INTERNATIONAL RESEARCH JOURNAL OF PHARMACY
www.irjponline.com ISSN 2230 – 8407
Research Article

DEVELOPMENT AND METHOD VALIDATION OF *AESCULUS HIPPOCASTANUM* EXTRACT

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Article Received on: 05/04/12 Revised on: 02/06/12 Approved for publication: 01/07/12

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ABSTRACT
Aesculus hippocastanum is highly regarded for their medicinal properties in the indigenous system of medicine. The objectives of the present study include the validation of *Aesculus hippocastanum* extract. Authenticated extract of seeds of the plant was collected and the method was developed for the validation. In this the extract was subjected to check the Accuracy, Precision, Linearity and Specificity. For the validation UV spectrophotometer was used. The proposed UV validation method for the extract is accurate, linear, precise, linear, specific and within the range. Further isolation and in-vitro studies are needed.
Keywords: *Aesculus hippocastanum*, Accuracy, Precision, Linearity, Specificity.

INTRODUCTION

Medicinal plants have been a major source of therapeutic agents or alleviation or cure human diseases since from longer time. According to an all India ethno biological survey carried out by the Ministry of Environment & Forests, Government of India, There are over 8000 species of plants being being used by the people of India.[1]. Not many of Indian herbal product are available in standardization form, which is the minimum requirement for introducing a product in the western market. So effort should be made to promote safety quality, integrity, standardization, validation of standard method in written document form and authenticity of the herbal products to match the requirements of WHO guidelines, which would inevitably help in raising the acceptance of Indian plant based products globally. New a days R&D thrust in the pharmaceutical sector is focused on validation of standard method. *Aesculus hippocastanum* belongs to family Hippocastanaceae also called as Horse Chest nut[2], indigenous to western asia, spreading to Europe and now widely cultivated around the world as an ornamental[2]. The major phytoconstituents of drug under study are saponins. Plant material containing saponins have long been used in many parts of the world for their detergent properties. The major phytoconstisuents present in *Aesculus hippocastanum* are, Saponins of $\hat{a}$-amyrin type known as aescin[3]and $\hat{a}$-aescin. The other phytoconstituents present are Flavonoids, tannins, tannis, and oligosaccharides[4]. Validation is a concept that has been evolving continuously, its first formal appearance is in the United States in 1978[5]. The validation of an analytical procedure is the process of confirming that the analytical procedure employed for a test of pharmaceutics is suitable for its intended use. In other word, the validation of an analytical procedure requires to demonstrate scientifically that risks indecision by testing caused by errors from analytical steps are acceptably small[6]. Validation is a basic requirement to ensure quality and reliability of the results for all analytical applications[7]. There are two important reasons for validating assays in the pharmaceutical industry. The first, and by for the most important, is that assay validation is an integral part of the quality – control system. The second is that current good manufacturing practice regulation requires assay validation[8].

Benefits of Method validation[9]

A fully validated process may require less in – process control and end product testing.It deepens the understanding of processes, decrease the risks of processing problems, and thus assure the smooth running of the process.

Steps in method validation[10]

Develop a validation protocol or operation procedure for the validation.
Define the application, purpose and scope of the method.
Define the performance parameter and acceptance criteria.
Define validation experiments.
Verify relevant performance characteristic of equipment.
Quality materials (for e.g. standard and reagents).
Perform pre – validation experiments.
Adjust method parameter or / and acceptance criteria if necessary.
Perform full internal and external validation equipments.
Developed SOP for executing the method in the routines.
Define criteria for revalidation.
Define type and frequency of system for suitability test and / or analytical quality controa (AQC) for the routine.
Document validation experiment and result in the validation report.

