Simultaneous Estimation of UHPLC Method Development and Validation for Kopexil and Minoxidil in Bulk and Topical Solution

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***Abstract*- Kopexil and Minoxidil belongs to a class of vasodilator drug used to treat Alopecia. A simple, accurate, precise method was developed for the simultaneous estimation of the Minoxidil & Kopexil in solid dosage form by using Ultra high-performance liquid chromatography (UHPLC). Retention time of Minoxidil was found 2.359 min & Kopexil was found 3.195 min. % RSD of the Minoxidil was found 0.15% & Kopexil was found 0.71%. % Assay was obtained as 100.09% for Minoxidil & 100.38% Kopexil. Regression equation of Minoxidil y = 145.3647x + 56.8594 R^2 = 1.0000 & Kopexil is y = 0.0789x + 0.0561 R^2 = 1.0000. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.**

***Keywords:* Minoxidil, Kopexil, UHPLC, Topical solution.**

1. INTRODUCTION:

1.1. PHARMACEUTICAL ANALYSIS

Pharmaceutical analysis is conventionally described as analytical chemistry which deals with drug analysis, involving the determination of active components, impurities, excipients, content uniformity, solubility, dissolution rate and stability of bulk and pharmaceutical dosage form. Pharmaceutical analysis plays a very vital role in quality assurance and quality control of bulk drug and their formulations. It also gives the information about the purity and safety of the products. [1,2]

1.1.1. Types

There are two main types of chemical analysis.

- Qualitative (identification)
- Quantitative (estimation)

1.1.1.1. Qualitative Analysis

These tests are performed to indicate whether the substance or compound is present in the sample or not. Various qualitative tests are detection of evolved gas, formation of precipitates, limit tests, colour change reactions, melting point and boiling point test etc. It is performed to establish composition of natural or synthetic substance.

1.1.1.2. Quantitative Analysis

These techniques are mainly used to quantify any compound or substance in the sample. The quantitative performance of suitable chemical reaction and either measuring the amount of reagent added to complete the reaction or measuring the amount of reaction product obtained. The characteristic movement of substance through defined medium under controlled conditions. [3]

1.2. ULTRA HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (UHPLC)

Ultra-High Performance Liquid Chromatography is new category of analytical separation science works on the similar principles of HPLC while increasing the overall interlaced attributes of speed, sensitivity & resolution.[4] It uses fine particles and saves time and reduces solvent consumption. [5]

1.2.1. Principle of UHPLC

The UHPLC is based on the principle of use of stationary phase consisting of particles less than 2 µm (while HPLC columns are typically filled with particles of 3 to 5 µm). The underlying principles of this evolution are governed by the van Deemter equation, which is an empirical formula that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). The Van Deemter curve, governed by an equation with three components shows that the usable flow range for a good efficiency with small diameter particles is much greater than for larger diameters.[6]

$H=A+B/v+Cv$

Where A, B and C are constants and v is the linear velocity, the carrier gas flow rate. The A term is independent of velocity and represents "eddy" mixing. It is smallest when the packed column particles are small and uniform. The B term represents axial diffusion or the natural diffusion tendency of molecules. This effect is diminished at high flow rates and so this term is divided by v. The C term is due to kinetic resistance to equilibrium in the separation process. The kinetic resistance is the time lag involved in moving from the gas phase to the packing stationary phase and back again. The greater the flow of gas, the more a molecule on the packing tends to lag behind molecules in the mobile phase. Thus, this term is proportional to v. [7]

Therefore, it is possible to increase throughput, and thus the speed of analysis without affecting the chromatographic performance. The advent of UHPLC has demanded the development of a new instrumental system for liquid chromatography, which can take advantage of the separation performance (by reducing dead volumes) and consistent with the pressures (about 8000 to 15,000 PSI, compared with 2500 to 5000 PSI in HPLC). Efficiency is proportional to column length and inversely proportional to the particle size. Therefore, the column can be shortened by the same factor as the particle size without loss of resolution. The application of UHPLC resulted in the detection of additional drug metabolites, superior separation and improved spectral quality.[7]

1.3. DRUG PROFILE

1.3.1 Kopexil

The compound kopexil with the molecular formula C4H6N4O (Fig. 1), is crystalline in nature, solid, and has the CAS number 113275-13-1. It is soluble in water, ethanol and propylene glycol and the melting point of kopexil is 218 °C. The mechanism of action of kopexil is which an enzyme (Lysyl hydroxylase) catalyses the formation of hydroxylysine, which contributes to the maturation of collagen synthesis. Increased level of collagen causes hardening and shrinking to occur around hair follicles greatly retarding their growth. It is used as to increase the volume of hair in the growth stage by working on the deep structure of the roots. It rejuvenates the hair roots so that healthy hair growth can persist. [8]

