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UV AND HPLC METHOD DEVELOPMENT OF AZADIRACHTIN AND GYMNEMIC ACID IN POLYHERBAL *CHURNA* AND ITS VALIDATION

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ABSTRACT

The present research work is associated with UV and HPLC method development of Azadirachtin and Gymnemic acid in polyherbal *churna* and its validation .UV method for simultaneous estimation of Azadirachtin and Gymnemic acid was developed using 95% methanol as a solvent. By scanning, the each solution was in the range of 200-400 nm. 210 nm was selected as a wavelength for Azadirachtin while 217 nm for Gymnemic acid. Method was validated by linearity ,range ,accuracy, precision (intraday and interday),LOD LOQ. HPLC method for simultaneous estimation of Azadirachtin and Gymnemic acid was developed using HPLC system of JASCO UV -2075 with C18Intresil, 4.6(i.d.) x 263 nm columns. Chromatogram for marker was developed using mobile phase methanol :acetonitrile in the ratio of 60:40 v/v. Separation was achieved with good resolution as 6.3 , Retention time as 1.9417 ,3.2083,asymmetry 1.15,0.87 and theoretical plates 1180,7955 for Azadirachtin and Gymnemic acid respectively. Method was validated by parameters as linearity, range, accuracy, precision (intraday and interday), LOD LOQ ,robustnees . *Churna* was analyzed in comparision with standard Azadirachtin and Gymnemic acid. The quantification of Azadirachtin and Gymnemic acid in *churna* chromatogram was done by comparing peak areas from chromatogram of standard Azadirachtin and Gymnemic acid.

KEYWORDS: UV, HPLC, method development, Azadirachtin, Gymnemic acid, polyherbal *churna*, validation.

INTRODUCTION

Quality control[1] of herbal preparation or proprietary products however is much more difficult than synthetic drugs because of the chemical complexity of the ingredients. As herbal preparation comprise hundreds of mostly unique, or species-specific, compounds, it is difficult to completely characterize all of these compounds. It is also equally difficult to know precisely which one is responsible for the herbs or herbal preparation's therapeutic action because these compounds often work synergistically in delivering therapeutic effects. Thus maintaining consistent quality in herbal preparation, both from batch to batch and over time is as problematical as it is necessary and has drawn serious attention recently as challenging analytical[2] task. Small scale and large scale producers of herbal products are proceed large numbers of Ayurvedic proprietary medicine. As our nation is except of high market potential in future standardization of such medicine by advanced analytical techniques is the most essential tool for quality assurance[3-5] of the same. Its quantitative and qualitative determination by UV and HPLC will be a choice of method development of two active marker compound. Such proposed work with its assurance of quality of such products in herbal industry is currently having great significance. There is no any precise and economic simulations estimation method has been reported for Azadirachtin and Gymnemic acid by UV and HPLC, Therefore it wasour intention to develop the suitable method for the same, which gives high degree of assurance with better strength, identity and purity of both the compounds. UV and HPLC method development and validation is important tool in analytical area .Hence simultaneous estimation of Azadirachtin and Gymnemic acid in MMC is challenging investigation in era of herbal drug analysis.

EXPERIMENTAL

UV Analysis[6,8]

It is must to observe pattern of UV absorbance with prior to HPLC method development for target materials.

Instrument Used

SHIMADZU 1800 UV/Visible double beam spectrophotometer, with pair of matched quartz cells corresponding to 1 cm path length was used for measurement of absobance. Elder digital balance used for weighing, ultra sonicator of Prama instrument was used for sonicating the drug and sample solution.

UV Method Development for Markers (Azadirachtin and Gymnemic acid)

Selection of Common Solvents: Solubility studies were carried out with a view to find a suitable solvent in which

the markers are completely soluble and stable. Solvents like water, 95% ethanol, methanol, acetonitrile were tried for checking solubility of AZA and GYM. After assessing the solubility of drug in different solvent 95% methanol has been selected and finalized as common solvent to observe spectral characteristics.

Selection of Sampling Wavelength for Simultaneous Analysis: The aliquot portions of stock standard solutions of AZA and GYM were diluted appropriately with solvent 95% methanol to obtain concentration 10 µg/mL of AZA and 10 µg/mL GYM solution, both the solutions were scanned in the range of 200-400 nm in 10 mm cell against solvent blank. The absorption maxima was determined, a representative spectrum of AZA and GYM in 95% methanol is shown in figure 29, 30 respectively. The study of spectrum revealed that Azadirachtin showed a well defined λmax at 210nm whereas Gymnemic acid showed at 217nm. These two wavelengths were selected for development of simultaneous equation.

Preparation of Standard Stock Solution and Study of Beer-Lamberts Law

Standard Azadirachtin Stock Solution: An accurately weighed quantity of Azadirachtin (AZA) 10 mg was dissolved in 95% methanol in 100 mL volumetric flask and volume was made up to the mark with the same solvent to get final concentration of 100 µg/ mL.

Standard Gymnemic acid Stock Solution

An accurately weighed quantity of Gymnemic acid (GYM) equivalent to 10 mg was dissolved in 95% methanol in 100 mL volumetric flask and volume was made up to the mark with the same solvent to get final concentration of 100 µg/ mL.

Study of Beer-Lambert's Law

Aliquots of working stock solution of AZA and GYM were prepared with 95% methanol to get concentration range of 2-12µg/ mL for AZA and 2-12µg/ mL for GYM. The absorbance of resulting solutions was measured at their respective wavelength. A calibration curve was constructed to study the Beer-Lambert's Law and regression equation. Dilution used for calibration curve as below.