The international Conference on Harmonization (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use[11]. Has developed a consensus text on the validation of analytical procedures. The document includes definitions for eight validation characteristics. An extension with more detailed methodology is in preparation and nearly completed[12].The United States Food and Drug Administration (US FDA) has proposed guidelines on submitting samples and analytical data for methods validation[13-15]. The Untied States Pharmacopoeia (USP) has published specific guidelines for method validation for compound evaluation[16].To validate biological fluids there are no official guidelines. The pharmaceutical

industry uses methodology published in the literature.[17, 18] The most comprehensive document was published as the 'Conference Report of the Washington Conference on Analytical Methods Validation : Bioavailability, Bioequivalence and Pharmacokinetic Studies held in 1990 (sponsored by the American Association of Pharmaceutical Scientists, the AOAC and the US FDA, among others)[18]. The report present guiding principles for validation of studies in both human and animal subjects that may be referred to in developing future formal guidelines.

MATERIALS AND METHODS

Instruments and Materials Instrument used was Shimadzu UV – 1700 UV visible spectrophotometer. The standard extract of Aesculus hippocastanum was collected from Pharmed medicare Banglore. The sample used was extract of Aesculus hippocastanum. All the apparatus, instruments and glass wares were calibrated before starting of experimental work.

Preparation of standard Standard solution of concentration 1 mg/ ml was prepared with water in 100 ml volumetric flask using standard extract of Aesculus hippocastanum. From this stock, throught a series of dilutions, working standards were prepared of concentration 30, 40, 50, 60, 70, 80, 90, 100 $\mu g/ml$ with water methanol in 10 ml volumetric flasks. These dilutions were scanned on a UV spectrophotometer between the wavelength 200-400 nm. Absorbance maxima wavelength for extract of Aesculus hippocastanum selected at 265 nm for the current study.

Preparation of Sample Accurately weighed 10 mg extract of Aesculus hippocastanum was taken in 50 ml volumetric flask with sufficient quantity of water and sonicated for 5 min. Volume was made up to 50 ml with water. The resulting solution was then filtered. From the filtered stock 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 ml of solution was taken in five separate 10 ml volumetric flasks and volume is made up to 10 ml with water. The absorbance of sample was recorded at 265nm using water as a reference.

Validation of the method

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics are expressed in terms of analytical parameters.

Design of experiment

Typical analytical parameters used in assay validation are :

a) Linearity / Beer's Law Range
b) LOQ (Limit of Quantification) and Standard calibration curve
c) Precision
d) Sensitivity
e) Accuracy

RESULTS AND DISCUSSION

Development of method

The UV spectrophotometric method for estimation of *Aesculus hippocastanum* extract has been developed. As the extract is purely soluble in water, it is selected as the best suitable solvent for estimation.

The wavelength maxima ($\ddot{a}_{max}$) is the peak maxima in the serial dilutions of working standards at the constant wavelength. For estimating *Aesculus hippocastanum* extract $\ddot{a}_{max}$ is selected at 265 nm (fig 1).

Determination of ^{0} (1 % l cm) of Aesculus hippocastanum extract at selected wavelength :

The absorbance for the standard dilutions of *Aesculus hippocastanum* extract was measured at 265 nm. From the absorbance readings the absorptivity (^{0}) of *Aesculus hippocastanum* extract was calculated with equation. ^{0} = A/C .... Eqn. 1,

Where A is absorbance and C is concentration in g/ 100 ml. The absorptivity *of Aesculus hippocastanum* extract at 265 nm was 16.39 $mole^{-1}$ cm^{-1} shown in table no 1.

Development of Equation for estimating *Aesculus hippocastanum* extract

The equation was developed for calculating the concentration of extract.

C_{xx} = A/ ^{0} Eqn.2

Where, A is absorbance of sample at 265nm, ^{0} is absorptivity of *Aesculus hippocastanum* extract at 265 nm. C_{xx} is concentration of extract in g/ 100 ml respectively. The equation 2 was modified for calculating the concentration of *Aesculus hippocastanum* extract in sample as c_{xx} = A/ 16.39. The assay calculation was shown in table no 2.

Validation of method

Linearity / Beer's Law Range

Determination of Linearity range for *Aesculus hippocastanum* extract shown in table no 3.

Linearity report of *Aesculus hippocastanum* extract shown in table no 4

Beer's law concentration range is shown in fig 2

Limit of detection (LOD)

Report : The Limit of detection for *Aesculus hippocastanum* extract was found to be 0.5 mcg/ml and Limit of Quantification was found to be l mdg/ml shown in table no 5.