1.3.2. Minoxidil

The compound minoxidil with the molecular formula C9H15N5O (Fig.2), is crystalline in nature and has the CAS number 38304-91-5. It is soluble in water, ethanol, acetone and chloro form and the melting point of Minoxidil is 248 °C. The mechanism of action of minoxidil is which it is thought to promote the survival of human dermal papillary cells (DPCs) or hair cells by activating both extracellular signal-regulated kinase (ERK) and by preventing cell death by increasing the ratio of BCl-2/Bax. Minoxidil may stimulate the growth of human hairs by prolonging anagen through these proliferative and anti-apoptotic effects on DPCs. Minoxidil, when used as vasodilator, acts by opening adenosine triphosphate-sensitive potassium channels in vascular smooth muscle cells. This vasodilation may also improve the viability of hair cells or hair follicles. It is used to stimulate hair growth. [9]

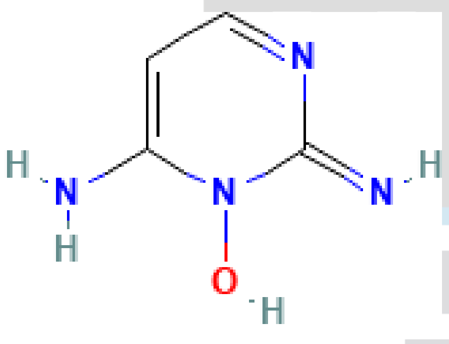

Figure 1. Chemical structure of Kopexil

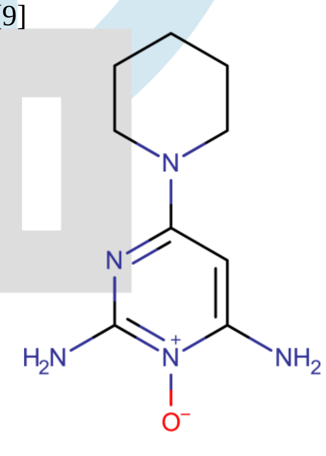

Figure 2. Chemical structure of Minoxidil

2. MATERIALS AND METHODS:

2.1. MATERIALS

2.1.1. Chemicals And Reagents

Kopexil and Minoxidil API was provided as gift sample from Synthiya laboratories LTD, Puducherry, India. HPLC grade water and methanol was purchased from Rankem laboratories. The pharmaceutical samples used in the present study is Androanagen tablets.

2.1.2. Instruments

The Agilent Infinity 1220 UHPLC system equipped with Agilent LC-20AT pump and UV detector was used. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 µl. For all parameters, high precision analytical balances of the Mettler and calibrated glassware were utilised.

2.2. METHOD DEVELOPMENT

2.2.1. Chromatographic Conditions
The column used in this method was LUNA C18; 4.6mm X 50 mm; 5Microns. The flow rate is set at 0.5 ml/min. Samples were injected through an injector valve with fixed loop at 20 µl. The buffer used in this method was 1g Potassium Dihydrogen Orthophosphate in 1000ml of water and add 3ml of Triethylamine mix well and adjust the pH: 3.0 with Orthophosphoric acid. The mobile phase comprised of Methanol: buffer pH 3.0 in the proportion of 40:60. Resulting solution was degassed by ultrasonication for 10minutes. The diluent used in this method was Mobile phase.

2.2.2. Preparation of Standard stock solution A: Weigh accurately and transfer about 100 mg of Minoxidil standard into 10 ml volumetric standard flask. Dissolve and makeup to the volume with Mobile phase.

2.2.3. Preparation of Standard stock solution B: Weigh accurately and transfer about 30 mg of Kopexil standard into 10 ml volumetric standard flask. Dissolve and makeup to the volume with Mobile phase.

2.2.4. Preparation of Standard solution: Transfer, 1ml from standard stock solution A and 1ml from standard stock solution B into 100ml volumetric flask makeup to the volume with Mobile phase.

2.2.5. Preparation of Sample solution: Weigh accurately equivalent to 50mg of Minoxidil sample into 50 ml volumetric flask. Dissolve and makeup to the volume with Mobile phase. Filter & further, dilute 5ml from the above solution into 50ml volumetric flask and makeup to the volume with Mobile phase.

2.3. METHOD VALIDATION

2.3.1. System suitability parameters: The system suitability parameters were determined by preparing standard solution of Minoxidil & Kopexil were injected Five times and the parameters like peak tailing, resolution and plate count were determined. The % RSD for the area of five standard injections results should not be more than 2%.