Determination of Absorptivity Values of Markers At Selected Wavelengths: Aliquot portions of AZA and GYM stock standard solutions were diluted with 95% methanol to obtain different concentrations of each drug. The absorbance of each solution was measured at 210 nm and 217 nm. A (1%, 1cm) values were calculated using following formula, Absorbance

A (1%, 1cm) =

Conc.(g/100ml)

Estimation of Concentration of Azadirachtin and Gymnemic acid In Polyherbal Formulation *(MMC)*

10 mg extract of *MMC* was dissolved in 10 mL 95% methanol in 10 mL volumetric flask, sonicated for 15 minutes. Filtered the solution; to get concentration of 1000 µg/mL. Further dilution was made to get final concentration of 10 µg/mL. Absorbance was determined at 210nm wavelength for Azadirachtin and 217nm wavelength for Gymnemic acid Concentration of both markers was estimated by using following formula,

C=A/ba

Whereas,

C = concentration of AZA/GYM µg/mL , b=path length

A = absobance of mixture at 210nm/217nm.

a = absorptivity of AZA at 210nm/absorptivity of GYM at 217 nm

Method validation[7,8]

Following parameters were used to validate the method.

Linearity Study: Linearity was studied by preparing serial dilutions using standard stock solution in 10 mL volumetric flask. I.C.H. Recommends that for the establishment of linearity, a minimum of 5 concentration normally used . The various dilutions used for linearity study are as follows. And the further study was carried out.

LOD and LOQ: Limit of detection (LOD) is the lowest amount of an analyte that can be detected but not necessarily as an exact value.

Limit of quantification (LOQ) is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were separately determined which is based on calibration curve. The S.D. of y intercept of regression line may be used as S.D. The given concentration was used for the this and the absorbance was taken and then calculate the standard deviation and slope.

LOD = 3.3 X σ/s

LOQ= 10 X σ/s

Where, σ =Standard deviation of y intercept of regression lines, S =Slope of calibration curve.

Accuracy: Accuracy of an analytical method is the closeness of test results obtained by the method to the true value. It was ascertained on the basis of recovery studies performed by standard addition method at 50, 100 and 150 % of test concentration. Known amount of standard drugs were added to analyzed sample and subjected to the developed UV method. Then the absorbance was taken and further calculation was carried out. The recovery study was performed three times at each level.

Precision: Precision of an analytical method is the degree of agreement among individual results when the method is applied repeatedly to multiple readings of a homogeneous sample. It is expressed as %R.S.D. of series of measurements. The interday's and intra

precision was determined using 10 µg/mL concentration of each marker.

Intraday's: It was carried out by estimating the corresponding responses (absorbance) three times on the same day between three hrs interval, measurement of responses (absorbance) was expressed in terms of % Relative Standard Deviation (%RSD).The given dilution was used and the absorbance was taken by different intervals in the same day like 11pm ,1pm ,3pm and further calculation was done.

Interday (Different days): It was carried out by estimating the corresponding responses (absorbance) of same sample was recorded on three different days, measurement of responses (absorbance) was expressed in terms of % Relative Standard Deviation (%RSD). The given dilution was used and the absorbance was taken by different intervals in a different day like first day, second day, third day and further calculation was done.

Robustness: The robustness of analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. By using this dilution the absorbance was taken by changing the concentration then the further calculation was carried out.

Ruggedness: The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days, etc. By using this dilution the absorbance was taken and the further calculation was carried out.

Analysis polyherbal formulation (MMC) simultaneous equation method: An accurately weighed quantity of polyherbal formulation *(MMC)* extract l00mg was taken in 100 ml volumetric flask and dissolved in 95% methanol by vigorous shaking. The volume was made up to the mark with same solvent and further dilutions were made to get final concentration of about 10 µg/mL of extract.The absorbances of the resulting solutions were measured at 210nm and 217nm gainst blank. From this extract amount of each drug was determined using simultaneous equation as mentioned as,

A2ay1-A1ay2
C x = Ax2ay1-ax1ay2, A1ax 2-A2ax1
C y = Ax2ay1-ax1ay2
Where,
C x = Concentration of AZA in µg/ml
C y = Concentration of GYM in µg/ml
ax1 = Absorptivity value of AZA at 210nm
ax2 = Absorptivity value of AZA at 210nm
ay1 = Absorptivity value of GYM at 217nm
ay2 = Absorptivity value of GYM at 217nm
A1 = Absorbance of sample at 210nm
A2 = Absorbance of sample at 217nm

HPLC Analysis[10,11,12]

Instrument used: HPLC system (Jasco UV-2075 model) consisting manual injector having capacity of 20 µL with detector LC-UV-2075 UV/VIS detector. The software used was chromenav .Column Intersil ODS-3V (250 x 4.6mm) C18Column packed with 5µm diameter particles size from GL science Inc. (made in Japan) was used for experiments. Elder digital balance used for weighing, pH meter of Hanna instrument, Ultrasonicator of Prama instrument was used for sonicating the drug and sample solution.

HPLC Method Development For Markers (Azadirachtin and Gymnemic acid)

Selection of Analytical Wavelength

The wavelength for detection was selected by preparing the individual solution of l0µg/mL of Azadirachtin and Gymnemic acid. Each solution was scanned in the range of 200-400 nm and they were overlaid. The wavelength selected for the analysis 263 nm at which both drug showed significant absorbance.

Selection of Stationary Phase

On the-basis of reversed phase HPLC mode, stationary phase column with C18 bonded phase i.e. - Intersil ODS-3V (250 x 4.6mm) with particle size 5 µm from GL science Inc (made in japan) was used for separation.