Precision

Precision of the instrument

Instrument precision Reports of *Aesculus hippocastanum* extract is shown in talbe no 6

Report

From the precision data of the instrument, the RSD for Aesculus hippocastanum extract was 1.823704% which is well within the acceptance criteria of not more then 2% . Hence the precision of the instrument is found to be satisfactory.

Precision of the method :

Method precision Report of *Aesculus hippocastanum* extract

Report

From the precision data of the method, the RSD for Aesculus hippocastanum extract was 0.1136% and which is within the acceptance criteria of not more then 2% . Hence the precision of the method is found to be satisfactory. Shown in table no 7

Sensitivity

Determination of sensitivity of *Aesculus hippocastanum* extract

Report

From the sensitivity data, sandell's sensitivity for Aesculus hippocastanum extract was found 0.061317. The results were in agreement as the sensitivity was found to be within he permissible limits. Shown in table no 8.

Accurany

Determination of accuracy

In case if assay of a drug substance accuracy may be determined by application of the analytical method to an analyte of known purity (e.g. reference standard) or by

comparison of the results of the method with those of a second well characterized method, the accuracy of which has been stated or defined. Accuracy is calculated as the percentage of recovery by the assay of the known added amount of analyte in the sample, or as the difference between the mean and the accepted true value, together with confidence intervals.

The ICH documents recommended that accuracy should be assessed suing a minimum of nine determination over a minimum of three concentrations levels, covering the specified range (i.e., three concentrations and three replicates of each concentration).

Recovery study data of *Aesculus hippocastanum* extract

The recovery calculation method is shown in table no 9

Recovery report for *Aesculus hippocastanum* extract

From the recovery data, the recovery for Aesculus hippocastanum extract was found in the range of 94.56 to 102.5%. The results were in agreement as the % recovery was found to be within he permissible limits of 92 – 108%. Shown in table no 10.

Optical Characteristics of *Aesculus hippocastanum* extract- Shown in table no 11.

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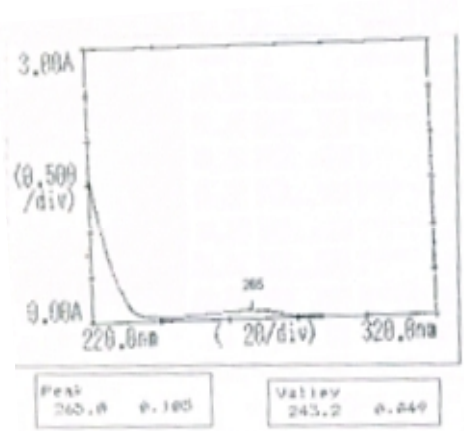
Fig 1



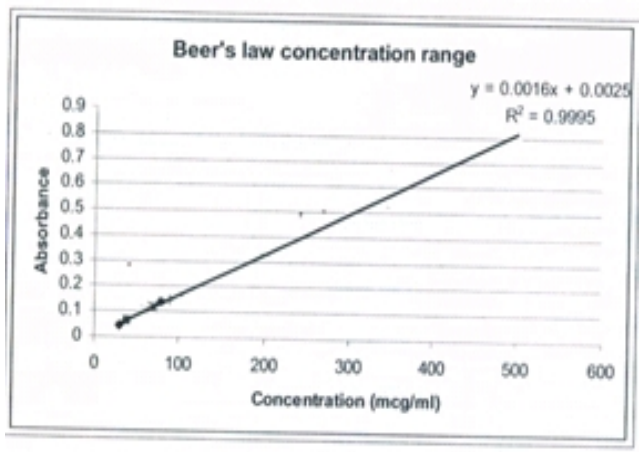


Fig 2 Beer's law concentration range

Table 1: Absorptive of *Aesculus hippocastanum* extract

Sl No.	Conc. Of Working Standard ($\mu g/ml$)	A	A $mole^{-1}cm^{-1}$
1	30	0.045	15
2	40	0.063	15.75
3	50	0.078	15.6
4	60	0.108	18
5	70	0.119	17
6	80	0.135	16.875
7	90	0.148	16.444
8	100	0.165	16.5
		Avg	16.39

