

**BIOANALYTICAL VALIDATION AND ACCURATE RP-HPLC  
METHOD DEVELOPMENT OF  
TRIAMCINOLONE:APPLICATION TO BIOEQUIVALENCE  
STUDIES**

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## Abstract

The present simple and sensitive method has been developed for the purpose of bioanalytical study of triamcinolone. The chromatography condition was optimized using a mobile phase 0.5% Tri-Ethyl Amine (TEA) (), pH- 3.48 and acetonitrile in the ratio of 50:50 at flow rate 1ml/min. and the stationary phase was used as thermo C<sub>18</sub> column. The plasma extraction method, protein precipitation technique was achieved 79.5% using 5% of trichloro acetic acid. The drug was eluted at 5.1 min. and no plasma endogenous materials were observed in the retention time. The limit of quantitation (LOQ) of the present method is 100ng/mL and linearity was in the range of 0.5-15.0 µg/mL. The range of drug r<sup>2</sup> had shown 0.999. The present successful development and validated method of triamcinolone is shown good accuracy, precision and linearity.

**Keywords:** Drug free human plasma, HPLC, Optimization, Triamcinolone, Validation.

### **Bioanalytical doğrulamave Triamsinolonun doğru RP - HPLC yöntemi geliştirme : biyo eşdeğerlik çalışmalarına Uygulama**

Bu yöntem triamsinolon basit ve hassas yöntem biyoanalitik çalışmanın amacı geliştirilmiştir. Kromatografisi şartları 1 ml / dakika 50:50 oranında bir hareketli faz , % 0.5 TEA (Tri- etilamin ) , pH- 3.48 ve acetonitril kullanılarak optimize edilmiştir. hareketsiz faz thermo C18 olarak kullanılmıştır. Plazma ekstraksiyon yöntemi , protein yağı tekniği trichloroasetik asit % 5 ile % 79.5 elde edildi . İlaç 5.1 dakikada elüte edilmiştir. herhangibir plazma endojen maddeler tutulması gözlemlendi. Bu yöntemin LOQ / ml ve doğruluk 0,5-15,0 mcg / mL aralığında , 100 ng olmasıdır. İlaç r<sup>2</sup> aralığı 0,999 göstermiştir. Triamsinolon mevcut başarıyla geliştirilmiştir ve onaylanmış bir yöntemi doğruluk , hassasiyet ve doğruluk gösterilir .

**Anahtar Kelimeler :** Triamsinolon , ilaçsız insan plazma , optimizasyon , doğrulama , HPLC

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## INTRODUCTION

Triamcinolone acetonide [9-Fluoro-11 $\beta$ ,16 $\alpha$ ,17, 21-tetrahydroxypregna-1, 4-diene-3, 20-dione cyclic 16,17-acetal with acetone (C<sub>24</sub>H<sub>31</sub>FO<sub>6</sub>)] is a long-acting corticosteroid with reported efficacy when given by intravitreal or subtenon injection as a treatment for diabetic macular edema, uveitis, retinal vein occlusion, and age-related macular degeneration (1-3). No method reported previously estimation of triamcinolone acetonide in plasma by using the protein precipitation technique (PPT) extraction in the literature. The present work was aimed at developing a sensitive High Performance Liquid Chromatographic (HPLC) for determination of triamcinolone acetonide in human plasma. The advantages of present method include single step optimized extraction procedure using PPT and short run time. The optimization of extraction procedure was carried out by comparing solid phase extraction, and liquid-liquid extraction for recovery and interference. The PPT was selected because it had huge advantages such as less processing time; very less organic solvent consumption, fewer steps and good plasma sample clean up. Only few methods have been described for the determination of TRE from plasma, urine, hair, bovine tissue and synovial and intraocular fluid, such as HPLC (4-10) and gas chromatography (11, 12). UV spectrophotometric methods for its pharmaceuticals have been described in British Pharmacopoeia (13) and United States Pharmacopoeia (14). Best of our knowledge, there has been no reported analysis method for the determination of triamcinolone in human plasma using PPT method. Therefore, the goal of this present study was to develop an HPLC method for the determination of triamcinolone in plasma which can be used for pharmacokinetic applications. No method reported previously estimation of triamcinolone in human blank plasma by using the protein precipitation (PPT) extraction in the literature. The present work was aimed at developing a sensitive High Performance Liquid Chromatographic (RP-HPLC) for determination of triamcinolone in human blank plasma. The advantages of present method include single step optimized extraction procedure using PPT and short run time. The optimization of extraction procedure was carried out by comparing

