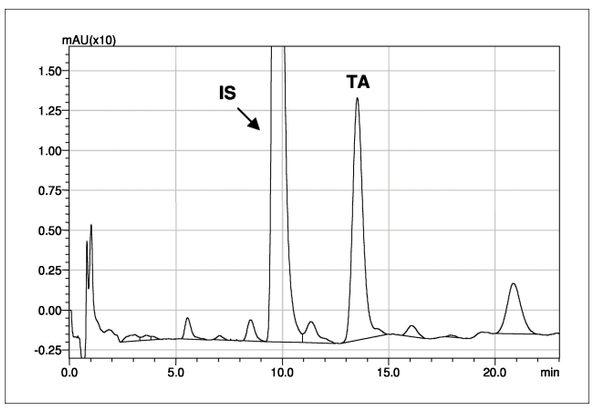

Fig. 2: Representative chromatogram of a stressed solution of triamcinolone acetonide (TA), detection at λ = 240 nm

Table 1: Results obtained for the precision study

Drug	Concentration (μg/mL)	Mean peak area (Drug/IS) ± R.S.D. (%)			
		Day 1	Day 2	Day 3	Intermediate precision
TA	6.80	0.12547124 ± 1.44	0.22937566 ± 1.49	0.23254531 ± 1.24	0.23383176 ± 2.30
	13.60	0.47412251 ± 0.61	0.47730127 ± 0.27	0.47428347 ± 0.26	0.47523575 ± 0.48
	27.20	0.95470815 ± 0.60	0.95014867 ± 0.57	0.95333057 ± 0.13	0.95272913 ± 0.47
	40.80	1.40543976 ± 0.33	1.40615710 ± 0.46	1.40696945 ± 0.12	1.40618877 ± 0.29
	54.40	1.88792507 ± 0.49	1.89405619 ± 0.11	1.88045426 ± 0.14	1.88747851 ± 0.41
BA	5.53	0.00508419 ± 6.92	0.00493869 ± 4.23	0.00480584 ± 6.59	0.00490145 ± 5.48
	22.12	0.01770867 ± 2.28	0.01747624 ± 2.24	0.01795603 ± 1.65	0.01771365 ± 2.14
	44.24	0.03483040 ± 2.65	0.03383565 ± 0.77	0.03469659 ± 1.33	0.03445422 ± 0.81
	66.36	0.05156696 ± 1.76	0.05082549 ± 1.28	0.05179352 ± 2.18	0.05139532 ± 1.76
	88.48	0.07568201 ± 1.46	0.07473259 ± 1.58	0.07309462 ± 4.83	0.07450307 ± 3.02

prepared as described in 3.3. Linear correlation was obtained between the peak area ratios of drug/IS and the corresponding concentration. Regression lines are y = 29.004x - 0.0690 for TA and y = 1192.8x + 0.4627 for BA and have coefficients of determination (r^2) that are 0.9999 for TA and 0.9962 for BA. Repeatability was determined by analyzing three replicate injections of the five standard solutions. For calculating intermediate precision this procedure was repeated on three different days (see Table 1). The accuracy of the method was determined by comparing a conventional true value and the value found. The values were measured at each standard solution level using nine replicates. The difference between the defined value and the value found was 98.17%-101.40% for TA and 84.59-112.03% for BA.

The limit of detection (LOD) was defined as a S/N ratio of 3:1, the limit of quantification (LOQ) was determined to be S/N ratio of 10:1. The LOD for TA was 0.15 μg/mL and for BA it was 0.55 μg/mL. The LOQ was established at 0.45 μg/mL for TA and 1.37 μg/mL for BA.

2.2. Quantitative analysis of triamcinolone acetonide suspensions

Different methods were applied to prepare a preservative free TA suspension, such as the filter technique, with and without using a three-way cock, and centrifugation (see 3.4). Furthermore, a completely new formulation without benzyl alcohol was compounded for intravitreal use (see 3.4). The developed and validated HPLC method was applied to assess the quality of different preparation methods of preservative free TA suspensions.

