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DEVELOPMENT AND VALIDATION OF HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHIC METHOD FOR SIMULTANEOUS ESTIMATION OF MUCUNA PRURIENS, WITANIA SOMNIFERA, AND BERBERIS VULGARIS IN POLYHERBAL FORMULATION.

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Abstract: The surge of interest in naturally occurring phytochemicals with anti-arthritic potential has led to the discovery of many molecules, few of them being Mucuna Pruriens (MU), Withania Somnifera (WI) and Berberis aristatata (BA). The aim of the present work was to develop and validate an HPTLC method for determination of commercially available polyherbal formulations. Analysis of (-) MU, WI and BE was performed on TLC aluminum plates pre-coated with silica gel 60F-254. Linear ascending development was carried out in twin trough glass chamber, saturated with mobile phase consisting of n-Hexane: Chloroform: Acetone: Formic acid (4: 1.6:4:0.6 v/v/v/v). Camag TLC scanner III was used for the scanning and analysis in absorbance mode at 585 nm. The method was found to give compact spots for MU, WI and BE. (R_f value of 0.22 ±0.01, 0.71±0.01 and 0.77±0.01, respectively) and was linear over the range 100–600 ng/spot. Accuracy, precision and repeatability were all within the required limits. The HPTLC method developed was found to be relatively simple, rapid and accurate for the routine analysis MU, WI and BE in polyherbal formulations.

Keywords: High-performance thin-layer chromatography; method validation; Mucuna Pruriens (MU); Withania Somnifera (WI) and Berberis Aristata (BA).

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INTRODUCTION

Depression is a common, debilitating, life threatening illness with an increasing morbidity and mortality. According to world health report, about 450 million people suffer from a mental or behavioral disorder [1]. By the year 2020, depression is expected to constitute the second largest source of global burden of disease after heart disease [2]. Depression is whole body illness which involves not only mood or emotion but also the physical body and thought process. The symptoms of depression are intense feelings of sadness, hopelessness, and despair, as well as the inability to experience pleasure in usual activities, changes in sleep patterns and appetite, loss of energy, and suicidal thoughts [3].

Depression is the most common of the affective disorders; it may range from a very mild condition to severe (psychotic) depression accompanied by hallucinations and delusions [4, 5]. Depression constitutes the second most common chronic condition in clinical practice. The causes of depression vary in different individuals. Psychosocial factors such as adverse living conditions can influence the onset and persistence of depressive episodes. Genetic and biological factors also play a part. It is estimated that 5.8% of men and 9.5% of women will experience a depressive episode in any given year [6].The primary clinical manifestations of major depression are significant depression of mood and impairment of function. Some features of depressive disorders overlap those of the anxiety disorders, including panic agoraphobia syndrome, severe phobias, generalized anxiety disorder, social anxiety disorders, posttraumatic stress disorder, and obsessive-compulsive disorder. The major disorders of mood or effect include the syndromes of major depression (formerly termed melancholia) and bipolar disorder (formerly termed manic-depressive disorder).Major depression is characterized by feelings of intense sadness and despair, mental slowing and loss of concentration, pessimistic worry, lack of pleasure, self-deprecation, and variable agitation or hostility. Physical changes also occur, particularly in severe, vital, or melancholic depression. These include insomnia or hypersomnia; altered eating patterns, with anorexia and weight loss or sometimes overeating; decreased energy and libido; and disruption of the normal circadian and ultradian rhythms of activity, body temperature, and many endocrine functions [7].The categories of drugs that are used for treatment of depression include nor-epinephrine reuptake inhibitors like amitriptyline , monoamine oxidase inhibitors like selegiline etc. Besides the drug treatment several types of psychotherapy or ''talk therapy'' and electroconvulsive therapy (ECT) can help people with depression.

Literature survey reveals that there is no single HPTLC method available for the simultaneous estimation of MU, WI and BE in polyherbal formulation. However, analytical methods have so far been reported for their quantification individually but not simultaneously. In present study, successful attempt has been made to develop a simple, accurate and rapid HPTLC method for

simultaneous estimation of MU, WI and BE in antidepressant polyherbal formulation and validated as per ICH guideline.