Solid phase extraction, and liquid-liquid extraction for recovery and interference. PPT was selected because it had obvious advantages such as shorter processing time; lesser organic solvent consumption, fewer steps, and good plasma sample clean up.

## **MATERIALS AND METHOD**

The HPLC grade of acetonitrile, *Tri-Ethyl Amine* (TEA) was obtained from Merck Darmstadt, Germany. The HPLC water obtained from HPLC Millipore unit, USA.

### *Method*

#### *Development of chromatographic condition*

The chromatogram was developing initially using separation condition such as mobile phase (0.5% TEA and acetonitrile in the ratio of 10:90 increasing order). Column such as C<sub>4</sub>, C<sub>8</sub>, C<sub>18</sub>& ODS in that C<sub>18</sub> column achieved good separation. The system was used shimadzu UFLC-20 AD model. The optimized chromatographic conditions were optimized using a mobile phase 0.5% TEA, pH- 3.48 and acetonitrile in the ratio of 50:50 at flow rate 1ml/min. the stationary phase was used as thermo C<sub>18</sub> column.

#### *Standard solution preparation*

Triamcinolone standard was prepared by using HPLC grade methanol and water in the ratio 1:1. Initially 100 mg of drug was weighted and transferred into the standard flask; the combined solvent (methanol and water) added and finally made the volume with the same. The stock solution further diluted was used for the analysis. The stock solution was maintained refrigerated at 8°C.

#### *Extraction method*

The sample was extracted using PPT, solid phase extraction and liquid-liquid extraction. In that solid phase extraction and liquid-liquid extraction achieved less than 60% with more interference in the drug retention time. Protein precipitation technique is achieved 79.5%. The healthy drug free human volunteer blood withdrawn and separated the plasma and stored at -

7°C. Initially 0.5ml of drug free plasma added in the 2ml RIA vial followed by 0.5ml of protein precipitant and 0.5ml of 1µg of drug added and vortexed for 2 minutes. The resulting solution was centrifuged at 4000rpm for 7 min. The supernatant was recentrifuged for 2 min at 4000rpm. The clear extracted solution is injected to UFLC system and chromatogram is recorded.

## **VALIDATION**

The validation (15) parameter like selectivity of the method was analysis for interference from plasma. The standard curve consisting of eight points was developed. Quality control samples of LQC (µg/mL), MQC (µg/mL) and HQC (µg/mL) were used to determine the intra and inter-day precision and accuracy of the assay. Peak area ratios of triamcinolone standard were fit to linear equation and drug concentration in test samples with the same day standard curve samples were calculated using this equation. For all the curves the correlation coefficients ( $r^2$ ) were never lower than 0.9.

### *Selectivity*

Selectivity of the method described was investigated by screening six different batches of human blank plasma. Under the proposed assay condition triamcinolone and internal standard had a retention time of 5.83min, rest of the peaks were due to the plasma components. Triamcinolone and internal standard were very well resolved under the proposed chromatographic conditions. None of the drug free plasma samples studied in this assay yield endogenous interference at these retention times (Figure1).

### *Accuracy and Precision*

The mean percent accuracy of the proposed method was found to be  $97.03 \pm 2.91\%$ . The precision of the inter day and intra-day assay were measured by the percent coefficient of variation over the concentration range of LOQ, low, middle and high quality control sample of triamcinolone during the course of validation. The results are given in Table 1.

