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RESEARCH ARTICLE

Standardization of Agnitundi Vati: An Ayurvedic Polyherbal Formulation

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ABSTRACT

Standardized herbal products of consistent quality and containing well defined constituents are required for reliable clinical trials and to provide consistent beneficial therapeutic effects. Pharmacological properties of an herbal formulation depend on phytochemical constituents present therein. Development of authentic analytical methods which can reliably profile the phytochemical composition, including quantitative analyses of marker/bioactive compounds and other major constituents, is a major challenge to scientists. Agnitundi Vati is an Ayurvedic tablet used in indigestion and fever. In the present work, attempt has been made to develop a chromatographic method for standardization of Agnitundi vati. Strychnine in *Strychnos nux vomica* and Gallic acid in *Terminalia chebula*, *Terminalia bellirica*, *Emblica officinalis* are selected as marker compounds. A simple, rapid, precise, accurate and reproducible high performance thin layer chromatographic (HPTLC) method was developed for quantification of strychnine and Gallic acid in Agnitundi vati. The proposed method was validated as per ICH guidelines.

KEYWORDS

Agnitundi Vati, Strychnine, Gallic Acid, HPTLC, Polyherbal Formulations, ICH

INTRODUCTION

Herbal medicine has been enjoying renaissance among the customers throughout the world, however one of the impediments in the acceptance of the Ayurvedic formulations is the lack of standard quality control profiles. Ayurvedic herbal formulations are generally available as single or mixture of more than one plant constituents.[1] Standardization is a system to ensure that every packet of medicine that is being sold has the correct amount and will induce its therapeutic effect. Standardization of natural product drugs, is based on chemical entities, "marker compounds" which are chemically defined constituents may or may not have therapeutic activity and are of high significance for control purpose.[2] Modern techniques such as HPTLC, HPLC, and GC etc. can be used to develop the methods for the quantification of marker compounds in these types of multicomponent herbal formulations[3].

Agnitundi Vati is an Ayurvedic tablet used in indigestion and fever. As the name suggests Agni means Digestive fire, hence the one which improves Digestive fire. It is Effective for gas problems, flatulence, all types of colicky pains, pains related to Vata imbalance. It contains Haritaki (*Terminalia chebula*), Chitrak (*Plumbago zeylanica*), Bibhitaki (*Terminalia bellirica*), Ajmoda (*Apium leptophyllum*),

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Amalaki (*Emblica officinalis*), Cumin (*Cuminum cyminum*), Vidang (*Embelia ribes*), Kuchala (*Strychnous nuxvomica*), Vatsanabh (*Aconitum ferox*)[4].

Strychnine is colorless, bitter crystalline alkaloid chemically known as strychnidin-10-one.Strychnine is a neurotoxin which acts as an antagonist of glycine and acetylcholine receptors. It primarily affects the motor nerves in the spinal cord which control muscle contraction[5]. Strychnine is also an important ingredient of homeopathic medication and is particularly recommended for digestive problems, feeling for cold as well as tetchiness. Powdered seeds of strychnine are used to treat atonic acid indigestion (dyspepsia), the tincture prepared with strychnine is frequently used in amalgams to invigorate the gastro-intestinal tract[6].

Gallic acid is chemically known as 3,4,5-trihydroxybenzoic acid ($C_6H_2(OH)_3COOH$ exist in two forms as the free molecule and as part of tannins. Pure gallic acid is colorless crystalline powder, salts and esters of gallic acid are termed as gallates. Gallic acid is commonly used in pharmaceutical industries. Gallic acid shows cytotoxic activity against cancer cells, without damaging healthy cells and it can be used to treat albuminuria and diabetes, it also has antifungal and antiviral properties, which is used as antioxidant and helps to protect the human cells against oxidative damage. It is also used as standard substance in many antioxidant assays. It has also shown to possess radical scavenging activity against several radicals. All these biological activities have indicated the potential use of gallic acid[7,8.] There are reports on extraction of strychnine and gallic acid using various extraction techniques and their individual estimation from single herbs and polyherbal formulations. However, no analytical method has been reported for simultaneous estimation of strychnine and gallic acid; which can be further applied for standardization of Agnitundi Vati.

