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BIOCHEMICAL INVESTIGATION AND DEVELOPMENT OF HPLC METHOD FOR TACROLIMUS

Ishraga Eltayeb M. A-Elbasit[1*], Mohd. Imran[2], Naira Nayeem[2]

[1]Department of Basic Health Sciences, Faculty of Pharmacy, Northern Border University, P.O. BOX 840, Rafha 91911, Kingdom of Saudi Arabia.

[2]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Northern Border University, P.O. BOX 840, Rafha 91911, Kingdom of Saudi Arabia.

Abstract:

The objective of this work was to develop a method for the quantification of tacrolimus in rabbit aqueous humor by UHPLC. UHPLC analysis was performed on a Waters Acquity UHPLC system (Milford, MA, USA). A 50 µL aliquot of rabbit aqueous humor was pipetted into a 2.0 ml Eppendorf tube and 100 µL of acetonitrile was added to precipitate the protein. The samples were vortex mixed for 2 min followed by filtration through 0.22 µm nylon filter. To this filtrate, 0.4 ml of 0.01M iodine was added and volume up to 1.0 ml was made with acetonitrile. Five microliter of this solution was injected into the UHPLC system. All the rabbit aqueous humor samples were stored at −20°C and were allowed to thaw at room temperature prior to sample preparation. Linearity was investigated by the assay in parallel of triplicate rabbit's aqueous humor samples spiked with TAC to concentrations of 10, 20, 50, 100, 200, 400, 600 and 800 ng/ml. Stability assessments under different conditions: bench-top, short-term, long-term storage stability and freeze–thaw were established. The results indicated that TAC had an acceptable stability under those conditions. A method was developed for quantification of tacrolimus in rabbit aqueous humor by UHPLC. This method with QL of 1.0 ng/ml was fast and just took 5 minutes. There were no interferences found from endogenous aqueous humor components or other sources. This assay has showed consistent precision and accuracy. The analytical method presented here will be useful for the determination of tacrolimus concentration in the ocular aqueous humor.

Keywords: *Tacrolimus, UHPLC, Validation, Stability Study, Ocular.*

Corresponding author:

Ishraga Eltayeb M. A-Elbasit,
Department of Basic Health Sciences,
Faculty of Pharmacy, Northern Border University,
P.O. BOX 840, Rafha 91911,
Kingdom of Saudi Arabia.
E-Mail: *ishraga20@yahoo.co.uk*
Mobile: *+966533506434*

QR code

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1. INTRODUCTION:

Tacrolimus (TAC) (Figure 1) is a 23-membered macrolide lactone, originally isolated from the bacterium *Streptomyces tsukubaensis*. As a potent immunosuppressant it is widely used for graft rejection prevention after organ transplantation [1]. TAC is also used in the treatment of autoimmune diseases such as multiple sclerosis, psoriasis, rheumatoid arthritis and atopic dermatitis [2]. The use of TAC is of special interest in ophthalmology because it was proved to be effective in the treatment of immune-mediated diseases of the eye such as dry eye syndrome, uveitis, scleritis, keratoconjunctivitis and corneal graft rejection [3-8]. The ability of TAC to suppress pathogenic pathway of inflammation at the site of action is an important goal and therefore the determination of TAC pharmacokinetics in the eye especially in aqueous humor is desirable and is needed. A review of literature reveals few bioanalytical methods currently available for the quantification of TAC in body fluids, and most of them are for determining drug in plasma or whole blood in a therapeutic monitoring study [9-14]. Presently, immunoassay [15] and LC–MS-MS [16] method has been reported for determination of TAC in aqueous humor. Immunoassays are widely used for the routine determination of TAC; however, they lack specificity due to cross reactivity of monoclonal antibodies with the metabolites and endogenous compounds [17]. Although, LC–MS-MS method described in the literature is highly specific and selective [16] but such performance is expected to remain the privilege of a few laboratories because of the onerous equipment required and complicated analysis. Pre-clinical pharmacokinetic investigations require the support of a fast and sensitive bioanalytical method for the measurement of the drug involved and usually for the analysis of drug in the aqueous humor, with small amount of proteins, HPLC-UV or UHPLC-UV method is preferred and extensively utilized [18]. HPLC method for determination of TAC in dosage form has been reported [19], however, to best of our knowledge, no validated HPLC or UHPLC method for the quantification of TAC in the aqueous humor, has been published in the literature. A weak chromophore group in the TAC and low drug concentrations achieved in the aqueous humor after topical drug instillation does not allow its detection by HPLC or with UHPLC [20]. The present work comes up from the lack of UHPLC methods that permit to determine TAC in aqueous humor. In the present study the sensitivity towards TAC was improved by pre-column formation of charge transfer complex with iodine followed by UHPLC-UV analysis. The method is simple and sensitive with a quantification limit sufficiently low to support aqueous humor pharmacokinetics.