Optimization of mobile phase

Different mobile phases were tried in order to find best condition for separation of AZA and GYM. Following composition mobile phases were tried,

1. Water: Methanol (60:40 v/v).
2. Water: Acetonitrile (30:70 v/v)
3. Water: Acetonitrile: Methanol (40:30:30 v/v/v)
4. Methanol: Acetonitrile (75:25 v/v)
5. Methanol: Acetonitrile (80:20 v/v)
6. Methanol: Acetonitrile (95:5v/v)
7. Methanol: Acetonitrile (90:10v/v)
8. Methanol: Acetonitrile (60:40v/v)

Buffer and mobile phase preparation

• Buffer preparation

10 mM phosphate buffer was prepared by adding 1.360 gm potassium dihydrogen phosphate in 1000 ml double distilled water. The solution was filtered through 0.45µm filter and sonicated for 15 min.

• **Preparation of mobile phase**: Mobile phase was prepared by mixing methanol: Acetonitrile in the ratio of 60:40v/v. pH of mobile phase was adjusted to 6.1 with othophosphoric acid.

• Degassing of the mobile phase

The prepared mobile phase was degassed by ultrasonication for 20 min, so as to avoid the disturbances caused by dissolved gases.

• Filtration of mobile phase
The degassed mobile phase was filtered through 0.45µ filter to avoid the column clogging due to smaller particles.

Final chromatographic condition
System used: JASCO UV -2075

Software: Chromnav

Column used: C18 intersil, 4. 6(i.d.)x 250mm

Mobile phase used: Methanol: Acetonitrile

Flow rate: 1ml/min

UV detection: 263nm

Condition: Gradient condition

Sample preparation
Standard stock solution containing Azadirachtin (AZA) and Gymnemic acid (GYM) was prepared by dissolving 10 mg of (AZA) and (GYM)separately in 100 mL of mobile phase and to get stock solution containing 100 µg/mL of AZA, 100 µg/ml of GYM in different 100 mL volumetric flasks. Suitable dilutions were made and the sample was filtered through 0.2 µ nylon membrane filter. Aliquots of 20 µL of the clear filtrate were injected into the HPLC column.

Priming of the system
Air in the conducting tubes was removed by manual method to obtain the continuous flow and to avoid the backpressure on the pump, avoiding the damage to the column.

Conditioning of the column
Before a new run on system, warm HPLC grade water was run at flow rate of 1 mL/ min-1 for 1 hr, so as to remove water soluble impurities from the column. Then methanol and water in 50:50 ratio was run at the same flow for 30 min. Conditioning of the column was done by passing methanol at 1 mL/min-1 flow rate for 30 min. So as to remove the remains of the previous run.

Loading of mobile phase
Filtered and degassed mobile phase was filled in the reservoir. Priming was done for each freshly prepared mobile phase.

Baseline stabilization
The detector was turned on for an hour before the actual run so as to obtain the stable UV light. The mobile phase run was started at required flow rate and the run was continued so as to obtain stable baseline.

Loading of samples
Properly prepared, filtered and sonicated samples were loaded into the manual injector port with the help of syringe and the sample was injected.

Calibration curve
Calibration curves were prepared by taking appropriate aliquots of standard AZA and GYM stock solutions in different 10 mL volumetric flask and diluted up to the mark with mobile phase to obtain final concentrations in the range of 10-60 µg/mL of AZA and 10-60 µg/mL of GYM The mobile phase was allowed to equilibrate with stationary phase until steady baseline was obtained. Then each dilution of both the markers was injected and peak area recorded. Calibration curve was constructed by plotting the peak area vs. the drug concentration and regression equation was computed, dilutions used for calibration curve are as below, (Mobile phase used - Methanol : Acetonitrile 60:40).

Method Validation[14,15]
The proposed method was validated as per ICH guidelines. The solutions of the drugs were prepared as per the earlier adopted procedure given in the experiment.

Specificity
Specificity was measured as ability of the proposed method to obtain well separated peak for AZA and GYM without any interference from other constituents of plant. Retention time for AZA -1.9417min. and GYM -3.2083 min. The each dilution of both the markers was injected and the peak area was recorded .The values obtained were very close to that in standard laboratory mixture indicates no interference from the other constituents of plant.(Mobile phase used - Methanol : Acetonitrile 60:40).

Linearity and Range: According to USP extract equivalent to 80, 90, 100, 110, 120 % of test concentration was taken and dissolved in mobile phase, diluted appropriately to obtain a concentration in the range of 80%-120% of the test concentration. Then each dilution of both the markers was injected and chromatogram was recorded. The chromatograms of the resulting solutions were recorded and by observing the chromatogram if this appears to be linear relationship, then the test result was appropriate . (Mobile phase used - Methanol: Acetonitrile 60:40).

LOD and LOQ: The LOD and LOQ were separately determined which is based on calibration curve. The S.D. of y intercept of regression line may be used as S.D.
LOD = 3.3 x σ/s
LOQ = 10xσ/s
Where, σ= Standard deviation of y intercept of regression lines
S = Slope of calibration curve.

Then each dilution of both the markers was injected and chromatogram was recorded. The chromatograms of the resulting solutions was recorded and further calculation was done. (Mobile phase used - Methanol: Acetonitrile 60:40).