2.2.1. Filtration technique

The preservative was removed by filtration through a 0.20 μm syringe filter. TA crystals were washed nine times with Ringer's solution in a tuberculin syringe by discarding and re-suspending the washing solution. TA and BA amounts remaining in the syringe as well as BA amounts found in each waste filtrate were analyzed.

The declared amount of TA and BA in 0.2 mL Volon® A suspension was 2 mg for BA and 8 mg for TA. The amount of BA and TA was measured initially before starting the purification process and was found to be 1.83 mg ± 0.70% (R.S.D.) for BA (n = 3) and 7.77 mg ± 0.30% (R.S.D.) for TA (n = 3).

After nine purification steps the amount of BA and TA remaining in the syringe was measured. The results covered a wide dosage range. TA (n = 34) concentrations ranged between 1.75-6.37 mg/0.2 mL, clearly indicating that the drug content deviates to an unacceptable degree after the purification process (compare Fig. 3). It can be assumed that TA crystals adhere to the filter membrane. Furthermore, TA crystals stuck to the syringe plunger could be observed. Fig. 4 shows the amount of BA still remaining after the 9th purification step. Resulting BA levels ranged between 0.17-0.78 mg/0.2 mL (n = 34).

The results of the mean BA amount in each waste are presented in Fig. 5. It was observed that the second washing solution showed the highest level of BA. It is interesting, that BA could not be eliminated completely. It seems that it levels off at 0.05 mg.

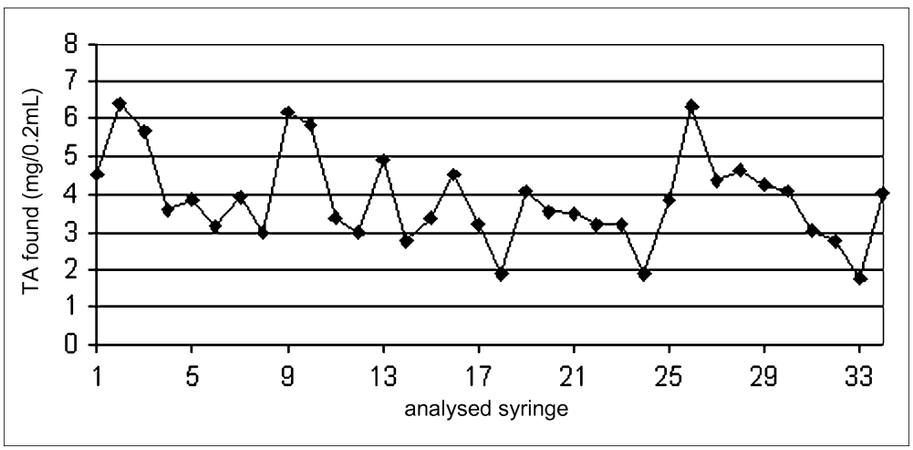

Fig. 3: Triamcinolone acetonide amount (mg) found in syringes (n=34) after nine purification steps by the filter technique

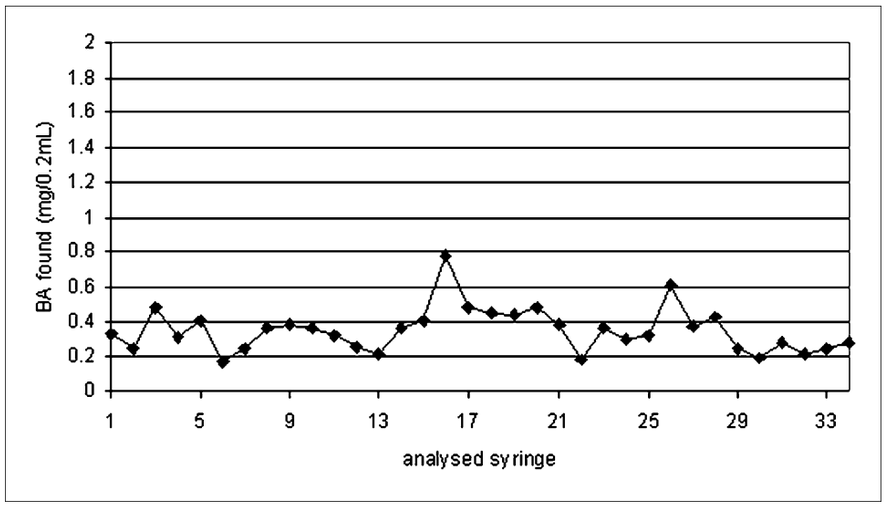

Fig. 4: Benzyl alcohol amount (mg) found in syringes (n=34) after nine purification steps by the filter technique

