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Determination of Triamcinolone in Subretinal Fluid by a Validated HPLC Method

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Introduction

Triamcinolone acetonide [9-Fluoro-11ß,16a,17,21-tetrahydroxypregna-1, 4-diene-3, 20-dione cyclic 16,17-acetal with acetone ($C_{24}H_{31}FO_6$)] (Fig. 1), 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]. Because duration and severity of effects and side effects of triamcinolone acetonide (TRE) dependent on its penetration to ocular fluids such as aqueous humor, vitreous, subretinal, thus the determination of TRE levels in human ocular media is important for the investigation of intraocular availability of TRE.

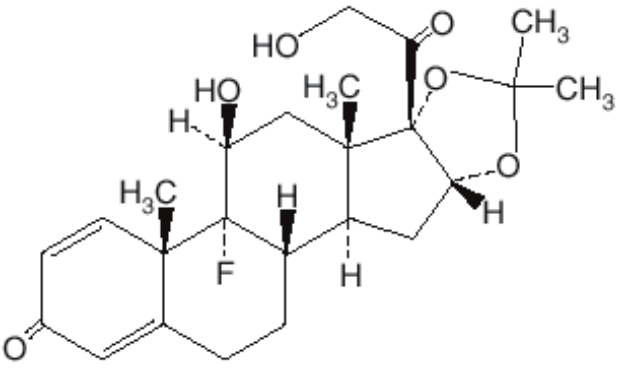

Figure 1
Chemical structure of TRE

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Several 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].

To our knowledge, there has been no reported analysis method for the determination of triamcinolone in subretinal fluids. Therefore, the aim of this study was to develop an HPLC method for the determination of TRE in subretinal fluids which can be used for ascertaining subretinal fluid levels of TRE after opthalmological applications.

Experimental

2.1 Apparatus

The HPLC equipment comprised of a solvent delivery system (Shimadzu 10 ATVP) and a photodiode array detector (Shimadzu M 10VP). The separations were achieved by a reversed phase column (Nucleosil 100-5 C_{18} 150 x 4.6 mm, 5 µm i.d.) at room temperature. The mobile phase was consisted of 60:40 (methanol : water, v/v) at a flow rate of 1.5 mL min^{-1} and the mobile phase was purged with Helium during chromatographic separation. UV detection was performed at 250 nm.

2.2 Chemicals and reagents

TRE and prednisolone (IS) were purchased from Sigma. Methanol was HPLC-grade and a Milli-Q water system (Barnstead, USA) was used. All other chemicals were of analytical reagent grade.

2.3 Standard and sample solutions

2.3.1 Standard solutions

Stock solutions of TRE (1000 µg mL^{-1}) and prednisolone (IS) (1000 µg mL^{-1}) were prepared in methanol. Working solutions were daily prepared by diluting stock solutions in mobile phase to desired concentrations. Stock solutions were stored at – 25 °C until analysis and protected from light.

2.3.2 Sample preparation

Subretinal fluid samples (100 µL) were added 25 µL (5 µg mL^{-1}) IS by mixing with vortex-mixer for 30 s. Then 175 µL ACN was added and

mixed on a vortex-mixer a for 2 min. After centrifugation (5000 rpm, 10 min), 150 µL supernatant was transferred micro vial and 50 µL of the solution was injected onto the column.

2.4 Calibration curve

The calibration curve was prepared by plotting the peak area ratio of TRE to IS against to the concentration of TRE at 0.017, 0.020, 0.030, 0.050, 0.070, 0.100, 0.200, 0.500, 0.700 and 1.000 µg mL^{-1} containing prednisolone (0.25 µg mL^{-1}) as an internal standard. Calibration curve standards and subretinal fluid samples were prepared for the analysis by the same procedure given in section 2.3.2. Because of the ethical restrictions the adequate blank subretinal fluid from patient can not be taken, then a calibration curve in drug-free subretinal fluid was not possible. Thus the calibration curve standards were prepared in ACN.

2.5 Sample collection

Patients were under topical TRE therapy and were given TRE before surgery by posterior subtenon injection. After 24 hours of TRE application, subretinal fluid samples were drawn during retinal detachment surgery. None of the patients had any ocular pathology other than retinal detachment. Local ethical committee of Numune Hospital approved this study and the written informed consent is taken from all patients accepted to the study. Samples were stored at – 25 °C until analysis and protected from light.