Precision: It is expressed as % R.S.D. of series of measurements. The interday and intra precision was determined using 10 µg/mL concentration of each marker. (Mobile phase used - Methanol: Acetonitrile 60:40)

Intraday
It was carried out by estimating the corresponding responses (peak area) three times on the same day between three hrs intervals. Measurement of responses (peak area) was expressed in terms of % Relative Standard Deviation (%RSD). Then each dilution of both the markers was injected and chromatogram was recorded at different intervals in the same day like 11 pm, 1pm, 3pm and further calculation was done.

Interday (Different days)
It was carried out by estimating the corresponding responses (peak area) of same sample were recorded on three different days. Measurement of responses (peak area) was expressed in terms of % Relative Standard Deviation (%RSD). Then each dilution of both the markers was injected and chromatogram was recorded at different intervals in a different day like first day, second day, third day and further calculation was done.

Accuracy: This involved the addition of known quantities of markers into analyzed sample .Three concentration levels were tested (50%, 100%, 150%). At each level, samples were prepared in triplicates and analyzed according to previously described procedure. Accuracy was expressed as % recovery. Then each dilution of both the markers was injected and The chromatograms of the resulting solutions was recorded and further calculation was carried out. The recovery study was performed three times at each level. (Mobile phase used - Methanol: Acetonitrile 60:40).

Robustness
It is measure of its capacity to remain unaffected by small but deliberate change in method parameters and provides an indication of its reliability in normal usage. The parameters for HPLC method include the variation in flow rate, wavelength and mobile phase composition. The Retention time and asymmetry were considered for robustness.

Effect of flow rate variation
Robustness of method was checked by changing flow rate from 0.9 mL/min-1 to 1.1 mL/min-1 instead of 1 mL/min-1 by injecting the three replicate injection of standard (l0µg/mL Azadirachtin and l0µg/mL Gymnemic acid) at 0.9 mL/min-1 and 1.1 mL/min-1.

Effect of wavelength variation
Robustness of method was checked by using wavelength 258nm and 268nm instead of 263nm by injecting the three replicate injections of standard (l0µg/mL Azadirachtin and l0µg/mL Gymnemic acid) at 258nm and 268nm.

Effect of mobile phase composition variation
Robustness of method was checked by using mobile phase composition 65:35and 70:30 instead of 60:40 by injecting the three replicate injection of standard (l0µg/ml Azadirachtin and l0µg/ml Gymnemic acid) at 65:35 and 70:30.

Ruggedness
The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days, etc. Then each dilution of both the markers was injected and The chromatograms of the resulting solutions was recorded and further calculation was carried out. (Mobile phase used - Methanol: Acetonitrile 60:40)

System suitability test
System suitability test is a pharmacopoeial requirement and is used to verify, whether the resolution and reproducibility of the chromatographic system are adequate for analysis to be done. The tests were performed by collecting data from five replicate injections of standard drug solution. Then each dilution of both the markers was injected and The chromatograms of the resulting solutions was recorded and then calculate the peak area, retention time, therotical plate, Asymmetry, Resolution along with their standard deviation .(Mobile phase used - Methanol : Acetonitrile 60:40).

RESULT AND DISCUSSUION

UV Analysis
Selection of analytical wavelength: Azadirachtin showed maximum absorbance at 210nm and 212nm and Gymnemic acid acid showed maximum absorbance at 217nm, 220nm but 210nm and 217nm was selected for Azadirachtin and Gymnemic acid respectively.

Absorptivity values of Markers at selected wavelengths
Aliquot portions of AZA and GYM stock standard solutions were diluted with solvent 95%methanol to obtain different concentrations of each drug . the absorbance of each solution was measured at 210nm and 217nm. A(1%1cm) values were calculated using following formula
Absorbance: A(1%1cm)= Conc.(g/100ml

Estimation Of Concentration Of Azadirachtin And Gymnemic Acid In Polyherbal Formulation By UV Spectroscopy
Concentration of both markers estimated by using formula
C=A/ba

Whereas,
C= Concentration of AZA/GYM µg/ml

A=Absorbance of mixture at 210nm/217nm
a= Absorptivity of AZA at 210nm/ Absorptivity of GYM at 217nm
b=path length

Method validation

Linearity study

Linearity was studied by preparing serial dilution using standard stock solution as shown in dilution schem. The linearity range for Azadirachtin and Gymnemic acid were found to be 2 µg/ml-20 µg/ml and 4µg/ml-36µg/ml ,respectively. Graph no 2 and 3.

Linear regression data for calibration curve of Azadirachtin and Gymnemic acid

The calibration plot Azadirachtin and Gymnemic acid follow Beer-Lambert's law at all selected wavelengths indicates response is linear function of concentration in the range of 2-20ug/mL for Azadirachtin and 4-36ug/mL for Gymnemic acid in UV spectrophotometric method . Graph no 1.

LOD and LOQ

Limit of detection is the lowest amount of an analyte that can be detected but not necessarily as an exact value. Limit of quantification is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were separately determined which is based on calibration curve. The S.D. of y intercept of regression line may be used as S.D.
LOD = 3.3 xσ/s
LOQ-10xσ/s
Where, σ = Standard deviation of y intercept of regression lines,
S = Slope of calibration curve.

The LOD was found to be 0.09 µg /mL and LOQ was found to be 0.27 µg /mL for Azadirachtin and LOD was found to be 0.105ug /mL and LOQ was found to be 0.318pg /mL for Gymnemic acid . The limit of detection (LOD) and quntification (LOQ) were near about 0.09µg/mL, 0.27 µg/mL for Azadirachtin and 0.105 µg/mL, 0.318 µg/mL for Gymnemic acid, which indicates adequate sensitivity of method.