These findings correspond to those of Rodriguez-Coleman et al. (2004), who pointed out that BA according to its octanol-water partition coefficient greater than one has a greater affinity to lipophilic substances, like TA.

The filtration technique applying a three-way cock indicates results that are very similar to those found applying the filtration technique, as the loss of BA (n = 6) ranged between 82.6–88.5% and the loss of TA (n = 6) ranged between 15.9–43% after nine purification steps. Therefore, only the results for the wastes are depicted in Fig. 5 giving a comparison of the mean BA amount in waste filtrates obtained from the filtration technique with and without using a three-way cock, respectively. Although the variability of the BA loss is not as large as in case of the filter technique, BA is still not satisfyingly eliminated by this method.

2.2.2. *Centrifugation*

The suspension was centrifuged (5000 rpm, 5 min) four times, the supernatant was extracted after each step and the residue was re-suspended with Ringer's solution. The amount of BA found in the supernatants after each centrifugation step (C_1–C_4) was: C_1: 7.91–8.57 mg/mL, C_2: 0.60–1.24 mg/mL, C_3: 0.06–0.08 mg/mL and in the last step C_4: 0.01–0.03 mg/mL. It was observed that after the second centrifugation step BA was almost completely removed. Although the BA concentration can be reduced adequately when applying this method, due to the centrifugal forces, the TA crystals agglomerate thus losing their dispersability. Particle sizes >100 μm were also found in a previous study, which necessarily correlate with the applied technique and do not fulfil BP 2007 requirements (Bitter et al. 2007).

2.2.3. *Analysis of the preservative free in-house formulation, dose uniformity and stability studies*

As endothelial cells are very sensitive to foreign substances, safety of selected ingredients has been evaluated by a literature survey (see introduction). Balanced salt solution was chosen not only to fulfill osmolarity and the target pH of 7.4 but also to mimic the aqueous humor and thus minimize damage to the tissues. A viscosity building agent is needed in order to help suspending TA particles as well as to protect the endothelium of the cornea and to preserve the shape of the intraocular cavity. Therefore, HPMC was chosen, as its formulations can easily be prepared and autoclaved. Furthermore, a surfactant was needed to disperse TA particles adequately. Hence, polysorbat 80 was used to wet the insoluble TA particles.

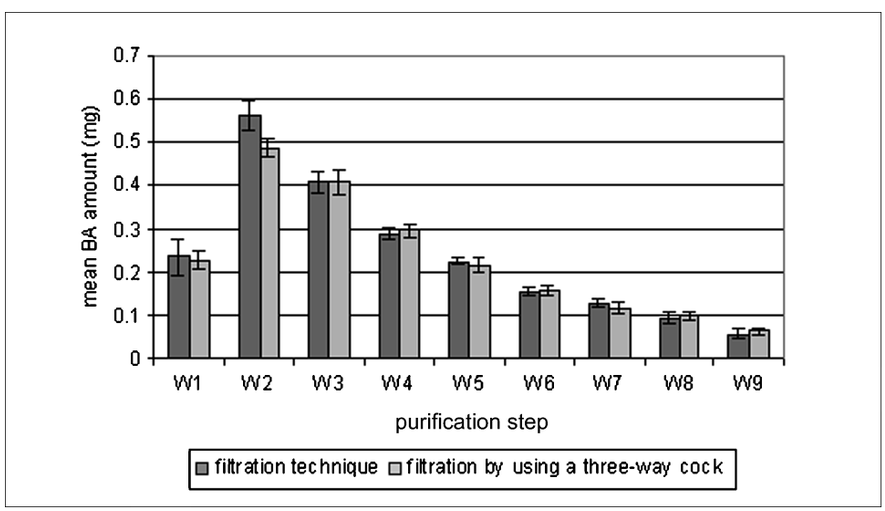

Fig. 5: Mean amount of benzyl alcohol (mg) (n=6) in the waste W1-W9 applying the filtration technique and the filtration technique using a three-way cock, respectively