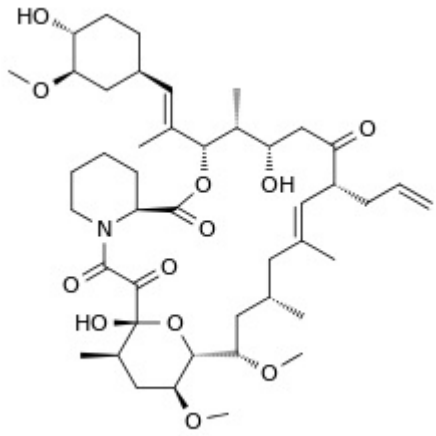

Fig.1: Tacrolimus

2. MATERIAL AND METHODS:

2.1. Chemicals

TAC (MW 803.5) was a gift from Biocon Pharmaceuticals (Bangalore, India). Transcutol P was obtained as a gift sample from Gattefosse India Pvt. (New Delhi, India). Tween 80 and PEG 400 were purchased from S.D. Fine-Chem Ltd. (Mumbai, India). Acetonitrile of HPLC grade was obtained from Qualigens Fine Chemicals (Mumbai, India). Distilled de-ionized water was prepared with the Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Chromatography

UHPLC analysis was performed on a Waters Acquity UHPLC system (Milford, MA, USA) equipped with a binary solvent manager, an auto sampler, column manager composed of a column oven, a pre-column heater and a photo diode array detector. Five microliter of the final analytical solution was injected into a Waters Acquity BEH C18 (50 mm × 2.1 mm, 1.7 µm) UHPLC column kept at 50°C and the chromatographic separation was performed by isocratic elution. The mobile phase consisting of a mixture of water and acetonitrile (1:1, v/v), with the flow rate of 0.4 ml/min was employed. The analysis was performed at 362 nm wavelength with a total run time of 8 min. Data acquisition, data handling and instrument control were performed by Empower Software v1.0.

2.3. Aqueous humor samples

A 50 µL aliquot of rabbit aqueous humor was pipetted into a 2.0 ml Eppendorf tube and 100 µL of acetonitrile was added to precipitate the protein. The samples were vortex mixed for 2 min followed by filtration through 0.22 µm nylon filter. To this filtrate, 0.4 ml of 0.01M iodine was added and volume upto 1.0 ml was made with acetonitrile. Five microliter of this solution was injected into the UHPLC system. All the rabbit aqueous humor samples were stored at −20°C and were allowed to thaw at room temperature prior to sample preparation.

2.4. Calibration

A stock solution of TAC (10µg/mL) was prepared by dissolving an appropriate amount of TAC in acetonitrile. Working standard solutions of TAC were prepared daily by diluting the stock solution with 0.4 ml of 0.01M iodine followed by volume make up with acetonitrile. To prepare the aqueous humor calibration standards, aliquots of 50 µl of aqueous humor were placed in each eppendorf tube and spiked with increasing concentrations of working standard solutions. The samples were vortex mixed for 2 min followed by filtration through 0.22 µm nylon filter. To this filtrate, 0.4 ml of 0.01M iodine was added and volume upto 1.0 ml was made with acetonitrile to give TAC concentrations of 10, 20, 50, 100, 200, 400, 600 and 800 ng/mL. Calibration standards were analyzed by UHPLC method.