The present research work deals with development of HPTLC method for standardization of Agnitundi Vati by detection and quantification of markers Strychnine and gallic acid simultaneously from in-house and marketed formulations. The proposed method was validated on the basis of its linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness according to ICH guidelines.

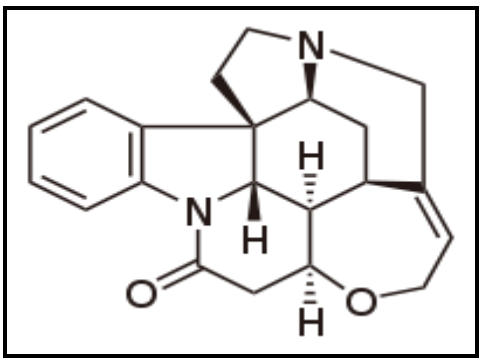

Figure 1: Structure of Strychnine

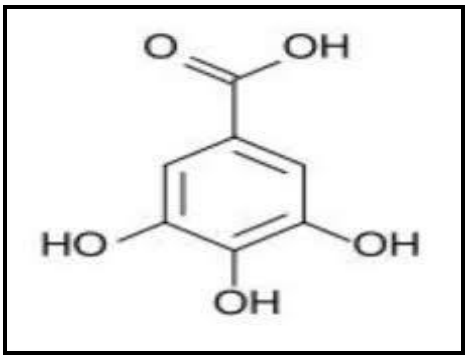

Figure 2: Structure of Gallic acid

MATERIAL AND METHODS

Materials

Raw materials used for the preparation of Agnitundi Vati and three different marketed brands (M-01, M-02, M-03) of Agnitundi Vati were procured from Ayurvedic medical shop, Mumbai and stored in air tight containers at room temperature. The stationary phase used was precoated with silica gel 60 F_{254} (20×20 cm) TLC plates of 0.2 mm thickness obtained from E. Merck Ltd. Mumbai, India.

Standards and Reagents

The organic solvents and chemicals of analytical grade were procured from S.D Fine chemicals Pvt. Ltd. Mumbai, India. Standard Strychnine and gallic acid were procured from Sigma Aldrich Pvt. Ltd. Mumbai, India.

Instrumentation

Camag Linomat 5 semiautomatic sample applicator equipped with a 100µl Hamilton syringe (Camag, Switzerland) and winCATS software (CAMAG Ver.1.4.1), Camag TLC Scanner 3, Twin trough chamber.

Experimental

Preliminary Studies

The quality of raw materials used in the preparation of Agnitundi Vati was assessed by determining the proximate parameters like ash value, extractive value and loss on drying using standard pharmacopoeial methods. Tablet extracts were qualitatively evaluated by chemical tests for the presence of various phytoconstituents like alkaloids, glycosides, saponins, phenolic compounds tannins and phytosterols.

HPTLC Method Development

Preparation of Standard Solution

Stock solutions of Strychnine and Gallic acid (1000µg/ml) were prepared separately by dissolving 10 mg of accurately weighed standard in 10 ml of methanol. From this stock solution 100µg/ml was prepared by transferring 1 ml stock solution to 10 ml volumetric flask and volume was then adjusted with methanol.

Preparation of In-House Formulation

All the ingredients were collected, dried and powdered separately, passed through 100 # sieve and then mixed together in specified proportions in a geometrical manner to get uniform mixture. To which citrus juice (*Citrus aurantium*) was added and grounded well to form a homogenous blend and compressed into tablets. The tablets were dried and packed in air tight containers for further analysis.

