

Original Research Articles**HPLC METHOD FOR DETERMINATION OF FLUOROMETHOLONE AND SODIUM CROMOGLYCATe IN BULK AND OPHTHALMIC SOLUTION****Hani M. Hafez, Abdulla A. Elshanawany, Lobna M. Abdelaziz, Mustafa S. Mohram**

Medicinal Chemistry Department, Faculty of pharmacy, Zagazig University, Zagazig, Egypt

ABSTRACT

Fluorometholone and Sodium Cromoglycate are used in the treatment of allergic conjunctivitis. A validated HPLC method was developed for the assay of them. The method was performed on BDS HYPERSIL C18 column (250x4.6 mm, 5 μ) and the mobile phase consisted of potassium dihydrogen phosphate (pH 4.5, 0.025M) - Acetonitrile (40:60, V/V) which pumped at a flow rate 1.0 ml/min at ambient temperature. 20 μ l of drugs sample solutions were monitored at two fixed wavelengths (λ = 240.0 nm for Sodium Cromoglycate and 330.0 nm for Fluorometholone). The proposed method was validated in terms of linearity, accuracy, precision and limits of detection and quantitation according to ICH.

Keywords: Sodium Cromoglycate; Fluorometholone; HPLC.**Corresponding author:** Hany M. Hafez Medicinal Chemistry Department, Faculty of pharmacy, Zagazig University, Zagazig, Egypt. T.: 020113231458; E.: hanyhaf_1982@yahoo.com**1. Introduction**

Sodium Cromoglycate (cromolyn sodium) inhibits the degranulation of sensitized mast cells that occurs after exposure to specific antigens. It acts by inhibiting the release of histamine and other mediators from the mast cell. It has no intrinsic vasoconstrictor, antihistaminic or anti-inflammatory activity. It is indicated for allergic condition of the eye including acute and chronic allergic conjunctivitis and vernal keratoconjunctivitis [1]. Fluorometholone exerts an anti-inflammatory action in hyperemia, cellular infiltration and vascularization. It inhibits inflammatory response to inciting agents of mechanical, chemical or immunological nature. It prevents kinin release, inhibits prostaglandin synthesis and inhibits lymphocyte and neutrophil function. It is indicated for treatment of steroid responsive inflammatory conditions of bulbar conjunctiva, allergic conjunctivitis and non specific keratitis [1]. Fluca® eye drop contains 0.1% of Fluorometholone and 2.0% of Sodium Cromoglycate are instilled twice daily to treat allergic conjunctivitis. Development of HPLC method for simultaneous estimation of this combination is our scope.

Sodium Cromoglycate is disodium 5, 5'-[(2-hydroxypropane-1, 3-diyl) bis (oxy)] bis (4-oxo-4H-1-benzopyran-2-carboxylate) (Fig.1), USP described spectrophotometric assay for determination Sodium Cromoglycate in ophthalmic solution [2]. BP estimated Sodium Cromoglycate potentiometrically [3]. Literature review reveals several methods which have been reported for the estimation of Sodium Cromoglycate by HPLC in pharmaceutical dosage forms [4-6], in urine [7 and 8] and by LC/MS/MS in plasma [8-10]. Other analytical techniques were reported for determination of Sodium Cromoglycate like TLC [11] and voltammetry [12].