2.5. Validation and stability

Linearity was investigated by the assay in parallel of triplicate rabbit's aqueous humor samples spiked with TAC to concentrations of 10, 20, 50, 100, 200, 400, 600 and 800 ng/mL. The TAC mean peak area was plotted against TAC concentration and regression analysis was performed. Precision was determined by repeat assay (n =6) of aqueous humor samples spiked with 1.0, 10.0, 50.0 and 100.0ng/mL TAC and expressed as the percentage coefficient of variation (CV) of the peak area. Accuracy expressed as percentage recovery was calculated as mean back calculated concentration/ theoretical concentration ×100. Quantitation limit (QL) in aqueous humor was defined as the lowest concentration on the calibration curve in which an acceptable accuracy of ±20% and a precision below 10% were obtained. The extraction efficiency was determined in triplicate at 50.0 ng/mL for TAC in aqueous humor. The peak areas obtained after extraction were compared with peak areas resulting from standard solutions at the same concentration. Recovery studies were carried out by applying the method to TAC-gel formulation to which known amount of TAC corresponding to 100% of the TAC label claim had been added. The analysis was done in triplicate. The bench-top stability (stored at room temperature for 4 hrs), short-term stability (stored at room temperature for 12 hrs), long-term storage stability (stored at −20 °C for 15 days) and stability after undergoing three freeze–thaw cycles of TAC in rabbit aqueous humor was evaluated.

3. RESULTS AND DISCUSSION:

3.1. Method development

The chemical structure of TAC and presence of the weak chromophore group does not allow detection by HPLC at therapeutic concentrations achieved in the aqueous humor (~40ng/mL) after topical ocular instillation of TAC [20]. This study attempted to overcome the above limitations by detecting TAC following pre-column formation of charge transfer complex followed by UHPLC detection at a wavelength of 362 nm. TAC being n-donor drug forms a stable charge transfer complex with π-electron acceptor iodine. The complex improves the sensitivity of the method by inducing unsaturated enone system thereby increasing potency of weak chromophore. The influence of various factors on absorbance viz. acceptor concentration, reaction time and choice of solvent was studied. The concentration of iodine must be suitable for quantitative reaction and should not be much higher than TAC concentration. It was found that 40% v/v of 0.01 M iodine solution 0.4 ml of 0.01M iodine was sufficient for the production of maximum and reproducible absorbance intensity. Higher concentrations of the iodine do not affect the absorbance intensity. The optimum reaction time was determined by monitoring the color developed at room temperature. Complete color development was attained after 20 min. This indicates that UHPLC analysis should be done 20 min after the addition of reactants to attain stable readings. The developed complex was found to be stable for at least 2 h without change in absorbance and peak area. In order to select the most appropriate solvent, the reaction was carried out in different solvents, acetonitrile, acetone, ethanol, water, Dimethylformamide and Dimethylsulfoxide. The results indicate that the acetonitrile was the most suitable dilution solvent because it affords excellent solvating power for iodine. High dielectric constant of acetonitrile promotes maximum yield of radical anions, high solvation of the acceptor and maximum sensitivity. In acetonitrile, complete electron transfer from TAC to iodine took place with the formation of complex with high molar absorptivity values. Further, the reaction of TAC

and iodine in acetonitrile is stoichiometric in the ratio 1:1 as determined by Job's method of continuous variation. Further, acetonitrile is also used as liquid extraction solvent which helps in protein precipitation. The use of UHPLC system with Bridged Ethyl Hybrid column packed with small sized (1.7 µm) particles, and ultra-high pressures further improves the sensitivity of the analysis. The developed method is sensitive, specific and rapid and is successfully applied to determine ocular pharmacokinetics of TAC after topical instillation.