Extraction of Strychnine and Gallic Acid from Marketed and In-House Formulations

Vati equivalent to 5g were triturated and extracted with 50 ml methanol, refluxed for 2 hours, filtered through Whatmann filter paper no. 41 and the final volume was then made up to 100 ml with methanol. This solution was used for quantification of Strychnine and Gallic acid.

Chromatographic Conditions

Chromatographic separation was achieved on HPTLC plates (10×10 cm) pre-coated with silica gel 60 F254 of 0.2 mm thickness with aluminium sheet support. Standard solutions of markers and extracts were applied to the plates as bands 6.0 mm wide, 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat 5 sample applicator equipped with a 100µl Hamilton syringe. Ascending development to a distance of 80 mm was performed at room temperature (24 ± 2°C) with mobile phase, in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 30 min. After development, the plates were dried and then scanned at 264 nm with a Camag TLC Scanner 3 using the deuterium lamp with winCATS software.

Optimization of Mobile Phase

Mobile phase composition was optimized to provide accurate, precise and reproducible results for the determination of Strychnine and Gallic Acid. The standard stock solution containing 100µg/ml of Strychnine and Gallic Acid was spotted on to TLC plate and developed in different solvent systems. Many preliminary trials were carried out for selection of mobile phase.

Calibration Curves of Strychnine and Gallic Acid

Serial dilutions were made in the concentration range of 20-80µg/ml of Strychnine and gallic acid. Aliquot of above solutions (10µl) were applied with the band width of 6 mm, in triplicate on TLC plate (10×10 cm) to obtain a concentration range of 200-800 ng/spot for Strychnine and gallic acid. Peak area for each band was recorded. Separate calibration curves were obtained by plotting a graph of peak area vs. concentration of Strychnine and gallic acid.

Assay

For assay purpose standard and sample (extract) solutions were applied on HPTLC plate in

triplicates. Standard solutions of 100µg/ml Strychnine and gallic acid were applied. Extracted solution was directly used for quantification of Strychnine and gallic acid. The amount of Strychnine and gallic acid present per gram of formulation was calculated by comparison of the areas measured for the sample with the calibration curves constructed from peak areas obtained from standard solutions of Strychnine and gallic acid.

Method Validation[9]

In accordance with ICH guidelines Q2 (R1) the optimized HPTLC method was validated with respect to following parameters.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample. It was determined by plotting a graph of peak area v/s concentration of standards to obtain correlation coefficient (r^2) and equation of the line.

Specificity

Specificity is the ability to assess the analyte in the presence of components that may be expected to be present in the sample matrix. The specificity of the method was ascertained by comparing the R_f value and the peak purity was assessed by comparing the spectrum of standard with Strychnine and gallic acid sample.

Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (%RSD) for a statistically significant number of samples. As per the ICH guidelines, precision should be performed at three different levels low quality control (LQC), medium quality control (MQC) and high quality control (HQC). Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed as intra-assay precision. It is assessed by using minimum of 9 determinations covering the specified range for the procedure. The intra-day assay precision was performed 3 times on same day, while inter-assay precision was performed on 3 different days.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of detection (LOD) is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. Limit of Quantification (LOQ) is the lowest amount analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. LOD and LOQ were determined by k x SD/s where k is a constant (3.3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal and s is the slope of the calibration curve.

Accuracy

Accuracy should be reported as percent recovery by the assay of known added amount of analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations /3 replicates each of the total analytical procedure). The percent recovery was calculated by performing recovery studies in triplicates of three concentration levels viz. 80%, 100%, 120% by adding known amount of standard mixture of Strychnine and gallic acid. These samples were then analyzed and the results obtained were compared with expected results.

Robustness

The robustness of an analytical procedure 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. It was studied in triplicate at 300 ng/spot and 400 ng/spot by making small changes in mobile phase composition, the mobile phase saturation time and amount of mobile phase. The final results were examined by calculation of %RSD of concentration.

RESULTS AND DISCUSSION

HPTLC Method Development

In situ HPTLC spectral overlain of Strychnine and gallic acid were taken. Isoabsorptive point was found at 264 nm and was selected as scanning wavelength (Figure 3).