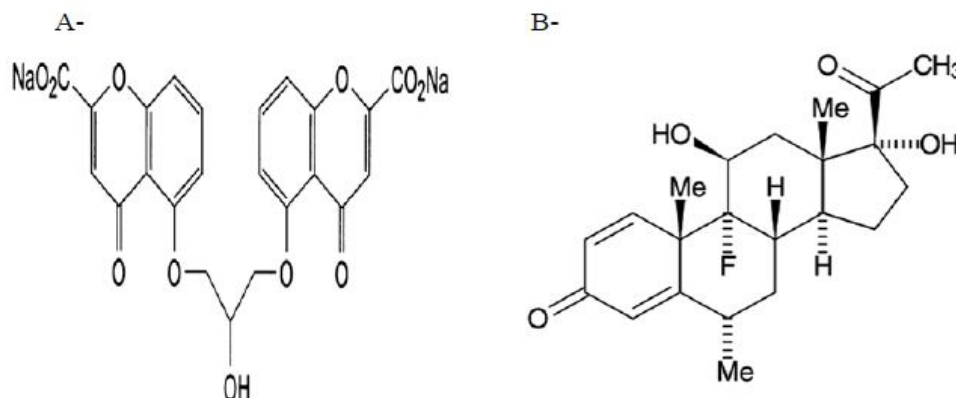


Fig.1. Structures of A-Sodium Cromoglycate and B- Fluorometholone respectively

Fluorometholone is 9a-fluoro-11b, 17a-dihydroxy-6a-methylpregna-1, 4-diene-3; 20-dione (Fig.1), USP and BP described RP-HPLC for determination Fluorometholone in eye drops [2, 3]. Literature review reveals several methods which have been reported for the estimation of Fluorometholone in pharmaceutical dosage forms by HPLC [13-14] and UV [15]. But literature review reveals that no method has been reported for estimation of both drugs together.

2. EXPERIMENTAL

2.1. Instrumentation

Analysis was performed on a chromatographic system of WATERS 2695 separation module connected to WATERS 2487 UV/VIS detector. The system equipped by Empower PC program. The chromatographic separation was achieved on BDS HYPERSIL C18 column (250x4.6 mm, 5 μ).

2.2. Chemicals and reagents

All reagents used were of analytical grade or HPLC grade. Potassium dihydrogen phosphate and ortho-phosphoric acid were supplied by (Merck, Darmstadt, Germany), Acetonitrile HPLC grade was supplied by (Fischer scientific, U.K.) and Distilled water. Water used in all the experiments was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA).

Sodium Cromoglycate and *Fluorometholone* working standard powders were kindly supplied by Egyptian international pharmaceutical industries company (EIPICO) (10th of Ramadan, Egypt), and were used without further purification.

2.3. Chromatographic condition

20 μ l of drugs sample solutions were monitored at two fixed wavelengths (λ = 330 nm for *Sodium Cromoglycate* and 240 nm for *Fluorometholone*). Liquid chromatography was performed on BDS HYPERSIL C18 column (250x4.6 mm, 5 μ) and the mobile phase consisted of Potassium dihydrogen phosphate (pH 4.5, 0.025M) - Acetonitrile (40:60, V/V) which pumped at a flow rate equals to 1.0 ml/min at ambient temperature.

Potassium dihydrogen phosphate (0.025 M) was prepared by dissolving 3.4 g Potassium dihydrogen phosphate in approximately 950 ml distilled water. The pH was adjusted to 4.5 with ortho-phosphoric acid. Water was added to 1000 ml. Mobile phase was filtered through a 0.45 μ l Nylon membrane filter (Millipore, Milford, MA, USA) under vacuum and degassed by ultrasonication (Cole Palmer, Vernon Hills, USA) before usage.

2.4. Pharmaceutical preparation

Fluca eye drops; Jamjom (K.S.A) contains (*Sodium Cromoglycate* 2.0% and *Fluorometholone* 0.1%) B.NO: MK097.

2.5. Preparation of stock standard solutions

Stock standard solutions containing 2.0, 0.1 mg/ml of *Sodium Cromoglycate* and *Fluorometholone* respectively were prepared by dissolving 200 and 10 mg of each in 50% methanol in 100 ml volumetric flask respectively. It was then sonicated for 5 minutes and the final volume of solutions was completed to 100 ml with 50% methanol to get stock standard solutions.

2.6. Preparation of calibration plot (working standard solutions)

To construct calibration plots, the stock standard solutions were diluted with 50% methanol to prepare working solutions in the concentration ranges (100-300 and 5-15 $\mu\text{g/ml}$) for of *Sodium Cromoglycate* and *Fluorometholone* respectively. Each solution ($n=5$) was injected in triplicate and chromatographed under the mentioned conditions above. Linear relationships were obtained when average drug standard peak area were plotted against the corresponding concentrations for each drug. Regression equation was computed.

2.7. Sample preparation

1 ml of *Fluca E/D* was taken into 100 ml volumetric flask then completed with 50% methanol.

Test solutions were analyzed under optimized chromatographic conditions and chromatogram is depicted in (Fig.2).