3.2. Method validation

Chromatography. Using the chromatographic conditions described, rapid elution of TAC from aqueous humor was achieved at 2.2 min (Figure2). UHPLC chromatograms of blank aqueous humor (Figure 2A) and the aqueous humor spiked with TAC (Figure 2B) were compared to show the selectivity of the proposed procedure. No interference was observed either by matrix or by formulation ingredients near the retention time, demonstrating the method's selectivity.

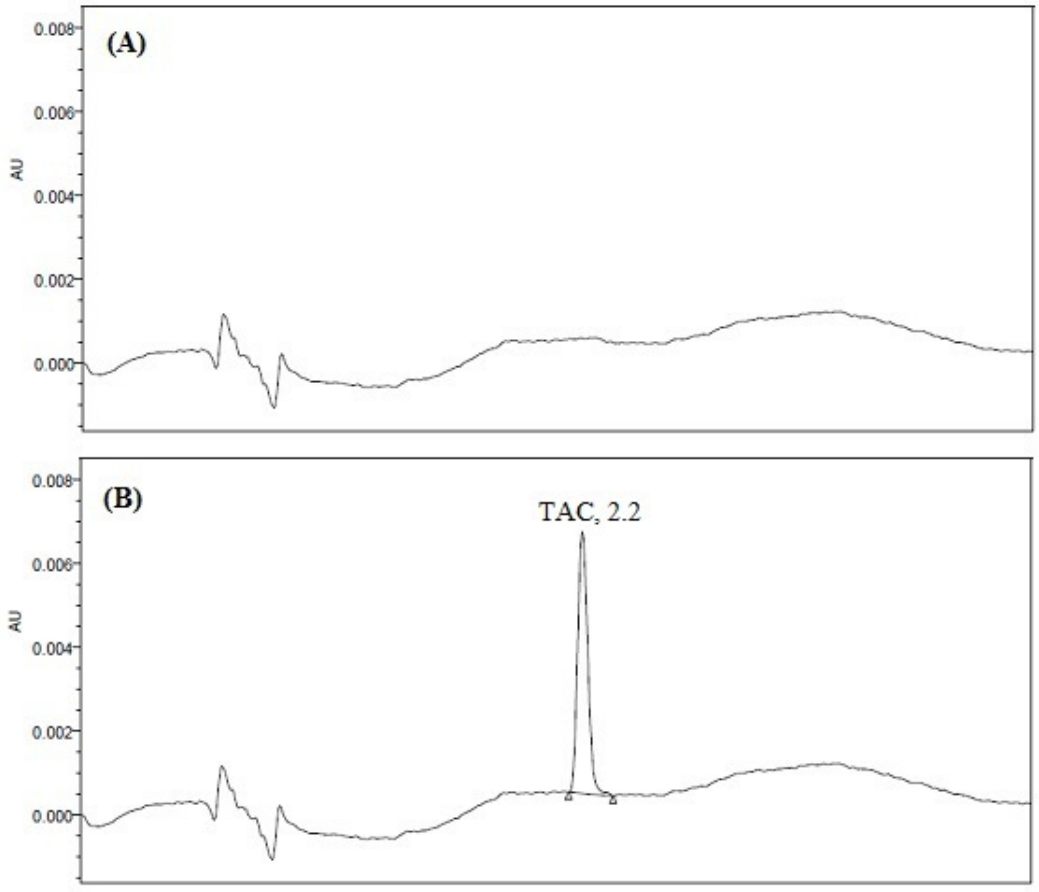

Fig. 2: UHPLC chromatograms of blank aqueous humor (Figure 2A) and the aqueous humor spiked with TAC (Figure 2B)

Method Validation. The results of the validation are summarized in Table 1. The method was found to be selective, precise, accurate and robust. The present method offered a quantitation limit (QL) of 1.0 ng/ml in the rabbit aqueous humor sample. Under the present QL of 1.0 ng/ml, the concentration of the TAC can be determined in aqueous humor samples after a single dose of 0.3% TAC eye drops which is sensitive enough for the pharmacokinetic study of TAC in the eye.

