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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR STRYCHNINE IN NUX VOMICA CONTAINING FORMULATIONS

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ABSTRACT

A selective, simple and efficient method-Reverse Phase High - performance liquid chromatography with UV detector was developed for the determination of active alkaloid, namely strychnine in Homeopathic formulations. The RP- HPLC separation was carried out using a Bischoff-chrombudget C18 (250mm x 4.6mm x5µm) column with a mobile phase consisting of Acetonitrile: Phosphate buffer pH-3.6 with 0.2%TEA and 0.02% W/V SLS(55:45) hence providing high efficiency, high resolution and excellent peak shape for the analyte standard. The method was validated over the range of 1-6 µg/ml for strychnine. Accuracy is determined by parallel Spiking method instead of % recovery method because of very low concentration of an analyte in the formulations that can not be detected and %RSD was 1.9844. Interday precisions and Intraday precisions were within range. With a simple and minor sample preparation procedure and short run-time (<3 min), the proposed method was applicable for the quantitative analysis of strychnine in various homeopathic formulations.

KEYWORDS: Validation, RP-HPLC, Strychnine, Homeopathic formulations.

INTRODUCTION

The plant kingdom is widely distributed into the world, about 2,50,000 species of pants recognized. These are some medicinal systems like Ayurveda, Siddha, Unani used traditionally since many countries.[1] Strychnos nux vomica L. (Loganiaceae) introduced in Chinese folk medicine used for inflammation, joint pains, allergic symptoms and treat

nervous diseases,[2] Nux Vomica was very reluctantly introduced into the European pharmacopoeia [3], Also placed in Japanese Pharmacopoeia (JP17) Database.[4] However, there are some active substances which are secondary metabolites or alkaloids are synthesized by the plants to save them from herbivorous animals and insects while high dose of the active alkaloids can induce convulsions of the central nervous system and finally death through respiratory or spinal paralysis or by cardiac arrest.[1][5] The lethal dose of strychnine is 10-100 mg. However, In some case 10-30 mg and for pediatrics LD 50 founded is 5-10 mg. The active dose of strychnine results into competitive blocks on the neuroinhibitory transmitter glycine at postsynaptic sites.[6][7]

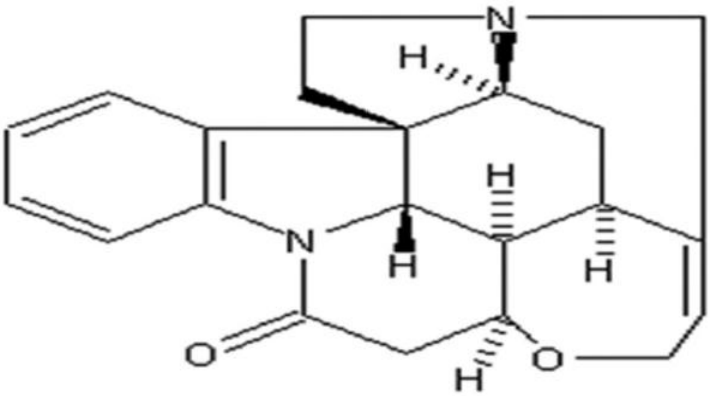

Fig-1.:-Structure of strychnine [8]

Mother tinctures are liquid preparations obtained by the solvent action of a suitable vehicle upon raw materials. The raw materials are usually in the fresh form but may be dried. Mother tinctures for homeopathic preparations may also be obtained from plant juices, with, or without the addition of a vehicle. For some preparations, the matter to be extracted may undergo a preliminary treatment.[9] Since homeopathy has become very popular as alternative or complementary medicine in many diseases, homeopathic tinctures need to be characterized following the regulations of the European Agency for the Evaluation of Medicinal Products of plant extracts.[10] Several analytical methods for quantitative determination of strychnos alkaloids have been described, including HPLC with UV detection, TLC, fluorescence spectrophotometric method , 1H NMR, GC–MS , LC–MS and LC–MS/MS ,etc.[11] However, few reports are available regarding determination of strychnine in mother tincture.