### *Linearity*

The linearity of each calibration curve was determined by plotting the peak area ratio of triamcinolone to internal standard versus nominal concentration of triamcinolone. For linearity study seven different concentration of triamcinolone were analyzed (0.5, 1, 2, 4, 6, 8 & 15 µg/mL). The peak area response was linear over the concentration range studied. Each experiment at all concentration was repeated three times on three separate days to obtain the calibration data. The coefficient of correlation  $r^2$  was found to be 0.9999.

#### *Recovery*

The mean extraction recoveries of triamcinolone determined over the concentration of 0.5, 4.0 and 15.0 ng/ml were 85.94, 94.59 and 85.71%. For the mean extraction recovery was  $88.74 \pm 5.06\%$ .

#### *Speed of Analysis*

The analysis is performed within 15 min in which 5 min for extraction of plasma and 10 min for chromatography.

#### *Stability studies*

Short-term and long-term stock solution stability study was evaluated, which proved no significant deviation from normal value when stored at 4°C. The stability of triamcinolone in plasma was determined by measuring concentration change in quality control samples over time. Stability was tested by subjecting the quality controls to three freeze-thaw cycles and compared with freshly prepared quality control samples. As shown in Table 2, the mean concentration of triamcinolone in quality control samples did not change significantly within the time period under the indicated storage conditions. Long-term stability study results concluded that triamcinolone is stable in plasma matrix at least for 30 days when stored at -20°C.

#### *System suitability*

System suitability test was performed daily before the run of analytical batch to check detector response to analyse. Both the formulations were well tolerated by all the volunteers in both the phases of study.

## RESULTS AND DISCUSSION

### *Development of bioanalytical method*

#### *Optimization of chromatographic condition in human plasma*

A Plasma study of the optimized method was carried out in healthy human plasma samples. samples was prepared by adding 0.5 ml plasma to 2.0ml centrifuge tube and added 0.5 ml of precipitating agent (10% v/v perchloric acid) vortexed for 2 min. The resulting solution was centrifuged at 4000 rpm for 7 min. The supernatant layer was separated and estimated by HPLC. The method has been developed using protein precipitation technique, achieved 79.5%. The supernatant solution was filtered and injected in HPLC system.

#### *Selectivity and sensitivity*

Endogenous interferences study was carried out using 10% of perchloric acid in protein precipitation method. The respective retention time of drug is 4.9 (Figure 2). No interference peak was recorded at the drug election time. Sensitivity of the method showed in nanogram level. Limit of detection and limit of quantification was recorded 30 and 100ng/mL, respectively.

#### *Accuracy and precision*

The accuracy of the proposed method was found to be 90 % as per FDA guidelines. The precision studies of the inter-and intra-day assay were measured percentage coefficient variation over the concentration of the quality control samples. The range has been selected 0.5 – 15 mcg/ml &  $r^2$  achieved 0.999. These results indicate the fitness of bioanalytical method. Over the concentration Vs mill absorbance unit (Table 1).

#### *Recovery*

In literature survey shown 70% achieved less than 90%. Our developed method has been achieved 90% (Figure 3). These percentage recovery indicates, more sensitivity when compare other methods such as solid phase extraction, liquid-liquid extraction and literature survey.

#### *Speed of analysis*

The optimized aqueous chromatographic condition was achieved less than 5 mins and bioanalytical method was achieved less than 15% min. in those 5 minutes for extraction of samples from plasma, 10 mins for elution of drug.

#### *Stability studies*

##### *Stock solution stability*

Stock solutions were prepared by using methanol and water in the ratio of 1:1. From the stock solution at the time of bioanalytical method validation preferred different quality control samples. All the quality control samples stored at suitable temperature.

##### *Short and long term*

Middle quality control and high quality control samples were prepared at time of method validation and injected in the system and compared with the short term and long term duration (Table 2).

##### *System suitability*

System suitability test was performed daily before the run of analytical batch to check detector response to the analyte. Both the formulations were well tolerated by all the volunteers in both the phases of study (Table 3).