Stability Studies. Stability assessments under different conditions: bench-top, short-term, long-term storage stability and freeze–thaw were established. The results indicated that TAC had an acceptable stability under those conditions, as shown in Table 2.

Table 1: Method validation data for TAC in aqueous humor

Parameter	**Value**				
Linearity (n=6)					
	Range (ng/mL)	Correlation coefficient (r^2)	Slope (SD)	Intercept (SD)	
	10 – 800	0.998	740.54 (3.241)	1162.30 (8.621)	
Sensitivity					
	QL (ng/mL)				
	1.0				
Reproducibility (n=6)					
Intraday	TAC spiked (ng/mL)	TAC found (ng/mL)	SD	**Precision** [a]	**Accuracy** [b]
	1.0 (QL)	0.92	0.08	8.69	92.0
	10.0	10.09	0.05	0.49	100.9
	50.0	51.35	2.60	5.06	102.7
	100.0	102.8	3.85	3.74	102.8
Interday					
	1.0 (QL)	0.93	0.09	9.68	93.0
	10.0	10.80	0.45	4.17	108.0
	50.0	50.50	2.97	5.88	101.0
	100.0	98.35	4.60	4.68	98.4
Extraction efficiency (n=3)					
	TAC spiked (ng/mL)	TAC found (µg/mL)	SD	Mean recovery (%)	
	50.0	45.5	5.5	91.0	
Recovery (SD; n=3)					
	TAC-gel				
	98.50 (1.9)%				

[a] Precision as CV (%) = 100 × Standard deviation/ Mean concentration found
[b] Accuracy (as % recovery) = 100 × Mean concentration found/Concentration spiked

Table 2: Summary of bench-top, short-term, long-term, and freeze–thaw storage stability

Condition	Nominal Amount (ng/mL)	Mean Amount Found [a] (ng/mL)	Accuracy [a] (%)
Bench Top	50.0	50.9	101.8
	100.0	99.2	99.2
Short Term	50.0	48.3	96.6
	100.0	100.4	100.4
Long Term	50.0	46.5	93.0
	100.0	96.8	96.8
Freeze-Thaw	50.0	45.8	91.6
	100.0	94.9	94.9

[a] Each concentration tested three times

[b] Accuracy (as % recovery) = 100 × Mean amount found/Nominal amount

4. CONCLUSION:

A method was developed for quantification of tacrolimus in rabbit aqueous humor by UHPLC. This method with QL of 1.0 ng/ml was fast and just took 5 minutes. There were no interferences found from endogenous aqueous humor components or other sources. This assay has showed consistent precision and accuracy. The analytical method presented here will be useful for the determination of FK506 concentration in the ocular aqueous humor.

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REFERENCES:

1. Scott LJ, McKeage K, Keam SJ, Plosker GL. Tacrolimus: a further update of its use in the management of organ transplantation. Drugs, 2003; 63(12):1247-1297.
2. Sádaba B, Azanza JR, García QE, Fernández V. Treatment with tacrolimus in autoimmune diseases. Rev Med Univ Navarra. 2004; 48(3):24-38.
3. Tinwala S, Shekhar H, Gupta S, Sinha R, Titiyal JS. Tacrolimus for Ophthalmic Use: An Update. Delhi J Ophthalmol. 2013; 23:211-215.
4. Lee YJ, Kim SW, Seo KY. Application for tacrolimus ointment in treating refractory inflammatory ocular surface diseases. Am J Ophthalmol. 2013; 155(5):804-813.
5. Zhai J, Gu J, Yuan J, Chen J. Tacrolimus in the treatment of ocular diseases. BioDrugs. 25(2):89-103.
6. Garg V, Jain GK, Nirmal J, Kohli K. Topical tacrolimus nanoemulsion, a promising therapeutic approach for uveitis. Med Hypotheses. 2013;81(5):901-904.
7. Pucci N, Caputo R, di Grande L, de Libero C, Mori F, Barni S, di Simone L, Calvani A, Rusconi F, Novembre E. Tacrolimus vs. cyclosporine eyedrops in severe cyclosporine-resistant vernal keratoconjunctivitis: A randomized, comparative, double-blind, crossover study. Pediatr Allergy Immunol. 2015;26(3):256-261.
8. Jung JW, Lee YJ, Yoon SC, Kim TI, Kim EK, Seo KY. Long-term result of maintenance treatment with tacrolimus ointment in chronic ocular graft-versus-host disease. Am J Ophthalmol. 2015;159(3):519-527.
9. Griffey MA, HockKG. Kilgore DC, Wei TQ, Duh SH, Christenson R, Scott MG. Performance of a no-pretreatment tacrolimus assay on the Dade Behring Dimension RxL clinical chemistry analyzer. Clin Chim Acta. 2007;384(1-2):48-51.
10. Bogusz MJ, Enazi EA, Hassan H, Abdel-Jawaad J, Ruwaily JA, Tufail MA. Simultaneous LC-MS-MS determination of cyclosporine A, tacrolimus, and sirolimus in whole blood as well as mycophenolic acid in plasma using common pretreatment procedure. J Chromatogr B. 2007; 850(1-2):471-480.
11. Chen YL, Hirabayashi H, Akhtar S, Pelzer M, Kobayashi M. Simultaneous determination of three isomeric metabolites of tacrolimus (FK506) in human whole blood and plasma using high performance liquid chromatography-tandem mass spectrometry. J Chromatogr B. 2006;830(2):330-341.

12. Brown NW, GondeCE, Adams JE, Tredger JM. Low hematocrit and serum albumin concentrations underlie the overestimation of tacrolimus concentrations by microparticle enzyme immunoassay versus liquid chromatography-tandem mass spectrometry. Clin Chem. 2005;51(3):586-592.
13. Ceglarek U, Lembcke J, Fiedler GM, Werner M, Witzigmann H, Hauss JP, Thiery J. Rapid simultaneous quantification of immunosuppressants in transplant patients by turbulent flow chromatography combined with tandem mass spectrometry. Clin Chim Acta. 2004;346(2):181-190.
14. Staatz CE, Taylor PJ, Tett SE. Comparison of an ELISA and an LC/MS/MS method for measuring tacrolimus concentrations and making dosage decisions in transplant recipients. Ther Drug Monit. 2002; 24(5):607-615.
15. Fujita E, Teramura Y, Shiraga T, Yoshioka S, Iwatsubo T, Kawamura A, Kamimura H. Pharmacokinetics and tissue distribution of tacrolimus (FK506) after a single or repeated ocular instillation in rabbits. J Ocul Pharmacol Ther. 2008; 24(3):309-319.
16. Yuan J, Chen JQ, Xie ZY, Zhai JJ, Zhou SY. Determination of tacrolimus in rabbit aqueous humor by liquid chromatography-electrospray ionization tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2008;868(1-2):34-41.
17. Tate J, Ward G. Interferences in Immunoassay. Clin Biochem Rev. 2004;25(2):105–120.
18. Ahuja S, Dong MW. Handbook of Pharmaceutical Analysis by HPLC. Amsterdam, Elsevier.
19. Namiki Y, Fujiwara A, Kihara N, Koda S, Hane K.,Yasuda T. Determination of the immunosuppressive drug tacrolimus in its dosage forms by high-performance liquid chromatography. Chromatographia. 40(5-6):253-258.
20. Yalçındag FN, Batıoğlu F, Arı N, Özdemir Ö. Aqueous humor and serum penetration of tacrolimus after topical and oral administration in rats: an absorption study. Clin Ophthalmol. 2007;1(1):61–64.