Table 2: Assay of Sample *Aesculus hippocastanum* extract

Sl.No	Vol. of Stock Taken (ml)	A	C_{xx}	Theoretical Conc.	% Practical Conc.
1	1.5	0.049	29.896	30	99.65426
2	2	0.061	37.217	40	93.04454
3	2.5	0.079	48.200	50	96.40024
4	3	0.104	63.453	60	105.7555
5	3.5	0.121	73.825	70	105.465
6	4	0.141	86.028	80	107.5351
7	4.5	0.155	94.569	90	105.0776
8	5	0.169	103.11	100	103.1117

Table 3 : Linearity data for *Aesculus hippocastanum* extract

Sl. No	Volume of *Aesculus hippocastanum* extract stock solution (ml)	Final Volume (ml)	Concentration µg/ml	Absorbance*
1	0.3	10	30	0.045
2	0.4	10	40	0.063
3	0.5	10	50	0.078
4	0.6	10	60	0.108
5	0.7	10	70	0.119
6	0.8	10	80	0.135
7	0.9	10	90	0.148
8	1.0	10	100	0.165
9	2.0	10	200	0.328
10	3.0	10	300	0.502
11	4.0	10	400	0.651
12	5.0	10	500	0.809

*Average of six readings

Table no 4 Linearity report

Parameter	Result observed	Acceptance criteria
Correlation co – efficient	0.9995	Not less than 0.99%
Percentage curve fitting	99.95	Not less than 99.7%
Regression Equation (Y)	0.0016x + 0.0025	-
Slope (b)	0.0016x	-
Intercept (a)	0.0025	-

Table 5 : Limit of detection for *Aesculus hippocastanum* extract.

Sl. No	Dilution volumes From 2nd stock Solution (ml)	Concentration meg / ml	Absorbance
1	2 ml	2	0.003
2	1 ml	1	0.001
3	0.5 ml	0.5	-

Table 6 – Instrument precision report

Parameters	Result observed	Acceptance criteria
Relative standard deviation for the method	1.823704	Not more than 2%

Table no 7 – Precision report of method.

Parameters	Result observed	Acceptance criteria
Relative standard deviation For the method	0.1136	Not more than 2%

Table no 8 - Determination of sensitivity of *Aesculus hippocastanum* extract

Sl.No	Conc. (mcg / 100 ml)	Absorbance	Sandell's Sensitivity
1	3000	0.045	0.066667
2	4000	0.063	0.063492
3	5000	0.078	0.064103
4	6000	0.108	0.055556
5	7000	0.119	0.058824
6	8000	0.135	0.059259
		Avg	0.061317

Table no 9- Recovery study data of *Aesculus hippocastanum* extract

Sl. No	Std *Aesulus hippocastanum* extract Conc (A) µg / ml	Samp *Aesculus hippocastanum* extract Conc . (B) µg / ml	Total Conc. (A + B) µg / ml	Absorbance	Total Amount (A + B) µg / ml	Amount Of std. Recovered	% Recovery Of standard
1	10	20	30	0.048	29.28615	9.29615	97.62
2	20	20	40	0.062	37.82794	17.82794	94.56
3	30	20	50	0.084	51.25076	28.25076	93.35

Table no 10- Recovery report.

Parameters	% recovery of *Aesculus hippocastanum* extract	Acceptance criteria
Level 1	97.62	92-108%.
Level 1	94.56	92-108%.
Level 1	93.35	92-108%.

Table no 11 - Optical Characteristics of *Aesculus hippocastanum* extract

Sl.No	Parameters	MS
1	Beer's Law limits (i g/ml)	30.500
2	Wavelength maxima (nm)	265
3	Molar extinction coefficient ($mole^{-1}$ cm^{-1})	16.39
4	Sandell's sensitivity (i g/cm^2 /0.001 absorbance units)	0.061317
5	Regression Equation (Y) Slope (b) Intercept (a)	0..0016x + 0.0025 0.0016x 0.0025
6	Coefficient of Variance	0.9995

Source of support: Nil, Conflict of interest: None Declared