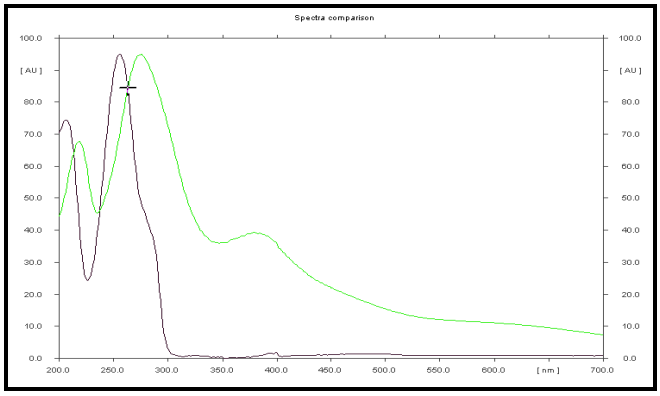

Figure 3: HPTLC Spectral overlay of Strychnine and Gallic acid

(Brown: Strychnine Green: Gallic acid)

Good resolution and sharp peaks with minimum tailing were obtained with mobile phase consisting of toluene: ethyl acetate: Methanol: formic acid 3.5:1.5:1 (v/v/v/v). Strychnine and gallic acid were satisfactorily resolved with Rf values at 0.21 ± 0.02 and 0.60 ± 0.02 respectively (Figure 4).

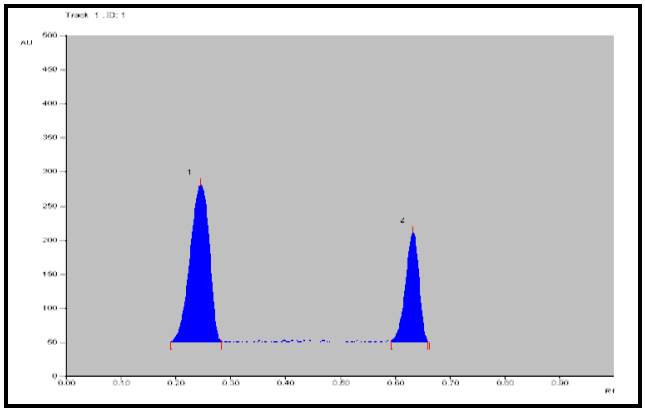

Figure 4: Densitogram of Strychnine and Gallic acid

HPTLC Method Validation

Linearity

Linear relationship was observed by plotting drug concentration against peak area for each compound. Strychnine and gallic acid showed linear response in the concentration range of 200-800 ng/spot.