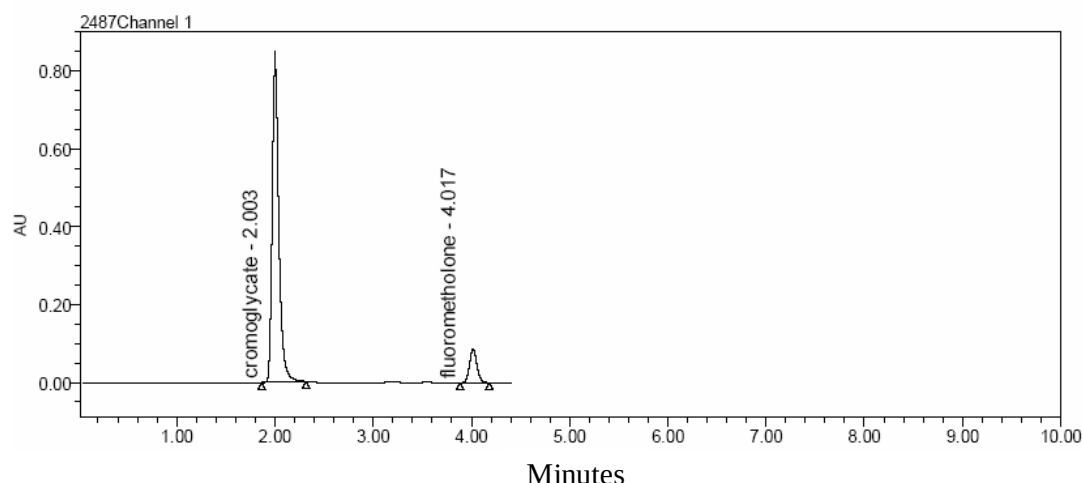


Fig.2. Typical HPLC chromatograms obtained from 20 μl injections of Sodium Cromoglycate (2.00 min.) and Fluorometholone (4.01 min.) respectively under optimized chromatographic conditions.

3. RESULTS AND DISCUSSION

3.1. Optimization of chromatographic condition

Several trials were carried out to obtain optimized chromatographic condition for simultaneous determination of *Sodium Cromoglycate* and *Fluorometholone* in their pharmaceutical preparations. Firstly, maximum absorption wavelengths (330,240 nm) for *Sodium Cromoglycate* and *Fluorometholone* were selected by scanning from 400-200 nm under UV (Fig.3). Potassium dihydrogen phosphate (pH 4.5, 0.025M) has no effect on absorption at wavelength more than 200 nm [16].

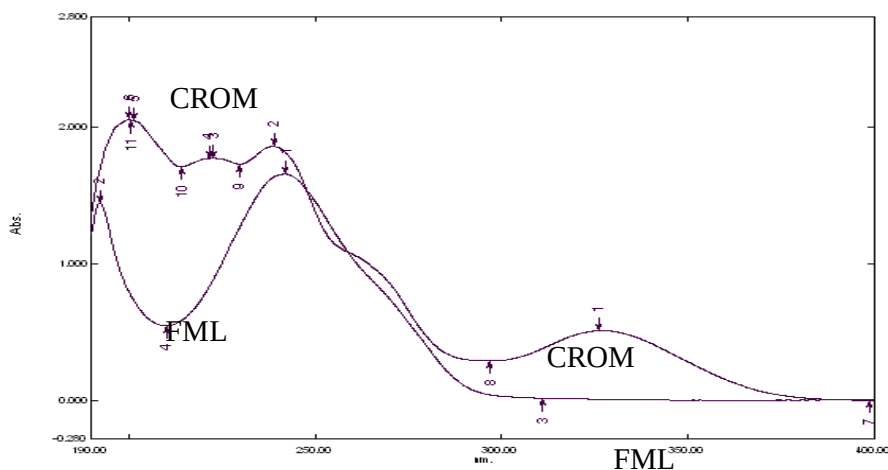


Fig.3. Typical UV spectrum of Sodium Cromoglycate (CROM) and Fluorometholone (FML)

Low concentration of buffer (0.025M) is adequate for most reversed phase applications. This concentration is low enough to avoid problems with precipitation when significant amounts of organic modifiers are used in the mobile phase [16]. PH of phosphate buffer was examined, it had no effect on separation because Sodium Cromoglycate is an ionized compound and Fluorometholone is a non ionized one. Different percentages of acetonitrile were tried with phosphate buffer to reach optimum separation with good resolution. 60% acetonitrile in mobile phase produced adequate separation and resolution. Using BDS HYPERSIL C18 column produced more sharper and symmetric peaks than Cyano column which produced poor, broad and asymmetric peak with Fluorometholone (Fig.4).