MATERIALS AND METHODS

Instuments

The pH of the mobile phase was checked on a pH/ion analyzer(Lab India PHAN, India). Instruments used in the study were For RP-HPLC SHIMADZU LC-20AT Prominence was used. Detection was performed with SHIMADZU SPD-20A Prominence UV/VIS Detector. The sample was injected using Rheodyne 7725 injector valve with a fixed loop at 20µl. Data acquisition and integration were performed using Spinchrom software (Spincho biotech, Vadodara). An ultrasonicator DTC 503 (Ultrasonics Selec, Vetra, Italy) was used for degassing the mobile phase.

Chemicals and Reagents

Stychnine gifted from Bakson drugs & pharmaceuticals Pvt. Ltd. India was subjected to Ultra-violet (UV), Infrared Proton spectral analysis to confirm their identity and purity. HPLC grade acetonitrile and methanol were purchased from Thermo-fisher scientific. Potassium dihydrogen phosphate AR (Analytical Reagent) and Triethylamine.Of HPLC grade were procured from Qualigens Fine Chemicals (Mumbai, India). Sodium Laurayl Sulfate.obtained from Sigma–Aldrich (St. Louis, MO, USA).

Preparation of standard solutions

A stock solution of strychnine 1 mg/ml was prepared by dissolving 10mg in 10 ml methanol. Primary Standard solution was prepared by withdrawing 1 ml aliquots of stoke solution diluted up to 10 ml with methanol. Working standard solutions of strychnine (1-6 µg/ml) was prepared by withdrawing suitable aliquots of corresponding primary standard into 10 ml volumetric flask and making up to the mark with mobile phase.

Sample preparation

To an aliquot 1 ml/20 ml(for mother tincture/ formulations), diluted to 20 times in water, Take this solution in separating funnel. The mixture was extracted with 20 ml of chloroform by thoroughly shaking for 3 min. After separation for 5min, the chloroform layer was taken and evaporated to dryness. The residue was reconstituted in 5 ml of Acetonitrile. The samples were further diluted and analyzed.

Chromatographic Condition

Chromatographic separation of strychnine obtained by Bischoff-chrombudget C18 (250mm x 4.6mm x5µm)column. The mobile phase used Acetonitrile: Phosphate buffer pH-3.6 with

0.2%TEA and 0.02% W/V SLS (55:45), filtered through a 0.2µm Nylon filter and degassing was performed by using ultrasonicator by 3 cycles of 5 minutes. Mobile phase pumping through the column was performed at 1ml/min, injection volume 20µl. The detection wavelength was selected as 254 nm. The temperature was Ambient.

Method Validation

A stock solution of the Strychnine was prepared at the strength of 1 mg/ml. It was diluted to prepare solutions containing1-6 µg/ml of strychnine. The solutions were injected in triplicate into the HPLC column, keeping the injection volume constant (20µl). Five injections, of three different concentrations (1,3 and 6 µg/ml), were given on the same day and the values of percentage relative standard deviation (R.S.D) were calculated to determine intra-day precision and these studies were also repeated on different days to determine inter-day precision. Accuracy was evaluated by the parallel spiking method. The specificity of the method was ascertained by analyzing standard strychnine and then comparing the sample retention time (Rt) of Strychnine in the homeopathic tincture with the Rt of the standard. To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution was recorded. The flow rate of the mobile phase was 1 ml/min.

RESULTS AND DISCUSSION

Linearity and sensitivity

The six-point calibration curves were found to be linear over the concentration 1-6 µg/ml for Strychnine(Table-3.1 and Figure-3.1,3.2). The linear equations for Strychnine was y = 41.448x + 0.2593 (R^2 = 0.9992). The LOD and LOQ for Strychnine 0.126 µg/ml And 0.383µg/ml respectively.