Table 2: Determination of dose uniformity before and after autoclaving in three batches of the new triamcinolone acetonide formulation

		Mean quantity of TA (mg/0.2mL) ± R.S.D. (%) (n=3)		
	Vial no.	Batch 1	Batch 2	Batch 3
Before autoclaving	1	7.79 ± 2.06	7.84 ± 2.03	7.89 ± 2.96
After autoclaving	2	7.69 ± 0.80	7.86 ± 2.72	7.85 ± 0.63
	3	7.74 ± 1.01	8.04 ± 1.80	8.05 ± 1.34
	4	7.84 ± 0.26	7.84 ± 1.97	7.84 ± 0.19

Sterility was assured for each batch. Bacterial endotoxins displayed <5 EU/mL for each batch, assuring its intravitreal applicability (European Pharmacopoeia 6th; United States Pharmacopeia 2010). Dose uniformity was proven for non-sterile and sterile vials of each batch. Table 2 gives the comparison of the results obtained before and after autoclaving. The autoclaving process did not affect stability, as amounts of TA were within the range of 95-105% (7.6-8.4 mg/0.2 mL) from the declared amount for each batch. No degradation was observed after light exposure. TA showed a mean initial concentration of 8.01 mg/0.2 mL ± 1.67% (R.S.D.). The mean TA concentration ± R.S.D. (%) measured after irradiation in the suntest was: for 8 min: 7.94 mg/0.2 mL ± 2.55% (R.S.D.), for 16 min: 7.93 mg/0.2 mL ± 1.12% (R.S.D.) and for 24 min: 8.08 mg/mL ± 0.03% (R.S.D.). This indicates that the formulations can easily be handled without special light protection. There were no substantial differences of the TA concentrations after storing the preservative free suspension at room temperature (21 ± 1 °C) for three months. Mean TA concentrations were found to be between 7.85-8.16 mg/0.2 mL. There was no microbial contamination during the storage period. Although the suspension is stable at room temperature, it is recommended to store it between 2-8 °C as it does not contain any preservative.

2.3. Conclusion

In view of the fact that BA is toxic to the eye, care should be taken that only preservative free TA suspensions are applied to patients for ocular use. A new simple HPLC analytical method has been developed for the simultaneous determination of BA and TA in parenteral formulations and was proven to be selective, linear, precise and accurate. The results of this study indicated that BA is not eliminated adequately from the commercial product by several filtration procedures. Moreover, uniform TA dosages are not guaranteed. The centrifugation method is not recommendable as TA crystals agglomerate, resulting in particle sizes potentially larger than 100 μm.

In conclusion, a preparation of a TA suspension completely without BA is proposed. The developed BA free formulation represents an adequate option as it is terminally autoclavable, stable, and provides uniform doses of TA. The new formulation may offer an alternative for the in-house production of intravitreally applicable TA preparations in hospital pharmacies and should enhance medication safety.

3. Experimental

3.1. Materials

Volon® A (40 mg/mL, Lot no. A050902, A071102, A070401), a TA suspension commercially available in Austria, was kindly provided by Dermapharma AG, Austria. TA (micronized, 1 g Lot no. 07D10-N19, 10 g Lot no. 09A16-N05) and HPMC (Hypromellosum E4 M, 100 g Lot no. 07K01-N04) were purchased from Fagron GmbH, Germany. BA (Lot no. 03510TX) was purchased from Aldrich Chemical Company Inc., Austria. Propylparaben (Lot no. 5662/1203 4706) was purchased from Gatt-Koller GmbH, Austria. Polysorbate 80 (Lot no. 835FO-231) was purchased from Elisabeth Schubert GmbH, Austria. Balanced salt solution (Lot no. 1622447) was kindly provided by Alcon Ophthalmika GmbH, Austria. Ringer's solution (Lot no. 8A2984) was supplied by Mayrhofer Pharmazeutika GmbH, Austria. 0.2 μm and 5 μm syringe filters (Minisart® NML 0.2 μm Lot no. 16534 080187 and Minisart® NML 5 μm Lot no. 1759480189103) were kindly provided by Sartorius Stedim, Austria. HPLC-grade methanol (Lot no. K40178707927) was purchased from Merck, Germany. The water used was HPLC-grade water (Lot no. 0017810023) purchased from Mallinckrodt Baker, Netherlands.