Accuracy

Accuracy of an analytical method is the closeness of test results obtained by the method to the true value. It was ascertained on the basis of recovery studies performed by standard addition method at 50, 100 and 150 % level, known amount of standard drugs were added to analyzed sample and subjected them to the proposed UV method . Results from recovery studies were within acceptable limits 98.50-99.39% and 99.01-99.37% for Azadirachtin Gymnemic acid respectively indicating accuracy of method was good.

Precision: The intraday and inter day's precision study of AZA and GYM was carried out by estimating the corresponding responses three times on the same day and on three different days and expressed as % RSD. The results for intraday and interday's precision of extract is shown in table as below. The low values of % R.S.D. (< 2%) for intra and inter day's variation, which suggested an excellent precision of method.

Robustness: The robustness of analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. By using this dilution the absorbance was taken by changing the concentration. Low values of SD and % RSD obtained after introducing small deliberate changes in the developed HPLC method indicated the robustness of method.

Ruggedness: The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperature, different days, etc. By using this dilution the absorbance was taken and the further calculation was carried out.

HPLC Analysis

Selection of analytical wavelength: After overlapping the individual spectra of AZA and GYM, 263 nm was selected for simultaneous analysis, at which both the marker shows significant absorbance**.** Graph no 4.

Optimization of HPLC method

Different mobile phases were tried in order to find best condition for separation of AZA and GYM.
Following mobile phases were tried,
1. Water:Methanol (60:40 v/v).
2. Water;Acetonitrile (30:70 v/v)
3. Water:Acetonitrile:Methanol (40:30:30 v/v/v)
4. Methanol:Acetonitrile (75:25 v/v).
5. Methanol:Acetonitrile(80:20 v/v)
6. Methanol:Acetonitrile:(95:5v/v)
7. Methanol:Acetonitrile(60:40 v/v)

Final mobile phase was developed as shown in Graph no 5.

Method validation[12]

Specificity: Extract was analyzed to the specificity of the optimized method in the presence of impurities and other ingredients. The representative chromatograms did not show any other peaks , which confirmed the specificity of method . In extract AZA and GYM eluted at the same retention time even in presence of the other constituents of plant.

Constituents of plant
Azadirachtin - 1.7414
Gymnemic acid -3.3734

Linearity and Range
According to USP extract equivalent to 80, 90,100,110,120 % of test concentration was dissolved in mobile phase,diluted appropriately to obtain a concentration in the range of 80%-120% of the concentration. The calibration plot Azadirachtin and Gymnemic acid follow Beer-Lambert's law, The correlation coefficient, intercept and slope were 0.9992, 0.125, 83644 and 0.994, 0.142, 63501 for Azadirachtin and Gymnemic acid respectively. The good correlation coefficient indicates the method is linear over the concentration range. It is shown in table no 1 to 5.

LOD and LOQ: Limit of detection is the lowest amount of an analyte that can be detected but not necessarily as an exact value. Limit of quantification is the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The LOD and LOQ were separately determined which is based on calibration curve. The S.D. of y intercept of regression line may be used as S.D.
LOD = 3.3 xσ/s
LOQ-10xσ/s
Where, σ = Standard deviation of y intercept of regression lines,
S = Slope of calibration curve.

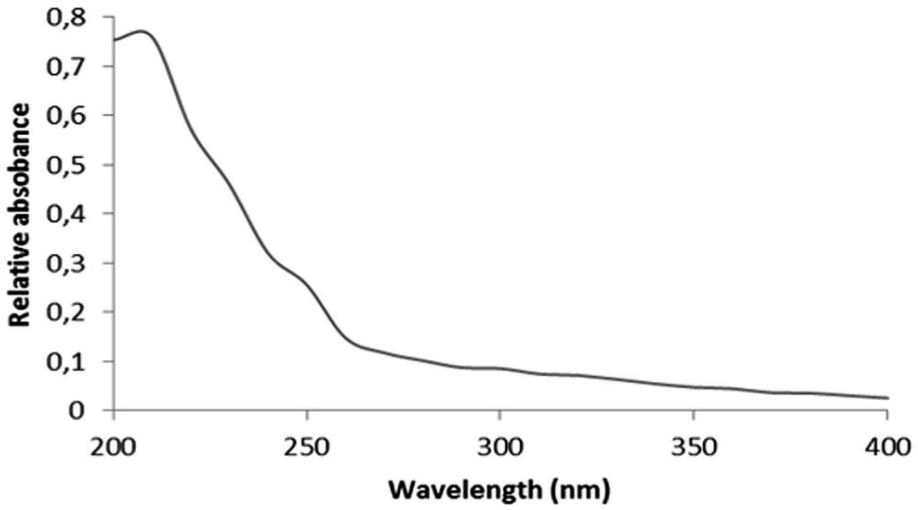
The LOD was found to be 0.26 µg /mL and LOQ was found to be 0.804µg /mL for Azadirachtin and LOD was found to be 0.25ug /mL and LOQ was found to be 0.78µg /mL for Gymnemic acid, which indicates adequate sensitivity of method. It is shown in table no 5.

Precision
The intraday and inter day's precision study of AZA and GYM was carried out by estimating the corresponding responses three times on the same day and on three different days and expressed as % RSD. The results for intraday and interday's precision of extract is shown in table as below. The low values of % R.S.D. (< 2%) for intra and inter day's variation, which suggested an excellent precision of method. It is shown in table no 10.