EXPERIMENTAL

Chemicals and Reagents: MU, WI and BE as reference standard were purchased from Natural remedies private limited.(Banglore, India). Capsule dosage form was also procured from local market. AR grade n-Hexane, Chloroform, Acetone, Formic Acid were obtained from Finar Chemicals. (Ahmadabad,India).

Apparatus: The Syringe with capacity of 100 µl (Hamilton, WInaduz, Switzerland), Linomat V Automatic Sample Spotter (Camag, Muttenz, Switzerland), TLC Chamber of glass twin trough chamber (10 x 10 x 4cm), TLC Scanner 3 linked to WinCats software (Camag,Muttenz, Switzerland) and HPTLC plates (10 x 10 cm, 0.2 mm thickness) precoated with silica gel 60 F254; E. Merck KgaA, (Darmstadt, Germany) were used for the analysis.

Chromatographic condition: The separation was performed on 10 cm×10 cm aluminum plates, precoated with 0.2 mm layers of silica gel 60 F_{254}. The TLC plate was pre-washed with methanol and dried. Samples were spotted on the TLC plate 10mm from the bottom edge and 15mm from side using Linomat V and analyzed using following parameters: band width 6 mm, spraying rate 10µL/s, volume of mobile phase,10.2 ml, chamber saturation time, 25 min, migration distance 85 mm, slit dimension 6.0× 0.45 mm, scanning speed 20 mm/s,Spraying reagent Anisaldehyde sulphuric acid, detection wavelength 585 nm. Mobile phase consisted of n-hexane–chloroform–Acetone-Formic acid (4:1.6:4:0.6 v/v).

Preparation of Stock solutions Standard stock solutions were prepared by dissolving 3 mg of accurately weighed MU, WI and BE, separately in 10 ml methanol using 10 ml volumetric flask.

Preparation of working standard solutions: Aliquots of 1.67 ml stock solution of each standard were transferred, mixed in 10 ml volumetric flask and BEluted with methanol upto the mark to get final concentration of 50.1 µg/ml for each standard.

Preparation of sample solutions: (MU and WI): Powder of 20 capsules, each containing MU, WI and BE were weighed and analysed: a quantity of powder (1 gm) was taken in 10 ml volumetric flask and was extracted with 2-3 ml of methanol for 2 or 3 times and then the solution was filtered. The filtrate was transferred to another 10 ml volumetric flask and sufficient methanol was added to adjust the volume upto the mark. For estimation of MU and WI, 0.1 ml of this solution was further diluted to 10 ml with methanol. From this final sample solution, 8 µl of the solutions was spotted on the TLC plate.

Preparation of sample solution (BE): Quantity of powder (1 gm) was again taken, transferred to the 50 ml volumetric flask and 50 ml 2N HCL was added. The mixture was refluxed for 2 hr at 100^0c, cooled and filtered through whattman filter paper. Then residue of sample was air dried and then it extracted with Chloroform. The Chloroform extract was collected in 10 ml volumetric flask and then volume was diluted upto mark with chloroform. From this final sample solution, 12 µl of the solutions was spotted on the TLC plate.

METHOD VALIDATION:

As per the conference on harmonization (ICH) guidelines, the method validation parameters checked were specificity, linearity, accuracy, precision, limit of detection and limit of quantification and robustness.

Linearity: Aliquots (2, 4, 6, 8, 10 and 12 µl) of working standard solution were applied on TLC plates using Linomate V and analyzed as described in chromatographic condition section. Calibration curves were obtained by plotting peak areas versus the concentration, and the regression equation were calculated. Each response was the average of three determinations.

Accuracy (% Recovery): The accuracy study was carried out by the standard addition method. Known amount of standard solution of MU, WI and BE was added to a pre-quantified sample solution of MU, WI and BE. Each solution was spotted in triplicate and the percentage recovery was calculated by measuring the peak areas and fitting these values into the regression equations of the calibration curves.

Precision: The intra-day and inter-day precisions of the proposed methods were determined by estimating the corresponBEng responses five times on the same day and on three BEfferent days over a period of one week for three BEfferent concentrations of MU, WI and BE.

Method Precision: This test is used to verify that the resolution and rMUeatability of the system were adequate for the analysis intended.The precision of the instrument was checked by rMUeatedly spotting (n=6) standard solution of MU,WI and BE for this method.