2.3.2. Specificity: Checking of the interference in the optimized method. We should not find any interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific.

2.3.3. Precision:

2.3.3.1. Preparation of Standard stock solution A: Weigh accurately and transfer about 100 mg of Minoxidil standard into 10 ml volumetric standard flask. Dissolve and makeup to the volume with Mobile phase.

2.3.3.2. Preparation of Standard stock solution B: Weigh accurately and transfer about 30 mg of Kopexil standard into 10 ml volumetric standard flask. Dissolve and makeup to the volume with Mobile phase.

2.3.3.3. Preparation of Standard solution: Transfer, 1ml from standard stock solution A and 1ml from standard stock solution B into 100ml volumetric flask makeup to the volume with Mobile phase.

2.3.3.4. Preparation of Sample solution: Weigh accurately equivalent to 50mg of Minoxidil sample into 50 ml volumetric flask. Dissolve and makeup to the volume with Mobile phase. Filter & further, dilute 5ml from the above solution into 50ml volumetric flask and makeup to the volume with Mobile phase.

2.3.4. Linearity:

2.3.4.1. 50% Minoxidil & Kopexil Standard solution: 0.5ml standard stock A and 0.5ml standard stock B solutions was pipette out and made up to 100 ml.

2.3.4.2. 75% Minoxidil & Kopexil Standard solution: 1.5ml standard stock A and 1.5ml standard stock B solutions was pipette out and made up to 200 ml.

2.3.4.3. 100% Minoxidil & Kopexil Standard solution: 1.0ml standard stock A and 1.0ml standard stock B solutions was pipette out and made up to 100 ml.

2.3.4.4. 125% Minoxidil & Kopexil Standard solution: 2.5ml standard stock A and 2.5ml standard stock B solutions was pipette out and made up to 200 ml.

2.3.4.5. 150% Minoxidil & Kopexil Standard solution: 1.5ml standard stock A and 1.5ml standard stock B solutions was pipette out and made up to 100 ml.

2.3.5. Accuracy:

2.3.5.1. For preparation of 80% solution: Weigh accurately equivalent to 40mg of Minoxidil sample into 50 ml volumetric flask. Dissolve and makeup to the volume with Mobile phase. Filter & further, dilute 5ml from the above solution into 50ml volumetric flask and makeup to the volume with Mobile phase.

2.3.5.2. For preparation of 100% solution: Weigh accurately equivalent to 50mg of Minoxidil sample into 50 ml volumetric flask. Dissolve and makeup to the volume with Mobile phase. Filter & further, dilute 5ml from the above solution into 50ml volumetric flask and makeup to the volume with Mobile phase.

2.3.5.3. For preparation of 120% solution: Weigh accurately equivalent to 60mg of Minoxidil sample into 50 ml volumetric flask. Dissolve and makeup to the volume with Mobile phase. Filter & further, dilute 5ml from the above solution into 50ml volumetric flask and makeup to the volume with Mobile phase.

2.3.5.4. Procedure: The standard solution, Accuracy -80%, Accuracy -100% and Accuracy -120% solutions were injected. The amount found and amount added for Minoxidil & Kopexil mean recovery values were calculated and the results were summarized.

2.3.5.5. Acceptance Criteria: The % Recovery for each level should be between 98.0% to 102.0%.

2.3.6. Robustness:

Small deliberate changes in method like Flow rate and Wavelength were made but there was no recognized change in the result and were within range as per ICH Guide lines. Robustness conditions like Flow minus, Flow plus, Wavelength decreasing and Wavelength increasing was maintained and samples were injected in duplicate manner. System suitability parameters were not affected and all the parameters were passed. %RSD was within the limit.

3. RESULTS AND DISCUSSION

3.1. METHOD DEVELOPMENT

3.1.1. Optimization of Chromatographic Conditions

To optimize the chromatographic conditions, the effect of chromatographic variables such as composition of mobile phase, ratio of mobile phase and flow rate were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetric factor, and theoretical plates were calculated. Finally, a simple and inexpensive method was developed by using a combination of Buffer and Methanol in ratio 60:40. observation: Minoxidil was eluted at 2.384 min & Kopexil was eluted at 3.126 min. Plate count and tailing factor was very satisfactory, so this method was optimized and to be validated. (Optimized chromatographic conditions are listed in Table 1 and fig.3)

Table 1

METHOD PARAMETER	OPTIMIZED VALUE
COLUMN	LUNA C18; 4.6mm X 50 mm; 5Microns
MOBILE PHASE	Buffer: Methanol (60: 40)
FLOW RATE	0.5 ml/min
DETECTOR WAVELENGTH	250 nm
INJECTION VOLUME	20 µl
RUN TIME	6 min
DILUENTS	Mobile Phase
RESULTS	Retention time and peak shape was good.