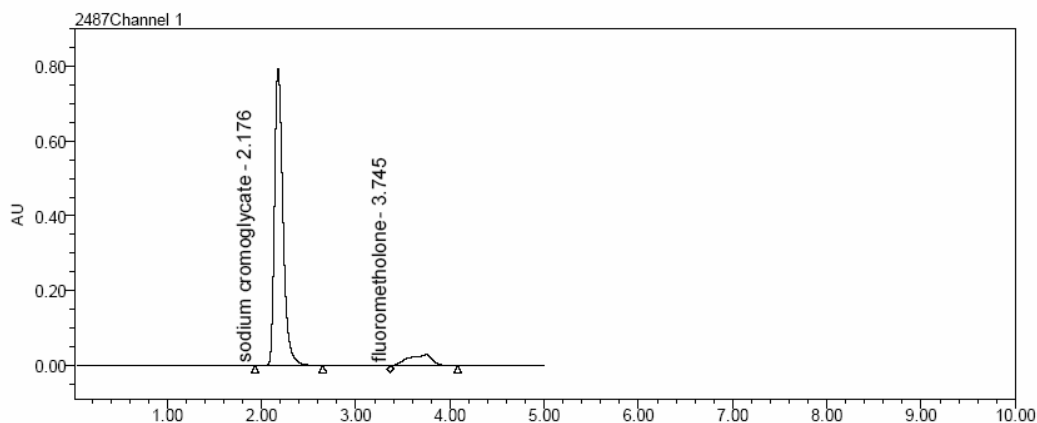


Fig.4. Typical HPLC chromatograms obtained from 20 µl injections of Sodium Cromoglycate (2.00 min.) and Fluorometholone (4.01 min.) respectively on BDS Cyano Hypersil column.

Some months later, another new method was developed and published for determination of *Sodium Cromoglycate* and *Fluorometholone* using TLC [17]. It has some drawbacks; first one is time consuming of TLC Technique for every run (about 1 hour). The second is using additional software to aid in calculating of indirect used UV spectroscopy. The third is the addition of known concentration of Fluorometholone to reach linearity range.

5. METHOD VALIDATION

5.1. Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc [18]. A Bulk of Fluca E/D (solution contains excipients only) had been prepared by mixing its excipients like benzalkonium chloride 0.005%; disodium edetate, polyvinyl alcohol, sodium phosphate monobasic monohydrate; sodium phosphate dibasic heptahydrate, polysorbate 80, sodium chloride and purified water then known concentration of studied drugs was added to the bulk then injected under previous condition. Recovery results showed that the bulk has negligible effect Which Means That the bulk did not interfere with developed method.

5.2. Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. For the establishment of linearity, a minimum of 5 concentrations is recommended [18]. Five Concentrations were chosen in the ranges (100-300 and 5-15 µg/ml) for corresponding levels of 50-150% w/w of the nominal analytical concentration of *Sodium Cromoglycate* and *Fluorometholone* respectively. The linearity of peak area responses versus concentrations was demonstrated by linear least square regression analysis. The linear regression equations were $\{Y = 21107X + 416.8 (r = 0.9999) \text{ and } Y = 48413X - 35.26 (r = 0.9998)\}$ for *Sodium Cromoglycate* and *Fluorometholone* respectively (Table 1). Where **Y** is the peak area of standard solution and **X** is the drug concentration.

5.3. Limits of detection and Limits of quantitation

According to the ICH recommendations, determination of limits of detection and quantitation was based on the standard deviation of the y-intercepts of regression lines (n=3) and the slope of the calibration plots [18] (Table 1).