3.2. HPLC conditions

The analysis was conducted with a HPLC system consisting of a Shimadzu (Shimadzu Corporation, Japan) HPLC equipped with 2 pumps (LC-10AS), autosampler (SIL-10AD), diode array detector (SPD-M20A), communications module (CBM-20A) and computer with software (Shimadzu LCSolution V1.21). The analytical column was a RP18-endcapped column, 5 μm particle size, (EcoCART 125-3, LiChrospher 60, Lot no. 411416, Merck KGaA, Germany). The column temperature was 30 °C. The mobile phase consisted of methanol and water at the ratio of 48:52 (v/v). The flow rate was set at 0.8 mL/min. The DAD was set at 254 nm to detect BA and at 240 nm to detect TA. Sample injection volume was 20 μL.

3.3. Preparation of stock- and standard solutions

All solutions were prepared with methanol-water (70:30, v/v) as solvent. Stock standard solution of BA was prepared at a concentration of 221.2 μg/mL. Stock standard solution of TA was prepared at a concentration of 136 μg/mL. Propylparaben was used as IS at a stock concentration of 210 μg/mL. Standard solutions of TA and BA were prepared by diluting the stock solutions with the solvent to produce concentrations of: 54.40 μg/mL TA and 88.48 μg/mL BA, 40.80 μg/mL TA and 66.36 μg/mL BA, 27.20 μg/mL TA and 44.24 μg/mL BA, 13.60 μg/mL TA and 22.12 μg/mL BA, 6.80 μg/mL TA and 5.53 μg/mL BA. The final concentration of the IS in each standard solution was 21 μg/mL.

3.4. Preparation of preservative free suspensions

3.4.1. Filtration technique

The filter technique is an ensuing modification of a method published previously (Jonas et al. 2001, 2004). 0.2 mL Volon® A was drawn into a 1 mL tuberculin syringe. The 0.20 μm syringe filter was first wetted with Ringer's solution and then placed on the tuberculin syringe. A 2 mL syringe filled with 0.8 mL Ringer's solution was connected on the other side of the filter by luer lock – to luer lock. The TA crystals were suspended in 0.8 mL Ringer's solution by back flushing through the filter. 0.8 mL of the supernatant, containing BA, was extracted through the filter, while the TA crystals remained in the syringe. The syringe was then refilled with Ringer's solution. This procedure was repeated eight times. Finally 0.2 mL suspension was left in the syringe. The amounts of TA and BA were measured at the beginning and after finishing the filtration process. Finally the amount of BA was measured in each waste filtrate.

3.4.2. Filtration technique applying a three-way cock

The filtration technique applying a three-way cock is an ensuing modification of a method published previously (Nishimura et al. 2003). A syringe filter and a tuberculin syringe filled with 0.2 mL Volon® A were prepared as described above. The 2 mL syringe filled with 0.8 mL Ringer's solution was connected to the three-way cock instead of directly being connected to the syringe filter. After suspending the pellets, the three-way cock was turned. Another 2 mL syringe was connected to the other outlet and the supernatant was extracted through the filter. The three-way cock was turned again and the tuberculin syringe was refilled with Ringer's solution. This process was repeated eight times. The amounts of TA and BA were measured at the beginning and after finishing the filtration process. Finally the amount of BA was measured in each waste filtrate.