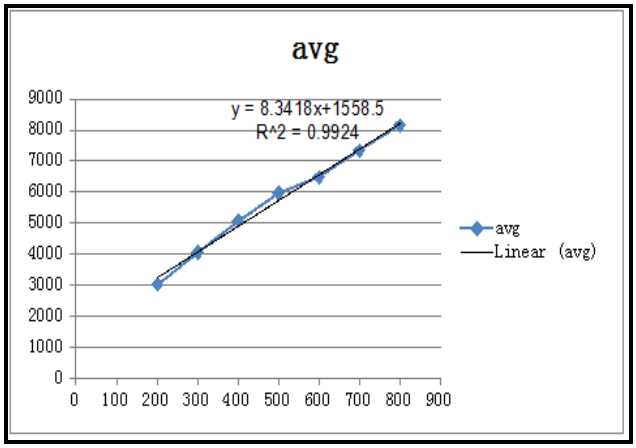

Figure 5A: Calibration Curve of Strychnine

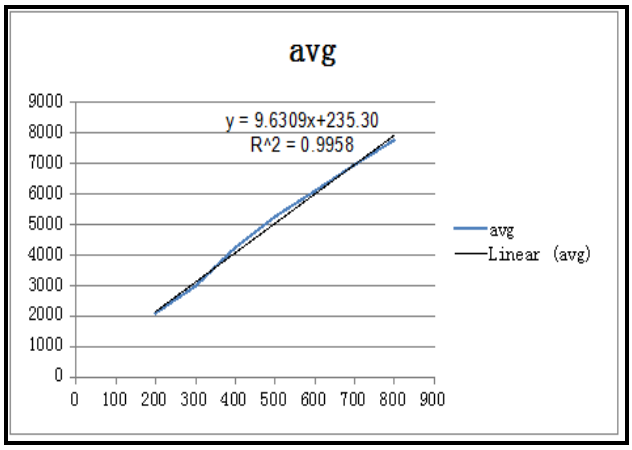

Figure 5B: Calibration Curve of Gallic acid

The linearity was validated by the high value of the correlation coefficients. The results are tabulated in (Table 1).

Table 1: Linear Regression Data for Calibration Plot for Strychnine and Gallic Acid

Parameter	Strychnine	Gallic Acid
Linearity range (ng)	200-800	200-800
Correlation Coefficient	0.9924	0.9958
Regression equation	y= 8.3418x + 1558.5	y= 9.6309x + 235.3
Slope	8.3418	9.6309
Intercept	15558.5	235.3

Specificity

When the spectra of standard Strychnine and gallic acid were overlayed or compared with extracts of Agnitundi Vati it was observed that constituents present in the extract did not interfere with the peaks of Strychnine and gallic acid. Thus the proposed method was proved to be specific. The spectra of the standard Strychnine (Figure 6A) and Gallic acid (Figure 6B) corresponded with extract of vati.

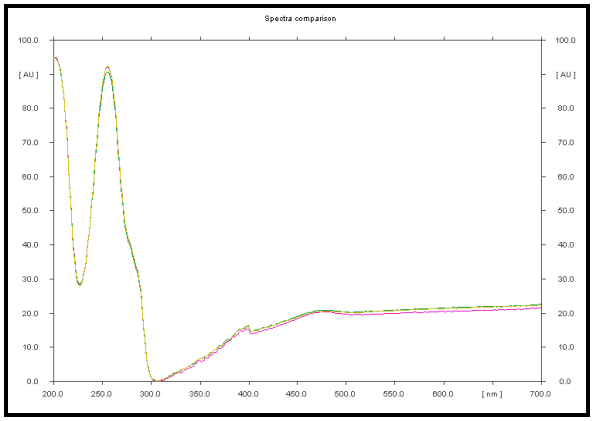

Figure 6A: Spectrum of standard Strychnine and strychnine from

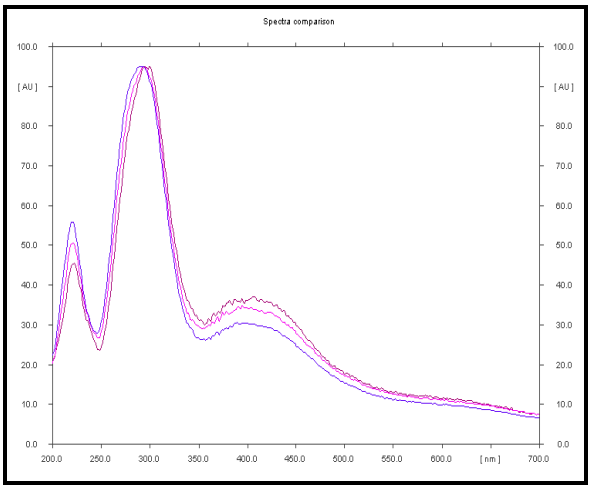

Figure 6B: Spectrum of standard Gallic acid and Gallic acid from extract

Precision

Intraday precision is used to describe the variation of the method, at three different concentration levels within the same day while interday precision is for variation between different days. The % RSD values for both intraday and interday precision were found within acceptable limit as shown in Table 2.