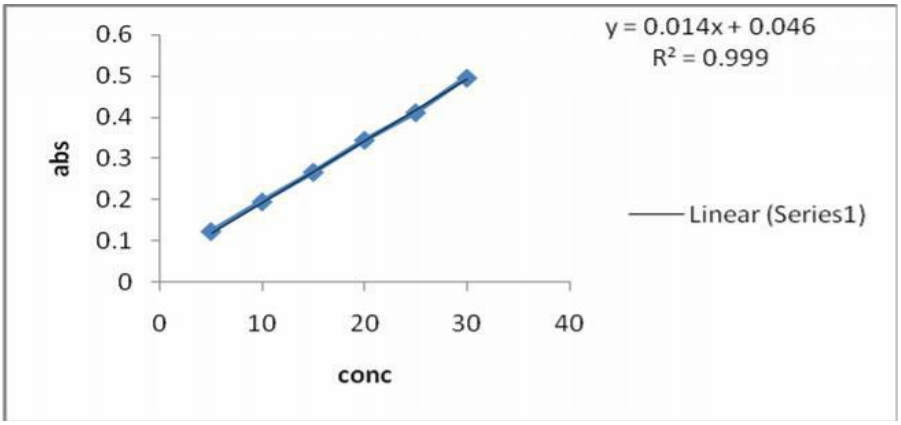
Accuracy
Accuracy of an analytical method is the closeness of test results obtained by the method to the true value. It was ascertained on the basis of recovery studies performed by standard addition method at 50, 100 and 150 % level, known amount of standard drugs were added to analyzed sample and subjected them to the proposed UV method. Results from recovery studies were within acceptable limits 98.26-99.35% and 98.32-99.27% for Azadirachtin and Gymnemic acid respectively indicating accuracy of method was good. It is shown in table no 11.

Robustness: Each factor selected was changed at three different levels. One factor at one time was changed to estimate the effect. The parameter included variation in flow rate, wavelength of detection and composition of mobile phase. Results are presented in table as below. Low values of SD and % RSD obtained after introducing small deliberate changes in the developed HPLC method indicated the robustness of method. It is shown in table no 12.

System suitability Test: This mobile phase gives resolved peaks with symmetry within limits and significant retention time. System suitability parameters were studied in order to determine the suitability of chromatographic system for analysis to be done. It is shown in table no 13.



Graph no 1



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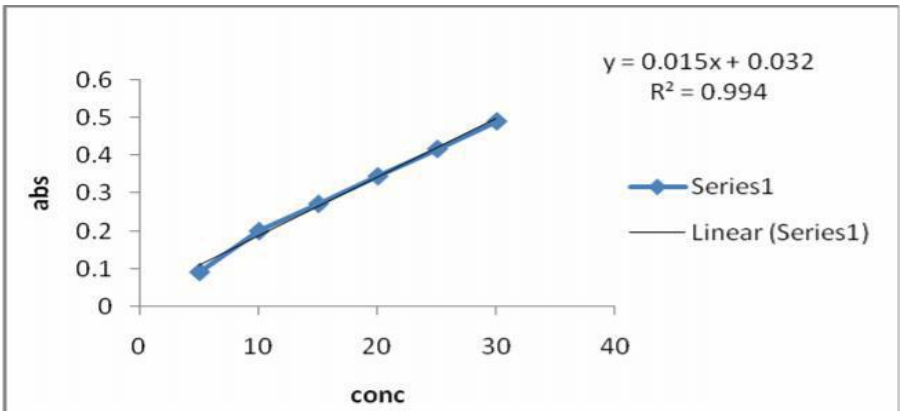

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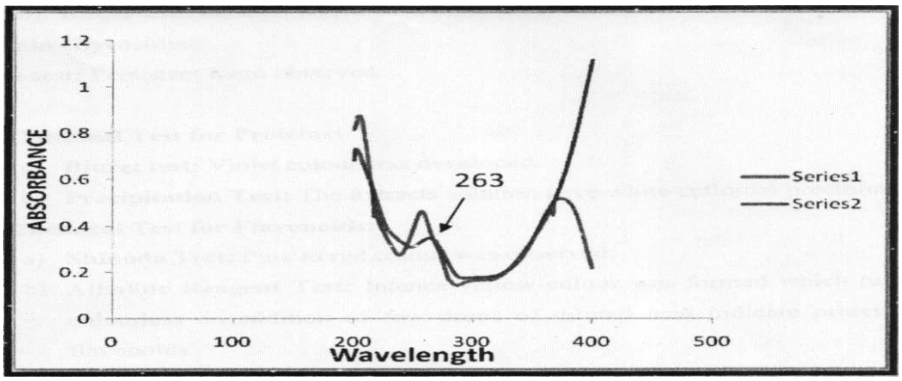
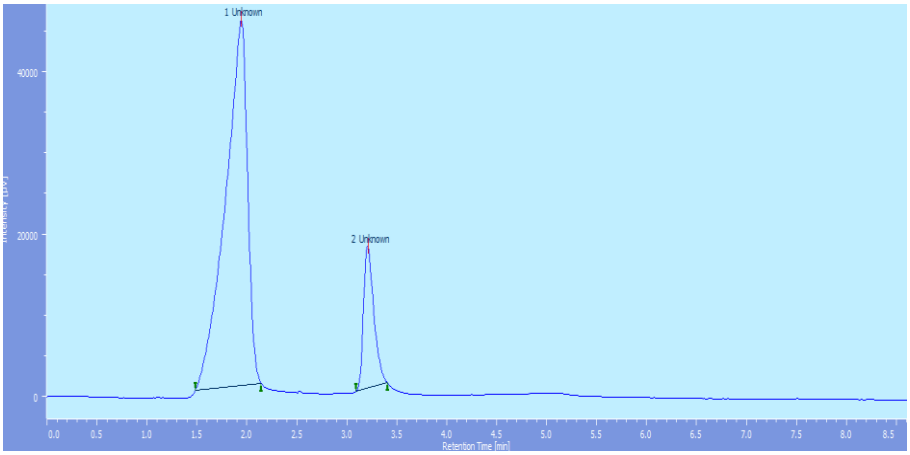