Limit of detection (LOD) and Limit of quantitation (LOQ): LOD and LOQ were calculated in accordance with the 3.3 s m^{-1} and 10 s m^{-1} criteria, respectively, where s is the standard deviation of the peak area and m is the slope of the corresponBEng calibration plot, determined from linearity investigation.

Specificity: The specificity of the method was checked by comparision of chromatograms obtained from sample, standard and corresponBEng placebo.

Robustness: Here, small deliberate changes in experimental conditions, such as amount of mobile phase, spotting time, saturation time, developed plate scanning, scanning speed, scanning wavelength were done. Obtained results were compared with original chromatographic conditions.

Analysis of dosage form: All capsules were purchase from a local market. The response of the dosage form was measured at 585 nm for quantification of MU, WI and BE by HPTLC as described as above. The amount of above mentioned drugs present in sample solution were determined by fitting the responses into the regression equation for MU, WI and BE.

RESULTS AND DISCUSSION:

For the HPTLC method, chromatographic conditions were optimized to achieve the best resolution and peak shape for MU, WI and BE. Several mobile phase compositions were tried but satisfactory separation for MU, WI and BE were obtained with mobile phase n-Hexane: Chloroform: Acetone: Formic acid in the ratio of 4:1.6:4:0.6 v/v/v/v. Quantification was achieved at 585 nm based on peak area.(Fig:01)

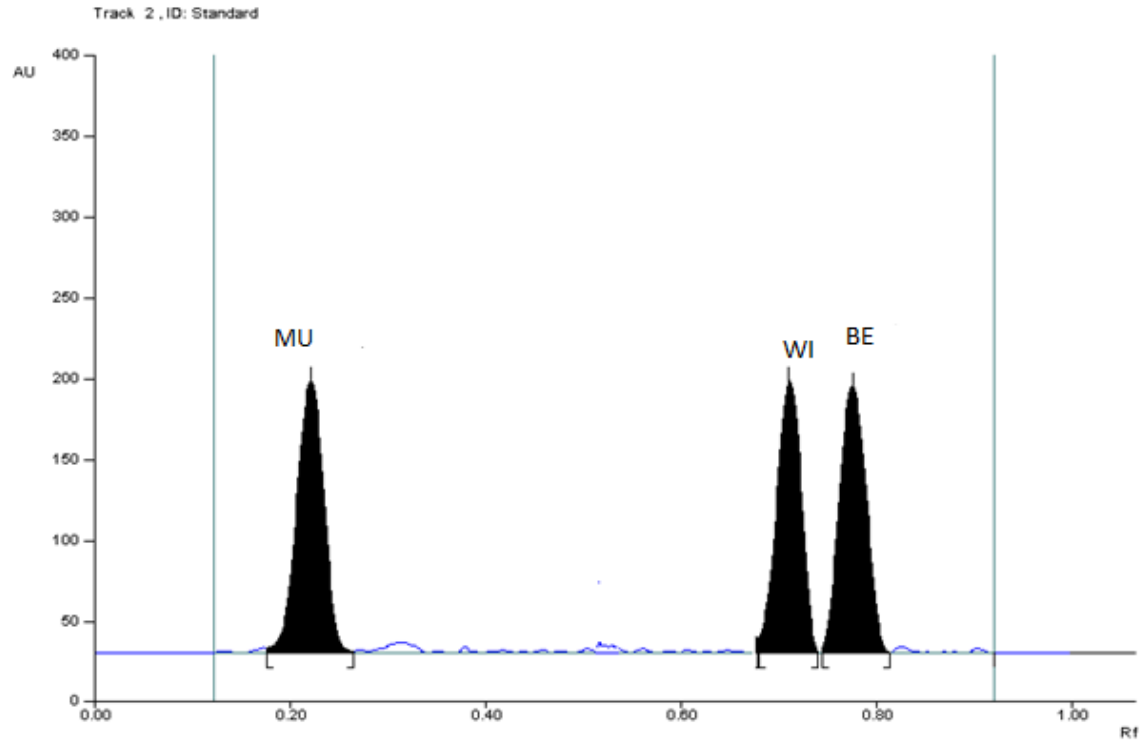

Fig.1. Chromatogram of standard MU, WI and BE for proposed HPTLC method.