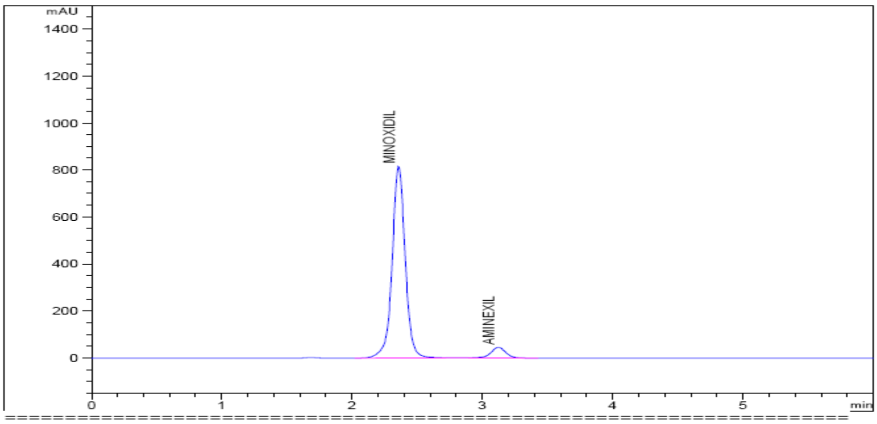

Figure 3: Optimized Chromatogram

3.2. METHOD VALIDATION

3.2.1. System Suitability

According to ICH guidelines plate count should be more than 2000, tailing factor should be less than 2 and resolution must be more than 2. All the system suitable parameters were passed and were within the limits which are shown in Table 2.

3.2.2. Specificity

Checking of the interference in the optimized method. We did not find any interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific. Retention time of Minoxidil was eluted at 2.359 min & Kopexil was eluted at 3.195 min which are shown in Table 3.

3.2.3. Linearity

Five linear concentrations (50,75,100,125 and 150) of Minoxidil & Kopexil were injected in a duplicate manner. Average areas were mentioned in fig.4 and fig.5. Linearity equations and Correlation coefficient obtained for the Minoxidil and Kopexil are show in Table 4, indicating an excellent fit of the compounds to the model within the range studied.

Table 2

DETAILS	KOPEXIL			MINOXIDIL		
Parameters	**Retention Time**	**Theoretical Plates**	**Tailing factor**	**Retention Time**	**Theoretical Plates**	**Tailing factor**
Average	3.123	3932	0.914	2.357	2941	1.0004
SD	0.00	1.92	0.00	0.00	2.70	0.00
% RSD	0.03	0.05	0.14	0.04	0.09	0.11

Table 3

DETAILS	KOPEXIL		MINOXIDIL	
Parameters	**Retention Time**	**AREA**	**Retention Time**	**AREA**
Blank	3.195	No peak observed	2.359	No peak observed
Standard	3.195	369.915	2.359	5698.456
Placebo	3.195	No peak observed	2.359	No peak observed

Table 4

DETAILS	KOPEXIL	MINOXIDIL
Linearity Equations	y = 0.0789x + 0.0561	y = 145.3647x + 56.8594
Correlation Coefficient (R^2)	1.0000	1.0000