Graph no 4



Graph no 5

Table 1: Absorbance values for calibration curve of Azadirachtin at λmax 210nm

Sr no	Concentration (µg/ml)	Mean absorbance *+SD
1	2	0.112
2	4	0.225
3	6	0.335
4	8	0.445
5	10	0.559
6	12	0.585

Table 2: Absorbance values for calibration curve of Gymnemic acid at λmax 217nm

Sr no	Concentration (µg/ml)	Mean absorbance *+SD
1	2	0.075
2	4	0.145
3	6	0.254
4	8	0.328
5	10	0.417
6	12	0.478

Table 3

Sr no		Conc.gm/100ml	Absorbance		A(1%1cm)
210nm		217nm	210nm		217nm
1	0.0010	0.575	0.456	544.71	456.7
2	0.0020	1.089	0.918	546	459.3
3	0.0030	1.657	1.378	546.3	459.2
4	0.0040	2.156	1.827	544.7	456.7
5	0.0050	2.748	2.295	549.0	459.0
6	0.0060	3.294	2.745	548.1	456.3
Mean		546.6		458.3	
SD		1.9572		1.2721	

Table 4

Sr no		Conc.gm/100ml	Absorbance		A(1%1cm)
254nm		273nm	254nm		273nm
1	0.0010	0.280	0.290	280.0	290.0
2	0.0020	0.558	0.578	279.0	289.0
3	0.0030	0.832	0.860	277.3	286.6
4	0.0040	1.110	1.418	277.5	287.6
5	0.0050	1.388	1.435	277.6	287.0
6	0.0060	1.683	1.741	280.6	290.1
Mean		278.6		288.2	
SD		1.4130		1.6055	

Table 5

Sr no	Parameter	Azadirachtin	Gymnemic acid
1	Slope	0.014	0.015
2	Intercept	0.046	0.032
3	R2	0.999	0.994
4	Range	2 µg/ml-20 µg/ml	4µg/ml-36µg/ml

Table 6: Linear regression data for calibration curve of Azadirachtin and Gymnemic acid

Recovery of Azadirachtin

Spiked Level (%)	Mean % Recovery*	SD	% RSD
50	99.39	0.1705	0.171
100	98.50	0.5701	0.578
150	99.50	0.0812	0.08

Recovery of Gymnemic acid

Spiked Level (%)	Mean % Recovery	SD	% RSD
50	99.39	0.1705	0.171
100	98.50	0.5701	0.578
150	99.50	0.0812	0.080

Table 7: Intra and interday´s precision for Azadirachtin

Formulation	Intra-day precision (n= 3)		Inter-day precision (n=3)	
SD of response	% RSD	SD of response	% RSD	
Extract	0.002	0.76	0.0041	0.56

Intra and interday´s precision for Gymnemic acid

Formulation	Intra-day precision (n= 3)		Inter-day precision (n=3)	
SD of response	% RSD	SD of response	% RSD	
Extract	0.001	0.132	0.0014	0.51

Table 8: Result of Ruggedness studies

Drug	Lable claim(µg/ml)	Amount Found (µg/ml)	% lable claim ±SD*
Analyst 1	Azadirachtin	0.007 µg	0.00699	99.95±0.0975
Gymnemic acid	10 µg	10.02	100.6±0.647
Analyst 2	Azadirachtin	0.007 µg	0.0070	100.08±0.427
Gymnemic acid	10 µg	10.03	100.57±0.457

Table 9: Analysis of formulation by simultaneous equation method

Sr no	Drug	Mean*% estimation	SD	%RSD
1	Azadirachtin	99.32%	0.9550	0.962
2	Gymnemic acid	99.65%	0.1573	0.151

Chromatographic parameter for Mixture

Name	R.T.(min)	Therotical plate	Asymmetry	Resolution
Azadirachtin	1.9417	2145	1.52445	0.0000
Gymnemic acid	3.2083	4332	1.15953	6.3084

Linear regression data

Sr no	Parameter	Azadirachtin	Gymnemic acid
1	Slope	0.014	0.015
2	Intercept	0.046	0.032
3	R2	0.999	0.994

Table 10: Intra and interday´s precision for Azadirachtin

Formulation	Intra-day precision (n= 3		Inter-day precision (n=3	
SD of response	% RSD	SD of response	% RSD	
Extract	46370.2	1.78	46499.5	1.43

Intra and interday´s precision for Gymnemic acid

Formulation	Intra-day precision (n= 3		Inter-day precision (n=3	
SD of response	% RSD	SD of response	% RSD	
Extract	29745.9	1.13	40137.4	1.22

Table 11: Recovery of Azadirachtin

Spiked Level (%)	Mean % Recovery*	SD	% RSD
50	98.26	0.31	0.31
100	99.35	0.314	0.314
150	98.45	0.298	0.298

Recovery of Gymnemic acid

Spiked Level (%)	Mean % Recovery	SD	% RSD
50	98.63	0.291	0.291
100	98.35	0.210	0.210
150	99.27	0.221	0.221