Specificity: The herbal formulation contains many constituents and during analysis they had shown small peak in chromatogram alongwith MU,WI and BE. But, the R_f values were different for each constituents and did not show any interference at peak of MU, WI and BE.

Linearity: The linear correlation was obtained between peak area and concentration in the range of 100.2 – 601.2 ng/spot for MU, WI and BE. The linearity of the calibration curves were validated by the value of correlation coefficient of regression (r). All the results are listed in table 1.

Table 1. Results from regression analysis of the calibration curves

Parameters	MU	WI	BE
Intercept	– 3097	70080	17321
Slope	12538	25225	4197
Correlation coefficient	0.9992	0.9996	0.9991

Accuracy (% Recovery): The recovery experiments were carried out by the standard addition method. The % recovery obtained were 99.13± 0.47, 99.32± 0.23, 99.63±0.80 for MU, WI and BE, respectively, which indicates the accuracy of method.

Precision: The low % RSD value of inter-day (0.34 – 0. 84) and intra day (0.29 – 0.81) implied that the reproducibility of the proposed method was good.

LOD and LOQ: LOD for MU,WI and BE were found to be 19.03, 24.65 and 29.22 ng/spot respectively by proposed method, while, the LOQ were found to be 57.69, 73.09 and 89.45 ng/spot, respectively.

Robustness: The method is found to be robust as the results were not significantly affected by slight variation in composition of mobile phase, spotting time, saturation time, developed plate scanning, scanning speed, scanning wavelength.

Method Precision: The % RSD for MU,WI and BE was found to be 0.36, 0.83 and 1.14 respectively. All the results were within the acceptable range.

Summary of all validation parameters for MU, WI and BE by proposed HPTLC method is given in table 2.

Table 2. Summary of validation parameters of the proposed method

Parameters	MU	WI	BE
Linearity (µg mL^{-1})	2-12	2-10	100-500
LOD (µg mL^{-1})	0.08	0.11	6.22
LOQ (µg mL^{-1})	0.25	0.35	18.84
System precision [a]	0.44	0.40	0.55
Method precision [a]	0.41	1.11	1.18
Intraday precision [a]	0.68	0.35	0.45
Interday precision [a]	0.77	0.39	0.39
Accuracy (%) [b]	99.13	99.32	99.64
Assay (%w/w) [b]	26.05	10.38	0.6

[a] n=6

[b] n=3

LOD limit of detection

LOQ limit of quantification

Assay of formulation: The proposed validated method was successfully applied to determine MU, WI and BE in herbal formulations.The procured formulation contains extract form of *Withania somnifera* (100 mg extract), *Mucuna* (50 mg Extract) and *Berberis* (100 mg extract). The mean percentage of MU, WI and BE were found to be 26.05, 10.38 and 6.0 %w/w, respectively, from the formulation. (Table:02)(Fig: 02 and 03).

