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Development and Validation of RP-HPLC Method for Simultaneous Estimation of Minoxidil and Aminexil in Topical Formulation

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A RP-HPLC method was developed and validated for simultaneous determination of minoxidil and aminexil in a combined topical liquid formulation. Isocratic separation was achieved on a C_{18} column (250 mm × 4.6 mm i.d., particle size 5 µm, ThermoHypersil BDS) using a mobile phase consisting of methanol-phosphate buffer (pH 3.0, 60:40, v/v) at a flow rate of 1 mL/min and UV detection at 250 nm. Linearity was observed over the concentration range of 1.0-2.0 mg/mL for both minoxidil ($r^2 = 0.99$) and aminexil ($r^2 = 0.99$). The average percentage recovery of the method was 101.0 % with a relative standard deviation of 0.14 %. The limits of detection (LODs) were 0.7346 µg/mL and 0.2423 µg/mL for minoxidil and aminexil and limits of quantification (LOQs) were 0.7346 µg/mL and 0.2423 µg/mL, respectively. The method was validated as per ICH guidelines.

Keywords: Minoxidil, Aminexil, Topical formulation, RP-HPLC, Validation.

INTRODUCTION

Minoxidil is a potent peripheral vasodilator, which was found orally effective in hypertensive disorders despite of having some serious side effects such as salt retention and hirsutism *etc*. Because of its proven potential in stimulating new hair growth, minoxidil has been increasingly used in the treatment of androgenic alopecia in both male and female. The topical administration of minoxidil shows promising response in inducing new hair growth in the affected areas of scalp because of its local cutaneous vasodilatory effect which results in increased local irritation and blood flow. Chemically, minoxidil is 2,4-diamino-6-piperidinopyrimidine-3-oxide (Fig. 1a). It is obtained as an odourless white crystalline powder, insoluble in water or alkaline solutions, freely soluble in alcohols and in acidic solutions [1,2]. Minoxidil is official in Chinese Pharmacopoeia, European Pharmacopoeia and United States Pharmacopoeia [3].

Aminexil, 2,4-diaminopyrimidine-3-*N*-oxide, (Fig. 1b), is a newer drug that is most commonly found in combination with minoxidil in marketed liquid formulations. Minoxidil and aminexil together exerts synergistic action by increasing the overall therapeutic outcome of the combined formulation, which therefore remains to be clinically more efficacious in individuals with any stage of hair loss. Aminexil occurs as an odourless white crystalline powder, slightly soluble in water.

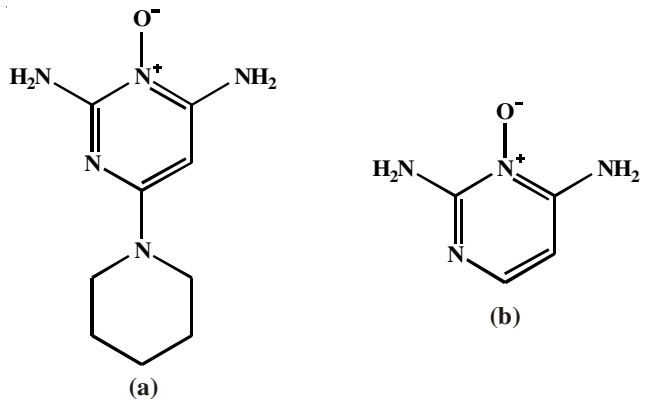

Fig. 1. Chemical structures of minoxidil (a) and aminexil (b)

Literature survey shows that no isocratic RP-HPLC methods have been reported so far for simultaneous estimation of minoxidil and aminexil in bulk drugs as well as in formulations. A RP-HPLC method for the estimation of minoxidil and related substances in topical formulation has been reported earlier [1]. The reference method for minoxidil quantification mentioned in the US Pharmacopoeia uses liquid chromatography [4]. Some other reported methods for the determination of minoxidil in pharmaceutical formulation and in human plasma include HPLC [5,6], differential pulse polarography [7,8], GC [9], radioimmunoassay [10].

To date, no study related to the isocratic analysis of minoxidil and aminexil have been reported. It was therefore an attempt to develop a RP-HPLC method for simultaneous determination of minoxidil and aminexil in the combined pharmaceutical formulation. In the present study, a simple, precise and accurate RP-HPLC method for simultaneous estimation of minoxidil and aminexil in topical formulation was developed and validated successfully according to the International Conference on Harmonization (ICH) guidelines.

EXPERIMENTAL

Minoxidil and aminexil (purity > 99.5 %) were procured from the Dr. Reddy's Laboratories Ltd., Hyderabad, India. Methanol (Merck Ltd., Mumbai, India) was of HPLC grade. Analytical grade di-potassium hydrogen phosphate, triethylamine, phosphoric acid, hydrochloric acid and sodium hydroxide were procured from S.D. Fine Chemicals Ltd., Mumbai, India. The water for HPLC was purchased from Qualigen Fine Chemicals, Mumbai, India. AMEXIDIL™ topical solution (Encube Ethicals Pvt. Ltd., Goa, India) with label claim of 5 % w/v of minoxidil and 1.5 % w/v of aminexil was purchased from the local medical store. All the other chemicals used were of analytical grade.