Results and Discussion

3.1 Analysis of TRE in subretinal fluid

In the presented method, TRE and IS are separated on Nucleosil 100-5 C_{18} column (150 x 4.6 mm, 5 µm i.d.) using mobile phase containing methanol:water (60:40 v/v) at a flow rate of 1.5 ml min^{-1}. Under optimized chromatographic conditions TRE and IS were accurately resolved from baseline and separated each other and there was no matrix effect from subretintal fluid (Fig. 2). Helium purge of mobile phase in the period of separation dramatically reduced baseline noise, which resulted in increased signal to noise ratio. Retention times for TRE and IS were 4.176 ± 0.066 (RSD=1.57 %, n=15) min and 2.923 ± 0.037 (RSD=1.28 %, n=15) min, respectively. The relative retention time of TRE/PRE was 1.428 ± 0.004 (RSD=0.31 %, n=15). Inter-day and intra-day variations of TRE and PRE retention times were less than 2 %. Additionally, the run time is adjusted to 20 min to avoid from effects of late eluting peaks.

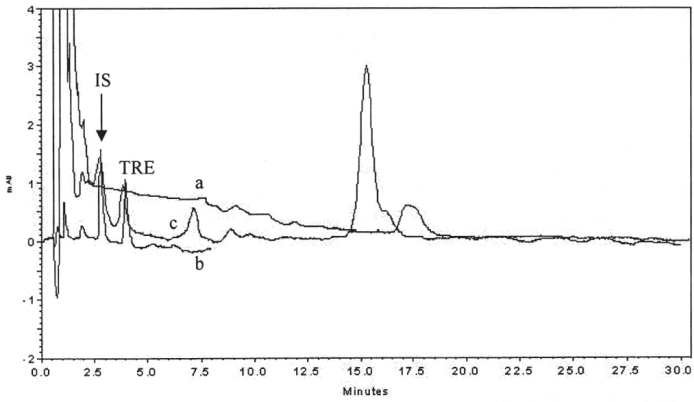

Figure 2
The representative chromatograms of a) drug-free subretinal fluid, b) 0.025 μg mL^{-1} TRE standard solution (IS: 0.25 μg mL^{-1}) and c) a patient subretinal fluid sample containing 2.21 μg mL^{-1} TRE (IS: 0.25 μg mL^{-1}).

3.2 Validation

The use of IS is an important parameter for reproducibility in HPLC in order to compensate the errors from injection and extraction process. In this study, prednisolone was selected as an IS. The method was tested with respect to validation parameters such as selectivity, linearity, precision and accuracy[15].

TRE and IS were well resolved from baseline and separated from each other and there was no matrix effect from subretinal fluid (Fig. 2). The spectrum obtained from PDA at the apexes and top of TRE peak from patient samples was identical thus the similarity for TRE peak was equal to unit. Same peak similarity was obtained for PRE. These results suggested that the method presented in this study was specific.

Developed method was linear in the range of 0.017-1.00 μg mL^{-1}. The regression equation was y = 3.6794x - 0.0362 (r = 0.9998, n=6) where y is the ratio of peak area (TRE/IS) and x is the concentration.

Limit of detection (LOD) as being the lowest concentration of TRE that can be distinguished from noise at a signal-to-noise ratio of 3:1 was 12 ng mL^{-1}. The limit of quantitation (LOQ) for TRE was found to be 17 ng mL^{-1} with a RSD value of 7.16 % (n=6).

Precision studies of the method were performed at three different concentrations of TRE (in the linear range) by using six independent series in the same day (intra-day precision) and six consecutive days (inter-day precision) within each series every sample was injected 3 times. The

RSD values of intra-day and inter-day studies varied from 1.19 % to 2.14 % showing that the intermediate precision of the method was satisfactory (Table 1).

TABLE I

Precision and accuracy of the HPLC method for the analysis of TRE (n=6).