Table 1: Calibration data was resulted from method validation of *Sodium Cromoglycate* and *Fluorometholone* respectively

Item	<i>Sodium Cromoglycate</i>	<i>Fluorometholone</i>
Linear range (µg/ml)	100-300	5-15
Detection limit (µg/ml)	1.11	0.07
Quantitation limit (µg/ml)	3.37	0.22
Regression data		
No.	5	5
slope (b)	21107	48413
Standard deviation of the slope	49.43	132.3
intercept (a)	416.8	35.26
Standard deviation of the intercept	7109.6	1058.8
correlation coefficient @	0.9999	0.9998
Standard error of regression	0.79	0.06

(Y = a + bC, where C is the concentration of the compound (µg/ml) and Y is the drug peak area)

5.4. Precision

The precision of the assay was investigated by measurement of both repeatability and Intermediate precision.

5.4.1. Repeatability

Repeatability was investigated by injecting a minimum of 6 determinations at 100% of the test concentration and percentage SD were calculated in Table 2.

5.4.2. Intermediate precision

In the inter-day studies, standard and sample solutions prepared as described above, were analyzed in triplicate on three consecutive days at 100% of the test concentration and percentage SD were calculated Table 2.

5.5. Accuracy

Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range (80,100 and 120%). Accuracy was reported as percent recovery by the assay of known added amount of analyte in the sample Table 2.

Table 2: Repeatability and Intermediate precision and Accuracy (Recovery %) of *Sodium Cromoglycate* and *Fluorometholone* respectively

Drug name		<i>Sodium Cromoglycate</i>		<i>Fluorometholone</i>	
		AV±SD mg/ml	AV±SD %	AV±SD mg/ml	AV±SD %
Repeatability		199.61±1.39	99.81±0.70%	9.95±0.01	99.49±0.12%
Intermediate precision		199.44±2.03	99.72±1.01%	9.95±0.07	99.45±0.68%
Accuracy & Recovery%	80%	161.99±0.12	101.24±0.08%	7.99±0.01	99.86±0.11%
	100%	200.93±0.34	100.47±0.17%	10.04±0.03	100.40±0.25%
	120%	241.53±0.18	100.64±0.07%	12.17±0.02	101.40±0.20%

5.6. Robustness

Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in method parameters and provides an indication of its reliability during normal usage [18]. Robustness was tested by studying the effect of changing mobile phase pH by ± 0.5 , the percentage of organic solvent (acetonitrile) in the mobile phase by $\pm 5\%$, temperature $\pm 5^\circ\text{C}$, wavelengths $\pm 5\text{ nm}$ and flow rate $\pm 0.1\text{ ml/min}$ had no significant effect on the chromatographic resolution of the method.

5.6.1. Stability of analytical solution

Also as part of evaluation of robustness, solution stability was evaluated by monitoring the peak area response. Standard stock solutions in methanol were analyzed right after its preparation 1, 2 and 3 days after at room temperature. The change in standard solution peak area response over 3 days was (1.03 and 0.68 %) for *Sodium Cromoglycate* and *Fluorometholone* respectively. Their solutions were found to be stable for 3 days at room temperature at least.

6.0. Application on pharmaceutical Preparation

The proposed methods were successfully used to determine *Sodium Cromoglycate* and *Fluorometholone* respectively in *Fluca E/D®*. Five replicate determinations were performed. Satisfactory results were obtained for each compound in good agreement with label claims. The results obtained were compared statistically with those from published method [4, 2] for *Sodium Cromoglycate* and *Fluorometholone* respectively by using Student's t-test and the variance ratio F-test. The results showed that the t and F values were smaller than the critical values. So, there were no significant differences between the results obtained from this method and published methods (Table 3).

Table 3: Statistical comparison of the proposed and published methods for determination of *Sodium Cromoglycate* and *Fluorometholone* respectively in their dosage forms by reported method (T- student test) and (F –test for variance)

Drug name	Recovery $\pm$ SD		Reference method number	Calculated t- values	Calculated F- values
	Proposed methods	Reference method			
<i>Sodium Cromoglycate</i>	99.13 $\pm$ 1.28	99.45 $\pm$ 1.72	4	0.57	0.55
<i>Fluorometholone</i>	101.51 $\pm$ 1.87	101.87 $\pm$ 1.56	2	1.46	1.44

(Where the Tabulated t-values and F -ratios at p = 0.05 are 2.57 and 5.