Table 3.1-Linearity of Strychnine

Concentration (µg/ml)	Area
1	42.334
2	81.163
3	123.83
4	170.054
5	206.796
6	247.796

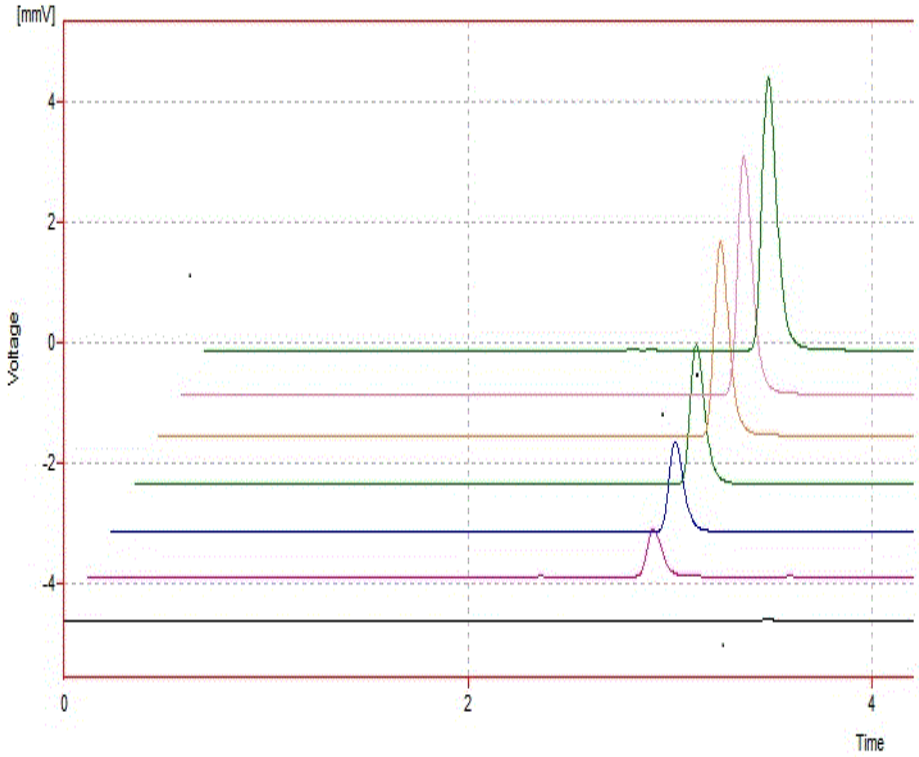

Figure-3.1: 3D chromatogram showing peaks of strychnine (1-6 ug/ml)