	Intra-day			Inter-day		
Added µg mL^{-1}	Found $\bar{x}$ (µg mL^{-1}) ± SE	Accuracy Bias (%)	Precision RSD (%)	Found $\bar{x}$ (µg mL^{-1}) ± SE	Accuracy Bias (%)	Precision RSD (%)
0.02	0.0199 ± 0.0002	-0.5095	2.1389	0.0201 ± 0.0020	0.02176	2.0696
0.05	0.0491 ± 0.0003	-1.8611	1.8252	0.0496 ± 0.0020	-0.8588	1.1889
0.10	0.1023 ± 0.0005	2.2551	1.6924	0.1001 ± 0.0007	0.1041	1.7132

$\bar{x}$: Mean, SE : Standard error, RSD : Relative standard deviation, Bias : [100 x (Found – Added) / Added]

The accuracy of a method is expressed as the closeness of agreement between the found value and reference value. It is determined by calculating the percentage relative error between the measured mean concentrations and added concentrations at the same concentration level of TRE. The results obtained for intra and inter day accuracy were ≤ 2.26% and ≤ 0.86 %, respectively (Table I).

In the literature, many solutions such as MeOH, ACN, EtOH or TCA were used for denaturation of proteins. In our study, ACN, EtOH and MeOH were tested and ACN was selected as denaturation solvent because of its high recovery. The recovery of TRE from subretinal fluid was 99.30 ± 0.63 (1.27 %, n = 4) at the concentration of 0.10 µg mL^{-1}.

3.3 Application to subretinal fluid samples

The method was applied to the analysis of subretinal fluid samples obtained from 10 patients. TRE levels in subretinal fluid after posterior subtenon injection can be detected by the method presented in this study (Fig. 2). TRE levels in subretinal fluid after topical administration were found between 0.97 µg mL^{-1} and 0.14 µg mL^{-1} and all concentrations were higher than the lower determination and quantitation levels of the developed method (Table II).

TABLE II

TRE levels of subretinal fluid samples obtained from patients after topical administration.

Subject No	Triamcinolone Level ($\mu g\ mL^{-1}$)
1	0.97
2	0.92
3	0.28
4	0.15
5	0.48
6	0.91
7	0.25
8	0.14
9	0.72
10	0.30

Conclusion

In this study, a simple, fast, efficient and reliable HPLC method was developed and validated for the analysis of TRE in subretinal fluid and its applicability to the samples obtained from patients was also demonstrated. Thus, the developed method was recommended for clinical investigations based on TRE level determination after opthalmological applications because of its high recovery, precision and accuracy.

Summary

A reversed-phase high-performance liquid chromatographic method is described for the determination of triamcinolone in subretinal fluids. Triamcinolone and the internal standard prednisolone (IS) were determined using a reversed-phase column (Nucleosil 100-5 C_{18}, 150 x 4.6 mm, 5 µm i.d.) eluted with a mobile phase containing methanol:water (60:40, v/v) at a flow-rate of 1.5 mL min^{-1}. Photodiode array detector was set to 250 nm. The retention times for triamcinolone and IS were 4.0 and 2.9 min, respectively. The linearity range was found to be in the range of 0.017-1.00 µg mL^{-1}. LOD and LOQ values were 12 ng mL^{-1} and 17 ng mL^{-1}, respectively. The recovery of triamcinolone from subretinal fulid was 99.30 ± 0.63 %. In intra-day and inter-day studies, the developed method was found to be accurate and precise with a bias value less than 2.5 % and the RSD value less than 2 %. In addition, the method was successfully applied to the analysis of triamcinolone in subretinal samples obtained from patients.

Keywords: Triamcinolone; HPLC; Validation; Subretinal fluid

Özet

Triamsinolonun Subretinal Sıvıda Valide Edilmiş HPLC Yöntemi ile Tayini

Triamsinolonun subretinal sıvıdan tayini için ters faz yüksek performanslı sıvı kromatografik yöntem tanımlanmıştır. Triamsinolon ve iç standart prednisolon (Nucleosil 100-5 C_{18} 150 x 4.6 mm, 5 µm i.d.) ters faz kolonu metanol:su (60:40, h/h) içeren hareketli faz ile elue edilmiştir. Hareketli faz akış hızı 1.5 ml dak^{-1} ve fotodiyot array dedektör dalgaboyu 250 nm dir. Triamsinolon ve IS alıkonma zamanları sırasıyla 4.0 and 2.9 dakikadır. Yöntemin doğrusallık aralığı 0.017-1.00 µg mL^{-1} arasındadır. Saptama sınırı (LOD) ve tayin alt sınırı (LOQ) sırasıyla 12 ng mL^{-1} ve 17 ng mL^{-1}'dir. Triamsinolonun subretinal sıvıdan geri kazanımı %99.30 ± 0.63 olarak hesaplanmıştır. Geliştirilen yöntemin gün içi ve günler arası tekrarlanabilirlik çalışmalarında yüzde bağıl hata %2.5'den küçük ve yüzde bağıl standart sapma % 2'den küçük bulunmuştur. Ayrıca, yöntem hastalardan sağlanan subretinal sıvı numunelerinin analizinde başarıyla uygulanmıştır.