HPLC instrumentation and analytical conditions: HPLC system (Water® e2695 PLC system, USA) equipped with a pump and a PDA detector was used in this study. For data acquisition and processing EMPOWER 2 software was employed. The chromatographic analysis was performed on a C_{18} column (250 mm × 4.6 mm i.d., particle size 5 µm) (ThermoHypersil BDS). The column temperature was maintained at 35 °C. Isocratic elution was performed using a mobile phase of methanol/phosphate buffer pH 3.0 (60:40, v/v) at a flow rate of 1.0 mL/min. The injection volume was 5 µL and the UV detection wavelength was 250 nm.

Standard and sample solutions: Phosphate buffer was prepared by dissolving 1 g of potassium dihydrogen phosphate in 1000 mL of water, adding 3 mL of triethylamine and adjusting the pH to 3 with dilute phosphoric acid solution.

Stock standard solutions were prepared by dissolving 250 and 75 mg of minoxidil and aminexil in 25 mL of mobile phase and 5 mL of each solution was further diluted to 100 mL using mobile phase in order to obtain a concentration of 0.5 and 0.15 mg/mL of minoxidil and aminexil, respectively. Working standard solution was prepared by mixing 50 mL of each of above standard solutions to obtain a solution having a concentration ratio of 5:1.5 (equal to that of marketed formulation) for minoxidil and aminexil. Working sample solution was prepared by diluting 5 mL of the formulated topical solution to 25 mL using mobile phase and 5 mL of the above prepared solution was further diluted to 100 mL using the same mobile phase.

RESULTS AND DISCUSSION

Method development: Preliminary studies were carried out in order to optimize a suitable method for simultaneous determination of minoxidil and aminexil in topical formulation. Trial runs were performed using C_8 and C_{18} reversed-phase columns, several mobile phase compositions and different flow rates for separation both the drugs with good chromatographic parameters (resolution, symmetry, tailing factor *etc*.). A C_{18} column (250 mm × 4.6 mm i.d., particle size 5 µm.) as a stationary phase with a mobile phase of methanol/phosphate buffer pH 3.0 (60:40,v/v) at a flow rate of 1.0 mL/min and a detection wavelength of 250 nm afforded the best separation with well-resolved and sharp peaks of both the drugs. The separation was carried out on an isocratic mode at room temperature and the injection volume was 20 µL.

Method validation: After method development, validation of the test method was performed in terms of the following parameters: linearity and range, accuracy and percentage recovery, precision, limit of detection (LOD), limit of quantitation (LOQ) and robustness [11-14].

Linearity and range: The linearity of the method was evaluated by analyzing six (n = 6) working solutions (calibration standards) of minoxidil containing 0.25, 0.375, 0.50, 0.625, 0.75 mg/mL and of aminexil containing 0.075, 0.112, 0.15, 0.187, 0.225 mg/mL. The plots of peak areas *versus* concentrations were linear in the range from 0.25 to 0.75 mg/mL and 0.075-0.225 mg/mL of minoxidil and aminexil, respectively. The regression equations were obtained as follows: $y = 16616x + 303.02$ ($r^2 = 0.99$) for minoxidil and $y = 19288x + 303.02$ ($r^2 = 0.99$) for aminexil, where y = peak area, x = concentration of solution; r = the square of determined correlation coefficient. The results implied that the method developed was linear over the specified range.

Accuracy and percentage recovery: Recovery studies were carried out at three different concentration levels (50, 100 and 150 %) in order to check the accuracy of the assay method. The study was performed three times (n = 3) at 0.025, 0.050 and 0.075 mg/mL. The average recovery of both minoxidil and aminexil were found 101 %, with RSD less than 1 % (Table-1), which indicates the accuracy of the method for determination.

TABLE-1
ACCURACY (% RECOVERY) RESULTS FOR THE DETERMINATION OF MINOXIDIL AND AMINEXIL

Drug	Spiked level (mg/mL)	Concentration (mg/mL)*		Recovery (%)	RSD (%)
		Spiked (amount added)	Measured (amount added)		
Minoxidil	50	0.2475	0.2498	101	0.10
	100	0.4955	0.4993	101	0.20
	150	0.7425	0.7484	101	0.20
Mean (%)				101	0.16
Aminexil	50	0.0742	0.0749	101	0.10
	100	0.1485	0.1498	101	0.20
	150	0.2227	0.2259	101	0.10
Mean (%)				101	0.13

*Mean of three determinations (n = 3) for each concentration.