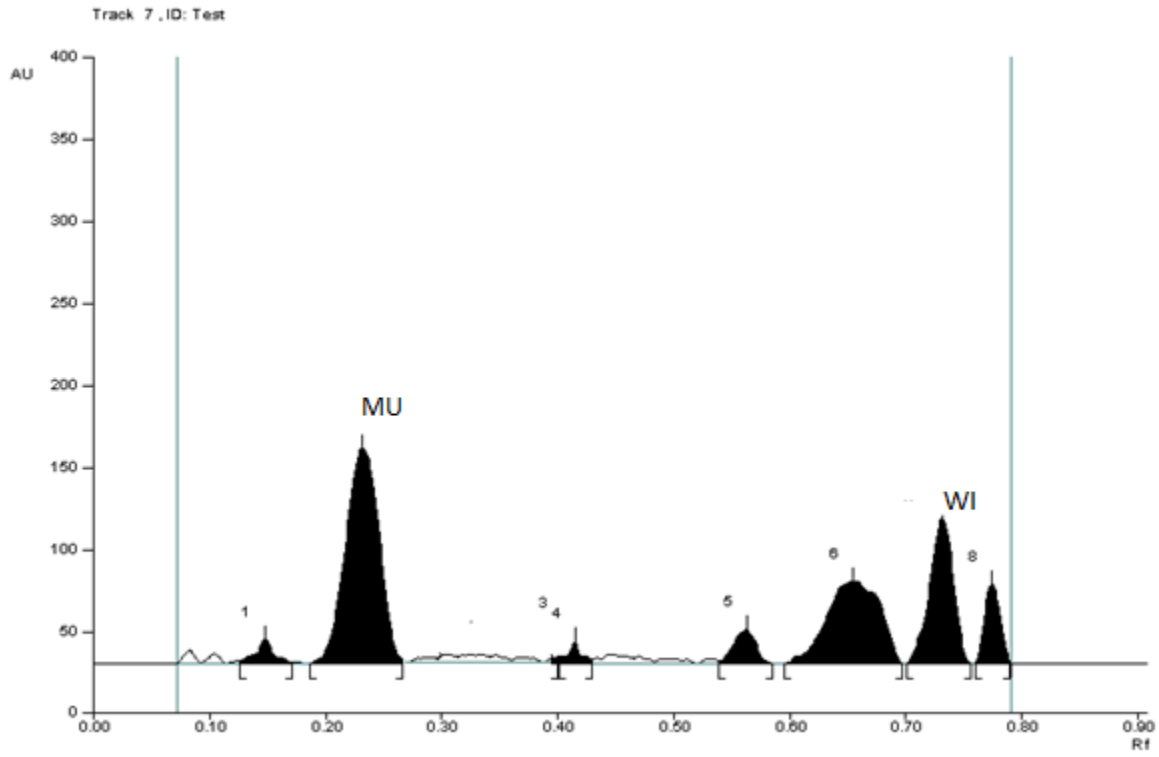

Fig.2. HPTLC chromatogram of formulation showing (-) MU and WI.

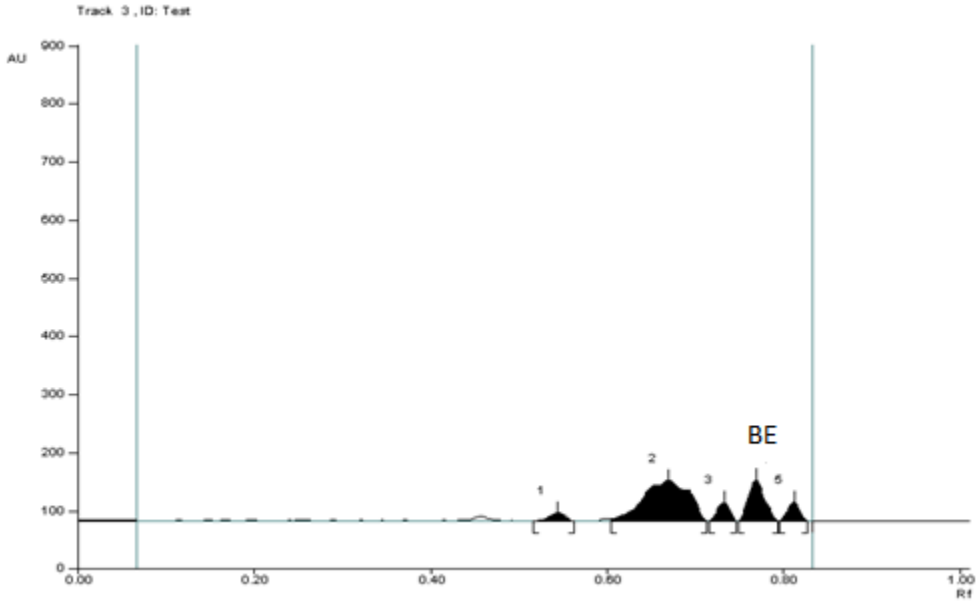

Fig.3. HPTLC chromatogram of formulation after hydrolysis of BE.

CONCLUSION:

The validated HPTLC method employed here proved to be simple, specific, accurate, precise, sensitive and robust. It can be successfully used for routine analysis of MU, WI and BE in combined dosage form. With the developed method, only single mobile phase is sufficient for quantification of MU,WI and BE either in combination or in single dosage form as per availability of formulation for herbal drug manufacturing industries.

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