##### *Freeze thaw stability*

At the time of method validation samples were taken from the deep freeze and kept for thaw. The different quality control samples compared with freshly prepared thaw samples.

## **CONCLUSION**



In summary, HPLC method quantification of triamcinolone in drug free human plasma were developed and validated as per FDA guidelines. This method has shown more significant merits over those previous available methods. This method offers significant advantages over those previously reported, in terms of improved sensitivity and selectivity, faster run time (5 mins) and lower sample requirements. Hence, this method may be useful for single and multiple ascending dose studies in animal and human subjects. The developed method has excellent sensitivity, reproducibility and specificity. The method has been successfully used to provide the bioavailability and bioequivalent study of triamcinolone in animal and human plasma. The developed assay showed acceptable precision, accuracy, linearity, stability, and specificity so applicable to clinical tool.

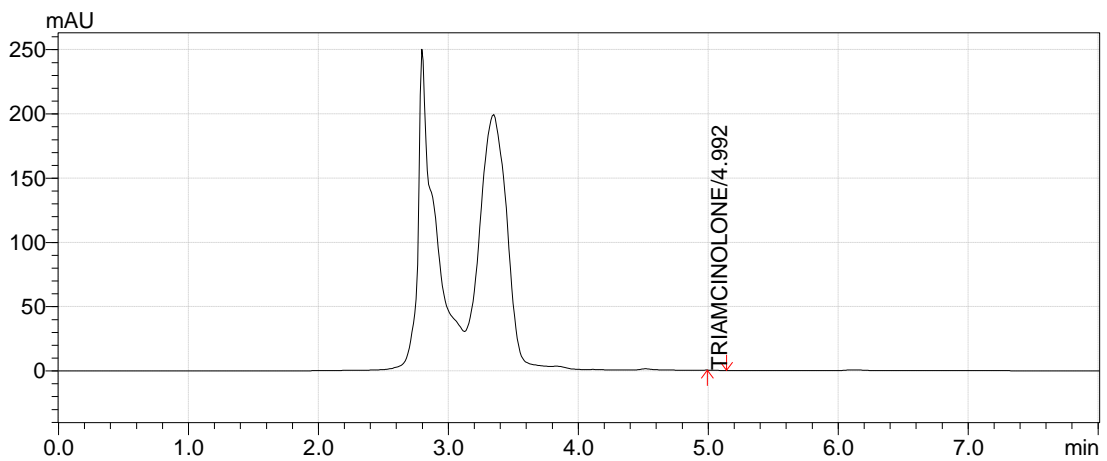
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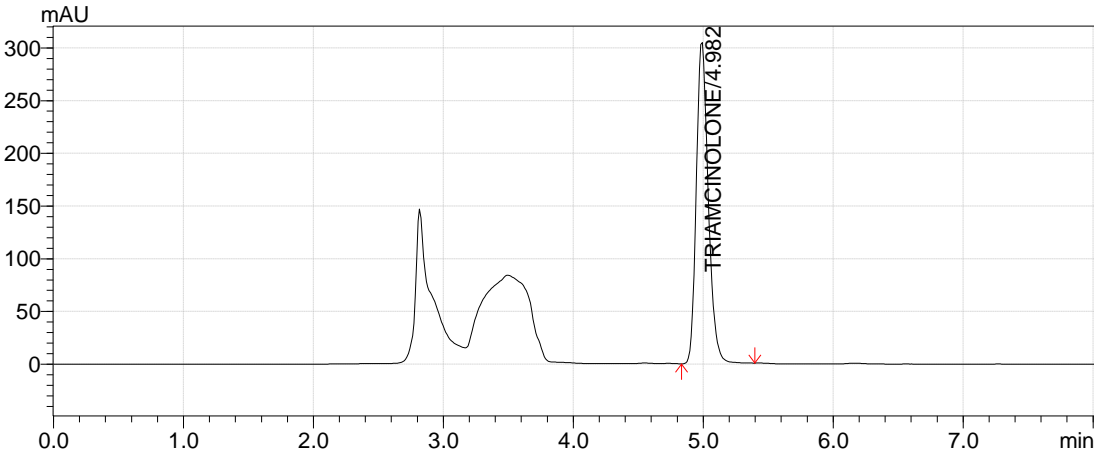
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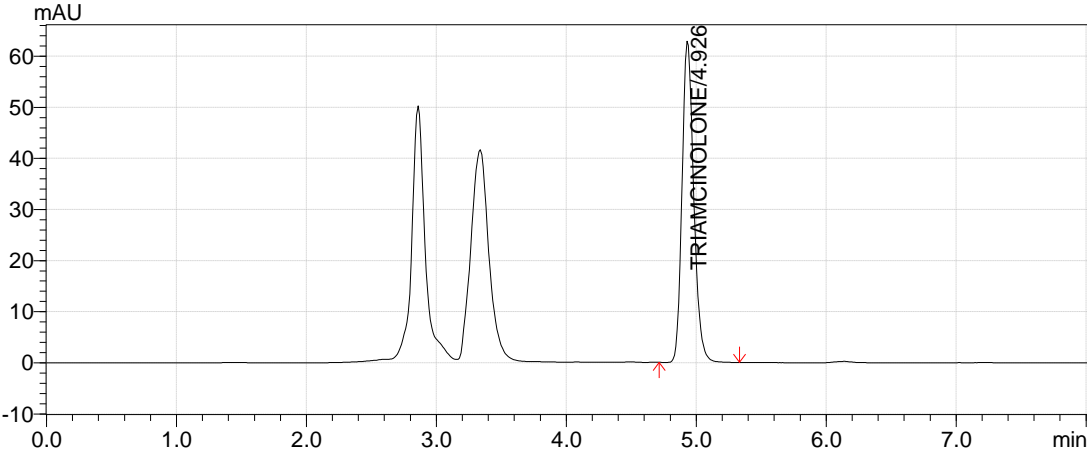
**Figure 1 Typical chromatogram of blank plasma**