Precision: Intra-day precision (repeatability) was evaluated by injecting six replicate (n = 6) of standard concentration (100 %) for each drug on the same day. Intermediate or inter-day precision (also called ruggedness) was performed on three

TABLE-2
RESULTS OF ROBUSTNESS INVESTIGATION

Standard condition	Modification	Recovery (%)		Retention time (min)		Resolution
		Minoxidl	Aminexil	Minoxidl	Aminexil	
Flow rate (mL/min)	0.8	101.2	101.4	3.640	6.993	13.985
	1.2	100.8	100.6	2.618	5.070	13.605
Column temperature (°C)	40	101.2	101.2	3.037	5.903	13.743
	50	101.1	101.2	3.024	5.523	13.605

*Mean of six determinations

consecutive days (*i.e.*, day 1, day 2 and day 3) by injecting six samples (n = 6) of standard solutions (100 %) for both the drugs. Precision was expressed as RSD value of the analyte peaks. The mean RSD values were obtained for the peak areas of minoxidil and aminexil on a single day (intra-day, day 1, n = 6) were 0.16 % and 0.13 %, respectively. The results of repeatability studies therefore confirm the reproducibility of the method. The mean RSD values on triplicate injections on three successive days (days 1-3, n = 9) were 0.02 % and 0.03 %, respectively. It indicates a good intermediate precision of the method.

Limits of detection and limits of quantitation: The limits of detection (LODs) for minoxidil and aminexil corresponding to a signal-to-noise ratio of 3 were 0.7346 µg/mL and 0.2423 µg/mL, respectively. The limits of quantitation (LOQs) corresponding to a signal-to-noise ratio of 10 were 0.7346 µg/mL and 0.2423 µg/mL for minoxidil and aminexil, respectively.

Stability of solution and robustness: The stability of the standard stock solutions (stored at 4 °C for 2 weeks) of each drug was determined at bench top, which showed no significant changes (< 2 %) relative to freshly prepared standards. The stability of sample solution was tested for every 2 h interval up to12 h. The RSD values of both minoxidil and aminexil were less than 1.0 %.

The robustness of the assay method was investigated by analysing six replicates (n = 6) of combined topical formulation of minoxidil and aminexil by introducing small changes in the chromatographic conditions (in developed method) which included changes of pH of the eluent, flow rate and column temperature. The % RSD value of the assay determined under robustness conditions was less than 1.0 %, indicating that the developed method was robust. System suitability was determined by six replicate injections of the system suitability solution. The results of system suitability parameters were found to be satisfactory. The results of robustness studies and system suitability parameters are given in Table-2.

Assay of minoxidil and aminexil in topical formulation: After successful development and validation of this method, it was employed for analysis of minoxidil and aminexil in combined topical formulation (Fig. 2). The method results in excellent separation with good resolution between the two analytes. Moreover, the higher percentage of recovery and non-interference of the formulation excipients in retention time of the drugs show the selectivity of the method for estimation of both the drugs in their combined dosage form.

Satisfactory results were obtained that the mean (± SD) percentage found (n = 3) for minoxidil 101.00 % (± 0.00) and aminexil 101.00 % (± 0.00) were in good agreement with the label claimed (Table-3).

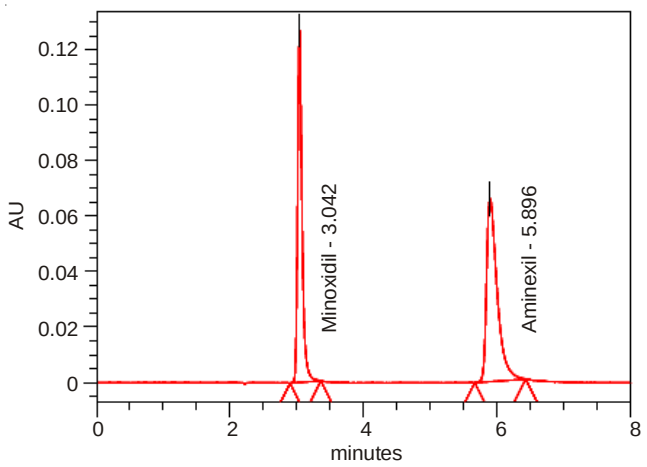

Fig. 2. Chromatogram of minoxidil and aminexil in combined topical formulation. Peak asymmetry and theoretical plates of minoxidil are 0.98 and 3100 and of aminexil are 0.98 and 3100, respectively

TABLE-3
ASSAY RESULTS OF MINOXIDIL AND AMINEXIL
IN COMBINED TOPICAL FORMULATION

Drug	Label claim (mg)	Amount found in mg* (mean ± SD)	Percentage estimated (mean ± SD)
Minoxidil	5000	4995.0 ± 1.1	101.0 ± 0.0
Aminexil	1500	1492.5 ± 0.6	101.0 ± 0.0

*Mean of three determinations (n = 3)

Conclusion

The developed method was successfully applied for simultaneous estimation of minoxidil and aminexil in combined topical formulation. The proposed method was found to be simple, accurate and precise. The method was free from interferences due to excipients present in the formulation. Therefore, this method may be useful for routine analysis of minoxidil and aminexil in bulk drugs and pharmaceutical dosage forms.

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