Table 2: Precision results

Compound	Concentration (ng/spot)	Interday		Intraday	
		Mean Area	%RSD	Mean Area	%RSD
Strychnine	300	4065	0.91	4039.7	0.50
	500	5972.45	0.50	5965	1.05
	700	7334.93	0.31	7321.8	1.47
Gallic acid	300	2987.2	0.24	2979.5	1.04
	500	5234.36	0.51	5215.9	0.88
	700	6958.56	0.45	6936.6	0.32

Table 3: LOD and LOQ

Marker	LOD (ng/spot)	LOQ (ng/spot)
Strychnine	20.24	61.32
Gallic acid	8.423	25.52

Table 4: Accuracy: recovery data for Strychnine

Formulation	Level	Total amount of marker	Measured amount	Recovery	%RSD	Mean Recovery
A	80	756	743.6	98.35	1.59	98.89%
	100	840	821.4	97.78	1.95	
	120	924	938	101.15	1.76	
B	80	810	799.6	98.71	0.67	98.72%
	100	900	887.8	98.64	1.42	
	120	990	978.5	98.83	1.71	

Table 5: Accuracy: recovery data for Gallic acid

Formulation	Level	Total amount of marker	Measured amount	Recovery	%RSD	Mean Recovery
A	80	612	598.6	97.81	1.02	98.89%
	100	680	668.2	98.26	1.51	
	120	748	752.7	100.62	1.67	
B	80	720	709.2	98.5	0.99	99.33%
	100	800	784.5	98.06	1.23	
	120	880	888.7	100.9	1.6	

Table 6: Robustness results for strychnine and Gallic Acid

Mobile phase composition	Strychnine (%RSD)		Gallic acid (%RSD)	
	400ng/spot	500ng/spot	400ng/spot	500ng/spot
2.8: 4.8: 1.5: 1	0.25	0.56	1.4	1.25
3: 5: 1.5: 1	0.34	1.02	0.89	0.74
3.2 :5.2 : 1.5 : 1	0.38	0.43	0.96	1.06
Saturation time				
30+5 min	2.01	1.43	1.98	1.87
30-5 Min	1.67	1.82	1.39	1.69

Accuracy

Accuracy of the method is reported as percent recovery of known added amount of analyte in the sample. The percent recovery was calculated by performing recovery studies in triplicates of three concentration levels viz. 80%, 100%, 120% by adding known amount of standard mixture of Strychnine and gallic acid. Results obtained were given in Table 4 and 5.

Robustness

The % RSD of the peak area was calculated in triplicate for changes in mobile phase composition and duration of saturation time for 400 and 500 ng/spot. The values of % RSD were less than 2% which indicated that the developed method is robust as shown in (Table 6).

Estimation of Strychnine and Gallic Acid in Marketed and In-House Formulations

The developed method was applied for the detection and quantification of Strychnine and gallic acid marketed and in-house formulations of Agnitundi Vati. The peaks for Strychnine and gallic acid were observed at Rf 0.21 ±0.02 and 0.60 ±0.02 respectively in the densitogram of extracts. The test samples of marketed formulations and in-house formulation were compared with the ingredients (Figure 7). There was no interference from other compounds present in the Vati. The total strychnine and gallic acid content in different marketed and in-house formulations of Agnitundi Vati was found to be satisfactory as shown in (Table 7).

Table 7: Strychnine and Gallic Acid Content in Polyherbal Formulations

	Formulation	Content in extract (ng/10µl)	Content (%w/w)
Strychnine	A	420	0.42
	B	450	0.45
Gallic Acid	A	340	0.34
	B	400	0.40

CONCLUSION

The HPTLC method was developed for standardization of Agnitundi Vati using Strychnine and gallic acid as biologically active chemical markers. HPTLC method was found to be rapid, simple, linear, precise and accurate for quantitative estimation of Strychnine and gallic acid from Agnitundi Vati. The proposed method was found to be useful, to evaluate various formulations available in the market containing both the drugs and also useful for determining the adulteration or substitution encountered in commercial market.

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