Table 12: Robustness evaluation of method of AZA and GYM

Parameter	Azadirachtin		Gymnemic acid	
Retention time	Asymmetry		Retention time	Asymmetry
A: Flow rate				
0.9ml/min-1	1.8424	1.23	3.1478	0.86
1 ml/min-1	1.9417	1.19	3.2083	0.87
1.1 ml/min-1	1.8447	1.19	2.9549	0.88
MeanSD(n=3)	1.8762±023	1.20±0.023	3.1036±0.12	0.87±0.01
B: Wavelength				
256nm	1.8742	1.39	3.4756	0.95
263nm	1.9427	1.19	3.2083	0.87
258nm	1.7434	0.95	2.8948	0.97
MeanSD(n=3)	1.8534±0.15	1.29±0.10	3.1929±0.18	0.93±0.052
C: mobile phase composition				
70:30	1.8546	1.20	3.2541	0.88
60:40	1.9427	1.19	3.2083	0.87
65:35	1.8475	1.23	3.4212	0.85
Mean SD(n=3)	1.8816±0.24	1.20±0.02	3.2945±0.11	0.86±0.015

Table 13: Result of System suitability Test

Sr no	Peak area		R.T.(Min)		Therotical plate		Asymmetry		Resolution
AZA	GYM	AZA	GYM		AZA	GYM	AZA		GYM
1	8364389	6350090	1.94	3.208	2042	4332	1.159	0.88	6.307
2	8364396	6350140	1.92	3.24	2040	4330	1.189	0.876	6.318
3	8364399	6350099	1.943	3.20	2046	4336	1.177	0.867	6.329
4	8364439	63500153	1.924	3.21	2048	4334	1.189	0.878	6.337
Mean	8364427	6350127	1.92	3.26	2044	4332	1.157	0.875	6.319
SD	25.2037	30.7457	0.0074	0.0027	1.3631	2.0945	0.010	0.049	0.0131
% RSD	0.0003	0.0004	0.318	0.0604	0.523	0.0284	0.9238	0.657	0.274

SUMMARY

In spite of the great advances observed in modern medicine in recent as medicines for the treatment of range of diseases. Madhu-Mehantak churna *(MMC)* is one of the polyherbal formulation as an antidiabetic and consist of nine ingredients including *Curcuma longa, Azardichata indica, Emblica officinalis, Syzygium jambulanum,, Gymnema sylvestre, Momordica charantia.*

UV method for simultaneous estimation of Azadirachtin and Gymnemic acid was developed by using the spectrum mode of analysis of SHIMADZU 1800 UV/Visible double beam spectrophotometer. 95 % methanol was selected as a suitable solvent The absorbance range of Azadirachtin and Gymnemic acid was found to be 0.1 to 1.0 and 0.07 to 1. The method, obeys Beer's and Lambert law. Method was validated with the help of parameter as linearity, range, accuracy, precision (intraday and interday's), LOD, LOQ. This method is useful as primary approach for HPLC method development.

HPLC method for simultaneous estimation of Azadirachtin and Gymnemic acid was developed by using HPLC system of JASCO UV-2075 with C18 Intersil, 4.6 (i.d.) x 250 mm column. During optimization of mobile phase number of trials were carried out as Water : Methanol (60:40 v/v), water : Acetonitrile (30:70 v/v), Water : Acetonitrile : Methanol (40:30:30 v/v/v),Methanol:Acetonitrile(75:25 v/v),Methanol: Acetonitrile (80:20 v/v), Methanol: Acetonitrile : (95:5v/v), Methanol: Acetonitrile (90:10v/v), Methanol:Acetonitrile(85:15v/v). Finally this method includes use of C18 Intersil, 4.6 (i.d.) x 250 mm column, mobile phase consist of mixture of methanol:acetonitrile (pH 6.1) in the ratio of 60:40 v/v at flow rate 1ml min". pH of mobile phase was adjusted with OPA. Retention time was found to be 1.9417,3.2083 for Azadirachtin and Gymnemic acid respectively. Theoretical plates was found to be 8210, 7955 for Azadirachtin and Gymnemic acid respectively. Method was validated with the help of parameter as linearity, range, accuracy, precision (intraday and interday's), LOD, LOQ, robustness. The developed HPLC method for Azadirachtin ,Gymnemic

acid and simultaneous estimation of Azadirachtin and Gymnemic acid is simple, accurate, precise, robust.

CONCLUSION

It is concluded that, Azadirachtin and Gymnemic acid identity and its estimation might be sufficient for standardization of *churna.* Any herbal dosage form may contain several constituent may have impossible and tedious work to a certain the quality of such product.

It will be possible to analyze one or each component in each and every crude drug by analytical technique and it will be better to get a satisfactory protocol for correct identification and standardization of the same. The present work done is partial fulfillment of research on analysis of crude material in polyherbal formulation.

FUTURE PROSPECTS

It is necessary to screen phytochemical investigation of marketed antidiabetic polyherbal formulation *Madhu-Mehantak churna* by various method like detailed macroscopy, microscopy, quantitative microscopy, physical determination, proximate chemical analysis, HPTLC fingerprint, estimation of volatile oil principles by gas chromatography, preclinical trial and clinical trial. It is also must to subject same for short term, accelerated, and long term stability study of such herbal dosage form as per ICH guidelines or any other regulatory recommendation. Stability studies may include detailed standardization by all applicable and critical parameters at different conditions. Real time stability study of such herbal dosage form may be carried out by keeping the packs of formulation at 30 ± 2 °c and 65 % relative humidity at an interval of 0, 3, 6, 9, 12 ,18, 24 months for long period. Stability data may give indication of presence of original phytoconstituents, any chemical degradation, microbial contamination, presence of active chemical entity throughout the period.

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