3.4.3. Centrifugation

The centrifugation technique is an ensuing modification of a method published previously (Hernaez-Ortega and Soto-Pedre 2004, 2006). 1 mL of Volon® A was put into an Eppendorf tube and was centrifuged at 5000 rpm for 5 min. The supernatant was removed with an Eppendorf pipette. The residue was resuspended with 1 mL Ringer's solution, the mixture was vortexed twice for 30 s. This procedure was repeated three times. The amount of BA was measured in each supernatant and after the last centrifugation step.

3.4.4. Preservative free formulation for intravitreal administration

Initially a polysorbate 80 solution and a HPMC solution were prepared by dissolving each compound in balanced salt solution. Under aseptic conditions the HPMC solution was filtered through a 5 μm syringe filter in order to remove particles from the raw material. The polysorbate 80 solution was filtered through a 0.2 μm syringe filter. Sterilized TA powder (160 °C, 2 h) was suspended in balanced salt solution. Subsequently the polysorbate 80 solution was added and mixed well. The HPMC solution was added to the mixture and stirred until a homogenous suspension was obtained. The resulting preparation contained TA in the defined concentration of 8 mg/0.2 mL, 0.5% HPMC and 0.02% polysorbate 80. The suspension was subdivided into sterilized 5 mL injection vials and autoclaved for 30 min (121 °C, 2 bar). Sterility was tested by the microbiology laboratory of the Hospital KA Rudolfstiftung, Vienna, Austria. Detection and quantification of bacterial endotoxins was assayed by the inspecting and certification authority MA 39 (Magistratsabteilung 39), Vienna, Austria, applying the Endosafe-PTS, FDA-licensed endotoxin detection system. Dose uniformity was assessed before and after autoclaving.

3.5. Sample preparation for HPLC analysis

All solutions were prepared with methanol-water (70:30, v/v) as solvent. The IS was used at a stock concentration of 210 μg/mL. TA and BA amounts measured in TA suspensions: 0.2 mL TA suspension was drawn into a tuberculin syringe. The content of the syringe was transferred to a 50 mL volumetric flask, and dissolved in 50.0 mL. This solution (4.0 mL) and 1.0 mL IS were diluted to 10.0 mL with the solvent. TA and BA amounts measured after nine filtration steps: the residue in the syringe was transferred to a 50 mL volumetric flask and dissolved in 50.0 mL. The solution (4.0 mL) and 1.0 mL IS was diluted to 10.0 mL with the solvent.

BA measured in waste solutions (W_1-W_9) after each filtration step: each waste was transferred to a 10 mL volumetric flask, 1.0 mL of IS was added and the solution was diluted to 10.0 mL with the solvent.

BA was measured in the supernatant after each centrifugation step (C_1-C_4): C_1 and C_2 had to be diluted one to ten to prevent overloading the HPLC column. 1.0 mL of the dilution of C_1 or C_2 and the supernatant of C_3 or C_4, respectively, were pipetted in 10 mL volumetric flasks, 1.0 mL of IS was added and the solution was diluted to 10.0 mL with the solvent.

3.6. Stability evaluation

Thermic stability and photostability were examined for the in-house preservative free compounded formulation. Thermic stability was tested after autoclaving (30 min, 121 °C, 2 bar). Assessment of photostability was based on forced irradiation using a Suntest CPS Accelerated Exposure Machine (Heraeus, Hanau, Germany; Art. no. 55007014): xenon burner NXE 1500, black panel temperature: 49 °C, radiation intensity (1300 W/m2); window-glass filter (Art. no. 56009562); time factor: 15 (1 min Suntest *15 min bright sunlight). Distance of source to specimen table 22 cm. 3.0 mL TA suspension was transferred into white glass vials and exposed to forced irradiation for 8, 16 and 24 min (corresponding to 2, 4 and 6 h of natural sun light, respectively). Samples 0.2 mL were taken before and after exposure and were prepared as described above. Furthermore, stability was determined after storing the preservative free suspension for three months under room temperature (21 ± 1 °C). The amount of TA remaining in the samples was determined by HPLC. Samples were considered stable if the TA concentration was within the range of 95-105% of the declared concentration (8 mg/0.2 mL).

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