7.0. CONCLUSION

A simple, accurate, precise, robust, valid, highly sensitive and reliable HPLC method has been established for simultaneous determination for *Sodium Cromoglycate* and *Fluorometholone* respectively in bulk and in their pharmaceutical dosage form. It is a new fast method about 4.5 min for determination both drugs simultaneously and it has low LOQ about 3.37 and 0.22 $\mu\text{g/ml}$ for Sodium Cromoglycate and Fluorometholone respectively.

8.0. REFERENCES

1. Bartlett JD et al (2003) ophthalmic drug facts, factors and comparisons. Missouri, USA
2. Health wise Knowledgebase (2012) US Pharmacopoeia, Rockville.
<http://www.healthwise.org>. Accessed 3 Jan 2012
3. British Pharmacopoeia, London (2012) with the monographs of the sixth edition of the European pharmacopoeia monograph <http://www.pharmacopoeia.co.uk>. Accessed 1 Jan.2012
4. Radulović D, Kocić-Pesić V, Pećanac D and Zivanović L (1994) HPLC determination of sodium cromoglycate in pharmaceutical dosage forms. *Farmaco* 49:375-6
5. Segall A, Vitale F, Ricci R, Giancaspro G and Pizzorno MT (1997) High Performance Liquid Chromatographic Determination of Sodium Cromoglycate. *Drug Develop Indust Pharm* 23: 839-842
6. Hassib ST, ElZaher AA and Fouad MA (2011) Development and Validation of RP-HPLC Stability-Indicating Methods for the Determination of Butamirate Citrate and Sodium Cromoglycate, *J Chem Pharm Res* 3:243-258
7. Mawatari K, Mashiko S, Sate Y, Usui Y, Iinuma F and Watanabe M (1997) Determination of disodium cromoglycate in human urine by high-performance liquid chromatography with post-column photo irradiation-fluorescence detection. *Analyst* 122:715-7

8. Aswania OA, Corlett SA and Chrystyn H (1997) Development and validation of an ion-pair liquid chromatographic method for the quantitation of sodium cromoglycate in urine following inhalation. *J Chromatogr B Biomed Sci Appl.* 690:373-8
9. Liu XY, Qu TT, Wang BJ, Wei CM, Yuan GY, Zhang R and Guo RC (2008) Determination of sodium cromoglycate in human plasma by liquid chromatography with tandem mass. *Biomed Chromatogr* 22:1021-7
10. Ozoux ML, Girault J, Malgouyat JM and Pasquier O (2001) Determination of sodium cromoglycate in human plasma by liquid chromatography–mass spectrometry in the turbo ion spray mode, *Journal of Chromatography B: Biomedical Sciences and Applications.* 765: 179–185
11. Radulović D, Kocić-Pesić V, Pećanac D and Zivanović L (1992), Determination of sodium cromoglycate in pharmaceutical dosage forms using TLC-densitometry. *Farmaco* 47:1583-7
12. Moreira JC, Foster SE, Rodrigues JA and Fogg AG (1992) Differential-pulse adsorptive stripping voltammetric determination of sodium cromoglycate at a hanging mercury drop electrode, *Analyst.* 117: 989-991
13. Altuntas TG, Korkmaz F and Nebioglu D (2000) Determination of tetrahydrozoline hydrochloride and Fluorometholone in pharmaceutical formulations by HPLC and derivative UV spectrophotometry. *Pharmazie* 55:49-52
14. Jonvel P and Andermann G (1983) Determination of Fluorometholone purity by very high-performance liquid chromatography. *Analyst* 108: 411-414
15. Vladimirov S, Cudina O, Agbaba D and Zivanov-Stakic D (1996) Spectrophotometric Determination of Fluorometholone in Pharmaceuticals Using 1,4-Dihydrazinophthalazine, *Analytical Letters.* 29: 921-927
16. Preparing Buffered Mobile Phases for Reversed Phase HPLC, <http://www.mac-mod.com/pdf/TR-BufferedMP.pdf>.
17. Saleh SS, Lotfy HM, Hassan NY and Elgizawy SM (2013) A comparative study of validated spectrophotometric and TLC- spectrodensitometric methods for the determination of sodium cromoglycate and Fluorometholone in ophthalmic solution, *Saudi Pharm J*, 21: 411-421
18. ICH Harmonized Tripartite Guideline, Validation of Analytical Procedures: Text and Methodology Q2 (R1).