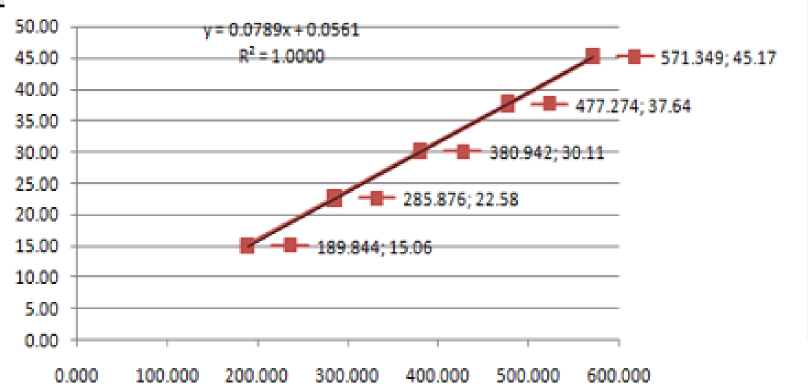

Figure 4: Calibration curve of Kopexil

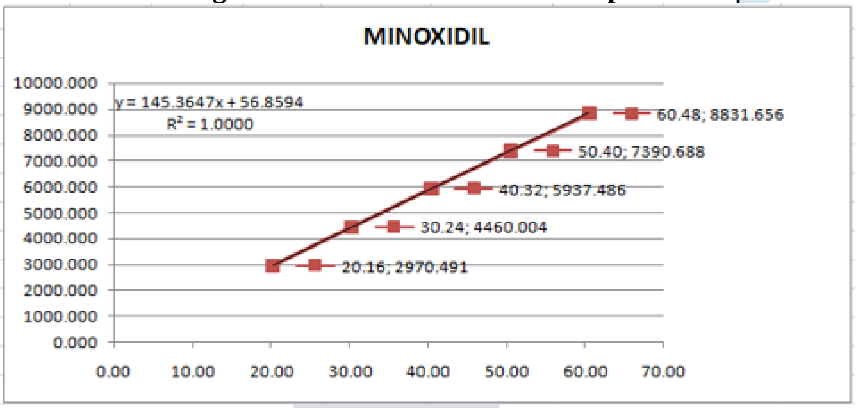

Figure 5: Calibration curve of Minoxidil

3.2.4. Precision

The precision of the proposed method was assessed as intermediate precision. Multiple samples were done as six working sample solutions of same concentrations were prepared, each injection from each working sample solution were injected into system for intermediate precision and were repeated on two different days for intermediate precision. The Average area, standard deviation and % RSD was calculated for Minoxidil & Kopexil and shown in Table 5. As the limit of Precision was less than "2" the system precision was passed in this method.

3.2.5. Accuracy

Three levels of Accuracy samples were prepared by different sample weight method (80%,100%and 120%). Triplicate injections were given for each level of accuracy and mean % Recovery was obtained for Minoxidil and Kopexil are shown in Table 6. As the % Recovery for each level should be between "98.0% to 102.0%" the accuracy was passed in this method.

3.2.6. Robustness

Robustness conditions like Flow minus (0.4ml/min), Flow plus (0.6ml/min), Wavelength minus (248nm) and Wavelength plus (252nm) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit. It is shown in Table 7.

3.2.7. Assay

Assay was performed with the above formulation. Assay Average percentage of Minoxidil for 100.09% & Kopexil 100.38% respectively are shown in Table 8. As the % Purity for each level should be between "98.0% to 102.0%" the assay was passed in this method.

Table 5

DETAILS	KOPEXIL		MINOXIDIL	
	Intermediate precision day-1	Intermediate precision day-2	Intermediate precision day-1	Intermediate precision day-2
Average	100.32%	100.48%	99.74%	99.54%
SD	0.672	0.65	0.63	0.43
% RSD	0.67	0.65	0.63	0.43

Table 6

DETAILS	KOPEXIL	MINOXIDIL
Average	100.77%	99.93%
SD	0.64	0.147
% RSD	0.64	0.15

Table 7

S. No	Condition	%RSD of Minoxidil Assay	%RSD of Kopexil Assay
1	Flow rate (-) _0.4	0.62%	0.62%
2	Flow rate (+) _0.6	0.27%	0.69%
3	Wavelength (-) _248	0.27%	0.80%
4	Wavelength (+) _252	0.16%	0.90%

Table 8

DETAILS	KOPEXIL	MINOXIDIL
Content in mg	15.057 mg	50.046 mg
Percentage Purity	100.38 %	100.09 %

4. CONCLUSION:

A simple, Accurate, precise method was developed for the simultaneous estimation of the Minoxidil & Kopexil in solid dosage form. Retention time of Minoxidil was found 2.359 min & Kopexil was found 3.195 min. % RSD of the Minoxidil was found 0.15% & Kopexil was found 0.71%. % Assay was obtained as 100.09% for Minoxidil & 100.38% Kopexil. Regression equation of Minoxidil $y = 145.3647x + 56.8594$ $R^2 = 1.0000$ & Kopexil is $y = 0.0789x + 0.0561$ $R^2 = 1.0000$. Retention times were decreased and that run time was decreased, so the method developed was simple and economical that can be adopted in regular Quality control test in Industries.

ABBREVIATIONS:

UHPLC - Ultra High Performance Liquid Chromatography
HPLC - High Performance Liquid Chromatography
HETP - Height Equivalent to a Theoretical Plate
CAS - Chemical Abstracts Service
DPC - Dodecylphosphocholine
ERK - Extracellular signal-regulated kinase
API - Active pharmaceutical ingredient
ML - Millilitre
µL - Microlitre
SD - Standard deviation
RSD - Relative standard deviation
% - Percentage

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