**Figure 2 Typical chromatogram of spiked plasma**



**Figure 3 Typical chromatogram of extracted plasma for recovery**



**Table 1.** Intra- and Inter-day accuracy and precision of triamcinolone in human plasma

<b>Standard concentration(µg/mL)</b>	<b>Average calculated concentration (µg/mL)</b>	<b>SD</b>	<b>% Accuracy</b>
<b>Inter-day (n=3)</b>			
<b>1.0</b>	0.96	0.38	
<b>6.0</b>	5.71	0.52	
<b>15.0</b>	14.86	0.47	98.5%
<b>Intra-day (n=3)</b>			
<b>1.0</b>	0.92	0.47	
<b>6.0</b>	5.73	0.82	
<b>15.0</b>	14.39	0.93	99.3%

**SD-Standard deviation**

**Table 2.** Stability study of triamcinolone

<b>Standard concentration (µg/mL)</b>	<b>Average concentration (µg/mL)</b>	<b>SD</b>
<b>Bench top (n=5)</b>		
<b>6.0</b>	5.94	0.46
<b>15.0</b>	14.25	0.82
<b>Freeze thaw stability (n=5)</b>		
<b>6.0</b>	5.12	0.76
<b>15.0</b>	14.21	0.75
<b>Long term stability (n=5)</b>		
<b>6.0</b>	5.63	1.05
<b>15.0</b>	14.14	0.93

**SD-Standard deviation**

**Table 3 System suitability studies**

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S. No.	Parameters	
	Triamcinolone	
1	Theoretical plate/meter	4752
3	Asymmetric factor	0.93
4	LOD (ng/mL)	47
5	LOQ (ng/mL)	90
6	Capacity factor ( <i>k</i> )	1.8
7	Asymmetry	1.25

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LOD-Limit of detection

LOQ-Limit of quantification