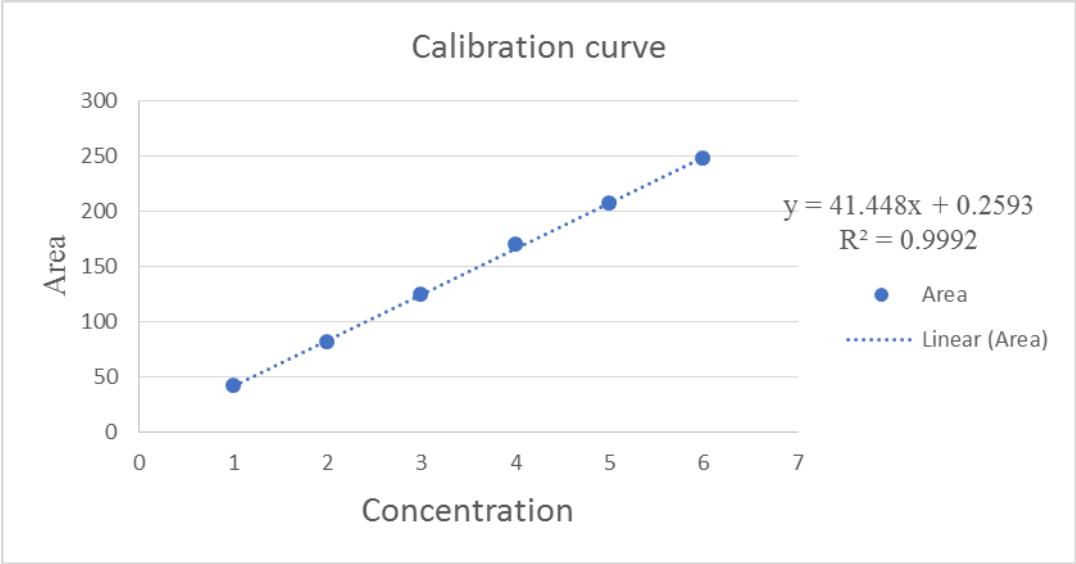

Figure -3.2: Calibration curve of strychnine (1-6 ug/ml)

Accuracy

Accuracy is determined by parallel Spiking method instead of % recovery method because of very low concentration of an analyte in a formulation that can not be detected The theoretical recovery of the target analyte from the spiked material is the sum of the amount of added

analyte plus an amount of naturally occurring analyte. The difference between the theoretical amount and the amount analytically determined in the spiked matrix provides and an estimate of accuracy.[12] The percentage RSD were calculated from the difference in area of linearity (Table-3.2) and area of spiked linearity shows parallel graph(figure-3.3) with regression value = 0.9992 and the results was satisfactory hence the method is accurate.

Table 3.2- Spiked Linearity of Strychnine

Concentration(µg/ml)	Area of linearity	Area of spiked linearity	Difference In Area
1	42.334	47.186	4.852
2	81.163	86.17	5.004
3	123.83	128.8	5.018
4	170.054	175.1	5.071
5	206.796	211.7	4.857
6	247.796	252.7	4.861
		Average	4.9438
		Standard deviation	0.0981
		%RSD	1.9844

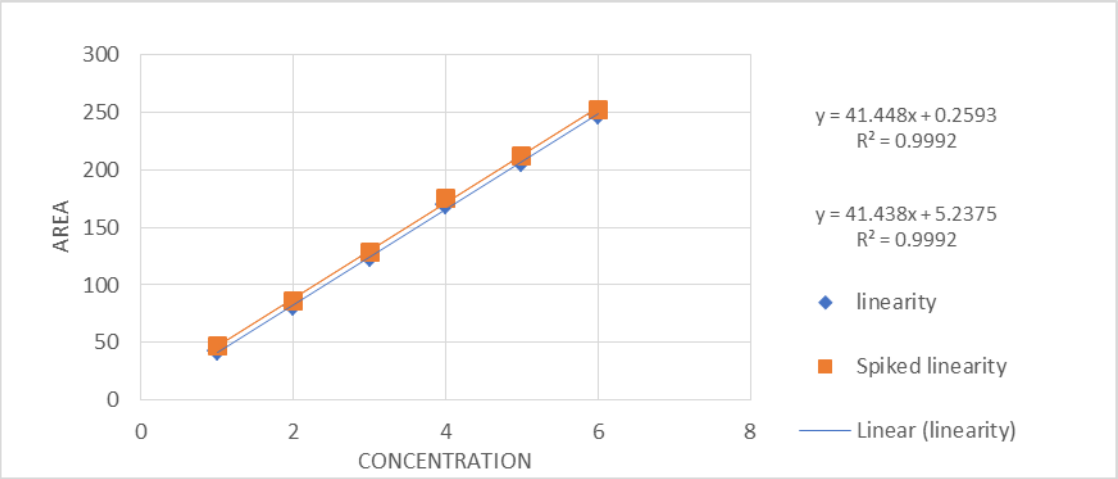

Figure-3.3: Spiked Linearity of Strychnine

Precision

Precision results represented in table 3.3.