Anahtar kelimeler: Triamsinolon ; HPLC; Validasyon ; Subretinal sıvı

REFERENCES

1. Martidis, A, Duker, J.S., Greenberg, P.B.: Intravitreal Triamcinolone for Refractory Diabetic Macular Edema. Ophthalmol, 109, 920 (2002).
2. Antcliff, R.J., Spalton, D.J., Stanford, M.R.: Intravitreal Triamcinolone for Uveitic Cystoid Macular Edema: An Optical Coherence Tomography Study. Ophthalmol, 108, 765 (2001).
3. Greenburg, P.B., Martidis, A., Rogers, A.H., Duker, J.S., Reichel, E.: Intravitreal Triamcinolone Acetonide for Macular Oedema Due to Central Retinal Vein Occlusion. Br. J. Ophthalmol, 86, 247 (2002).
4. Huopalahti, R.P., Henion, J.D.: Application of Supercritical Fluid Extraction and High Performance Liquid Chromatography/Mass Spectrometry for The Determination of Some Anabolic Agents Directly From Bovine Tissue Samples. J. Liquid Chromatog. Related Technologies, 19(1), 69 (1996).
5. Derendorf, H., Rohdewald, P., Hochhaus, G., Mollmann, H.: HPLC Determination of Glucocorticoid Alcohols, Their Phosphates And Hydrocortisone in Aqueous Solutions and Biological Fluids. J. Pharm. Biomed. Anal., 4(2), 197 (1986).
6. Althaus, Z.R., Rowland, J.M., Freeman, J.P.: Separation of Some Natural and Synthetic Corticosteroids in Biological Fluids and Tissues By High-Performance Liquid Chromatograph. J. Chromatog. B: Biomed. Sci. Appl. 227(1), 11 (1982).

7. Cirimele, V., Kintz, P., Dumestre, V., Goullé, J.P., Ludes, B.: Identification of Ten Corticosteroids in Human Hair By Liquid Chromatography-Ionspray Mass Spectrometry. Forensic Sci. Int. 107, 381 (2000).
8. Mora, P., Eperon, S., Felt-Baeyens, O., Gurny, R., Sagodira, S., Breton, P., Guex-Crosier, Y.: Trans-Scleral Diffusion of Triamcinolone Acetonide, Current Eye Research, 30, 355 (2005).
9. Beer, P.M., Bakri, S.J., Singh, R.J., Liu, W., Peters III, G.B., Miller, M.: Intraocular Concentration and Pharmacokinetics of Triamcinolone Acetonide After A Single Intravitreal Injection. Ophthalmol., 110(4), 681 (2003).
10. Gaillard, Y., Vayssette, F., Pépin, G.: Compared Interest Between Hair Analysis And Urinalysis In Doping Controls Results For Amphetamines, Corticosteroids And Anabolic Steroids In Racing Cyclists. Forensic Sci. Int., 107, 361 (2000).
11. Amendola, L., Garribba, F., Botrè, F.: Determination Of Endogenous And Synthetic Glucocorticoids in Human Urine By Gas Chromatography-Mass Spectrometry Following Microwave-Assisted Derivatization. Anal.Chim. Acta, 489, 233 (2003).
12. Hubbard, W.C., Liu, M.C., Bickel, C., Argenti, D., Heald, D., Schleimer, R.P.: Measurement of Low Picomolar Levels of Triamcinolone Acetonide in Human Bronchoalveolar Lavage Fluid By Gas Chromatography-Electron-Capture Negative-Ion Mass Spectrometry. Anal. Biochem. 290(1), 28 (2001).
13. Ganderton, D.: British Pharmacopoeia, Her Majesty Stationary Office, London, 1998, pp. 1321, 1978.
14. Novitch, M.: The United States Pharmacopoeia XXIV. Pharmacopoeial Convention Inc, USA, 2000, p. 1685.
15. ICH Topic Q2A, Validation of Analytical Procedures: Methodology, CPMP/ICH/281/95.