Table 3.3 -Precision of Strychnine

Concentration (ppm)	Intraday Precision Area		Interday Precision Area	
	Mean±SD	%RSD	Mean±SD	%RSD
1	43.3866±0.6413	1.4782	42.6616±0.1743	0.4087
3	123.2523±0.0738	0.0599	123.467±0.2601	0.2106
6	250.2337±1.0528	0.4207	250.5847±1.5355	0.6127

Robustness

Robustness is measured by making small changes in method parameters. The method is considered robust when it remains unaffected by small changes in parameters. Three factors are varied at 3 levels which include (a) Flow rate (0.9, 1, 1.1), (b) Ratio of Acetonitrile in mobile phase (50%, 55%, 60%), (c) Detection wavelength (252nm, 254nm, 256nm)(Table-3.4). One factor was changed at a time. 3 replicate injection of bulk drug mixture at 3 consecutive levels were injected. Results were reported in terms of Relative Standard Deviation (RSD).

Table 3.4-Robustness of Strychnine

Factors		Mean Rt (min)	%RSD of Rt	Mean Area (mV.s)	%RSD of Area
Flow Rate (ml/min)	0.9	3.1043	0.0743	135.4704	1.1481
	1.0	2.6733	0.6504	123.7707	0.8657
	1.1	2.57	0.3891	114.106	0.3148
Ratio of ACN.(ml)	50	3.1505	0.8090	123.2483	0.7569
	55	2.6733	0.6504	123.7707	0.3148
	60	2.3353	0.2487	123.21	0.7090
Wavelength (nm)	252	2.797	0	121.432	0.8269
	254	2.6733	0.6504	123.776	0.3148
	256	2.803	0	108.0183	0.4102

Limit of detection (LOD)

Limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected but not definitely quantitated, under the asserts experimental conditions.

The detection limit (DL) may be expressed as:

$$DL = 3.3\sigma/S$$

Where, σ= standard deviation of the response

S=slope of the calibration curve.

The parameter LOD was determined on the basis of response and slope of the regression equation. The LOD for this method was found to be 0.126 µg/ml(Table-3.5).

Limit of quantification (LOQ)

LOQ is the smallest amount of the analyte in a sample matrix that can be quantified with acceptable accuracy and precision under the stated experimental conditions. It is expressed as the concentration of an analyte (e.g. parts per million) in the sample.

The Quantitation limit (QL) may be expressed as:

$$QL = 10\sigma/S$$

Where σ= standard deviation of the response

S=slope of the calibration curve.

The parameter LOQ was determined on the basis of response and slope of the regression equation. The LOQ for this method was found to be 0.383µg/ml (Table-3.5).

Table-3.5: Summary of validation parameters

PARAMETER	RESULT
Analytical Wavelength	254 nm
Retention time	2.79Min
Linearity Range	0.1 to 6 ppm
Regression Equation	y = 41.448x + 0.2593
Correlation Coefficient	0.9992
LOD((µg/ml)	0.126 µg/ml
LOQ(µg/ml)	0.383µg/ml
Intraday precision(%RSD)	0.090101
Interday precision(%RSD)	0.068898954

APPLICATION OF METHOD IN MARKETED FORMULATIONS

The method was applied in marketed formulations labelled as nux vomica 2x/D3 along with other constituents and the results were represented in table 3.6.

Table-3.6: Quantitation of strychnine in formulations

Product	Concentration of strychnine present
Formulation -1 (mother tincture)	**367.925** µg/ml
Formulation-2 (Nux Vomica 2x with other constituents)	**3.89** µg/ml
Formulation-3 (Nux Vomica D3 with other constituents)	**3.1793** µg/ml

CONCLUSION

From the above experiment, the developed method was found to be sensitive, specific, robust, and inexpensive. The extraction procedure is simple and effective; the measurement range is sufficient to determine active concentrations for strychnine. This simple method is applicable in most of the laboratories equipped with HPLC. This paper includes the analysis of strychnine in homeopathic formulations which contains other active constituents as well as strychnine mention in their dilutions concentration but not in definite µg/ml concentration level.

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