Research Article

www.ijrap.net (ISSN:2229–3566)

A HPTLC STUDY OF VATSANABHA AND SWASANANDAM GUTIKA TO EVALUATE THE ACONITINE CONTENT

Sugeena P [1]*, Tharalakshmi S [2]

[1] Assistant Professor, Department of Rasa Shastra and Bhaishajya Kalpana, KMCT Ayurveda Medical College, Kozhikode, Kerala, India

[2] Associate Professor, Department of Rasa Shastra and Bhaishajya Kalpana, Government Ayurveda College, Thiruvananthapuram, Kerala, India

Received on: 08/05/20 Accepted on: 23/06/20

***Corresponding author**
E-mail: sugeenapalakkara@gmail.com

DOI: 10.7897/2277-4343.110496

ABSTRACT

The drug Vatsanabha (*Aconitum ferox*) is a toxic plant belonging to Ranunculaceae family containing many toxic alkaloids. Aconitine is one of the alkaloid presents in this drug. Vatsanabha is used therapeutically in Ayurveda after subjecting it to certain purification techniques known as shodhana. The toxicity allegations against Ayurvedic formulations containing Vatsanabha are a matter of concern nowadays. Hence it will be crucial to estimate the Aconitine content in Vatsanabha after shodhana procedure and the formulations containing it. Swasanandam gutika is a pill formulation containing Vatsanabha along with Hingula (Mercury Sulphide) and Karpura (*Cinnamomum camphora*). High Performance Thin Layer Chromatography (HPTLC) is an advanced form of thin layer chromatography which is helpful in analyzing the natural products. In this study, the Aconitine content in Vatsanabha before and after shodhana and in Swasanandam gutika is analyzed by HPTLC. It was found that the Aconitine content in Vatsanabha was reduced after shodhana and was negligibly less to estimate in Swasanandam gutika.

Keywords: Vatsanabha, Aconitine, Sodhana, Swasanandam gutika, HPTLC

INTRODUCTION

The drug Vatsanabha identified as *Aconitum ferox* is considered to be one of the deadliest poisonous plant in the world. Vatsanabha or Mahavisha, *Aconitum ferox* is a species of monk's hood from the family Ranunculaceae which is a deciduous perennial with tall and erect stems crowned by racemes of large eye catching blue, purple, white zygo-morphic flowers with numerous stamens[1].

The main chemical constituents in Aconitum are aconitum alkaloids, mainly diester-diterpenoid alkaloids, monoester-diterpenoid alkaloids and amine-diterpenoid alkaloids. Diester-diterpenoid alkaloids include Aconitine, mesaconitine and hypaconitine; monoester-diterpenoid alkaloids include benzoylaconine, benzoylmesaconine and benzoylhypaconine; and amine-diterpenoid alkaloids include aconine, mesaconine and hypaconine. Aconitum alkaloids are supposed to be the main toxic ingredients in Aconitum and may cause severe cardio-neuro and cyto-toxicities. The diester-diterpenoid alkaloids were reported to be toxic and can lead to manifestation of arrhythmia[2].

The major alkaloid among diester-diterpenoid alkaloid is Aconitine which causes severe toxicological reactions. Even though Aconite is considered to be a cardio-toxic drug, patients with Aconitine poisoning generally present with a series of systemic disturbances such as gastrointestinal, cardiovascular and neurological manifestations. The gastrointestinal symptoms include nausea, vomiting, diarrhea and abdominal pain. Numbness in mouth and limbs, paraesthesia, central nervous system depression, respiratory muscle depression, convulsions and seizures are the principal neurological manifestations. The perioral numbness is the first and foremost symptom observed in patients experiencing accidental Aconitine poisoning due to herbominerals containing improperly purified Vatsanabha. Cardiovascular manifestations predominantly include hypotension, palpitations, chest pain, bradycardia, sinus tachycardia, ventricular tachycardia and ventricular fibrillation. Depending on the doses and time of exposure to Aconitine, the severity of the symptoms escalates. The cardiotoxicity and neurotoxicity caused by inadequate consumption of Aconitine have been occasionally reported in recent years. Therefore, the public should be warned about the risk of Aconitine poisoning resulting from over the counter medication. Moreover, the drug control authorities should scrutinize improper shodhana techniques adopted by the pharmaceutical firms by ensuring proper quality control measures. Aconitine is also quickly decomposed or eliminated with a short half-life[3]. Fatal dose of Aconite is 1 gm of root or 250 mg of extract or 25 drops of the tincture or 4 mg of Aconitine. The fatal period ranges from 45 minutes to 6 hours[4].

Many Ayurvedic formulations has Vatsanabha as a constituent, but it is used therapeutically after subjecting it to certain purification techniques emphasized in classical treatises known as shodhana. Swasanandam gutika is such a herbo-mineral drug mentioned in the textbook Arogyakalpadruma[5] which contains Vatsanabha. It contains only three ingredients and hence the proportion of Vatsanabha is higher in Swasanandam gutika when compared to other multi -ingredient herbo-mineral formulations.

Thin layer chromatography (TLC) studies are among the key identity tests in most pharmacopoeia monographs. An extension of TLC is HPTLC (High Performance Thin Layer Chromatography) is a robust, simple, rapid and efficient tool in quantitative analysis of compounds. HPTLC also remains as one

of the most flexible, reliable and cost-efficient separation techniques ideally suited for the analysis of herbal drugs. It is one of the sophisticated instrumental techniques having advantages of automation, scanning, full optimization, selective detection principle, minimum sample preparation, hyphenation and so on. This makes it a powerful analytical tool in providing chromatography information regarding complex mixtures of pharmaceuticals, natural products, clinical samples, food stuff etc[6].

HPTLC is adopted as a tool in this study to analyse the changes occurring to the Aconitine content in raw Vatsanabha and the sample after shodhana. As Aconitine is responsible for the toxic effects, its presence in the Swasanandam gutika is also evaluated.

MATERIAL AND METHODS

The drugs were procured pharmaceutically processed; methanolic extract was prepared and subjected it to HPTLC analysis. It involved the following steps.

Sodhana of Vatsanabha

Aconitum ferox (Figure 1) was procured from SKM Siddha and Ayurveda Company Erode. It was botanically authenticated by pharmacognosists at Drug Standardization Unit, Trivandrum. The purification procedure adopted was Swedana with cow's milk in Dolayantra for 1 yama (time which is equal to ¼ of day or night; time that is four times of muhurta plus 1/4th of muhurta) i.e. 3 hours[7].

62 g of Vatsanabha was taken and then cut into small pieces. It was then placed in a cloth and tied to prepare a poultice. It was then suspended on an iron rod placed over the neck of a mud pot containing 750 ml of milk. The poultice was immersed in it without touching the sides and bottom of the vessel. The pot was kept on the stove and subjected to Swedana (Figure 2). After one and a half hours the level of milk in the pot was noticed to be reduced and it was replaced by an additional 250 ml of boiled milk. After 3 hours of Swedana, the poultice was taken out (Figure 3) and the contents were washed in warm water in order to remove the adhered milk scum and other impurities. Then it was kept in sunlight to dry for 10 days. 52 g of drug was obtained after shodhana.

Preparation of Swasanandam gutika (SG)

Shodhita Hingula, Shodhita Vatsanabha and Karpura (*Cinnamomum camphora*) were taken 10 g each. For the purpose of Bhavana, 30 ml of triphala kwatha was prepared. Shodhita Vatsanabha and Karpura were powdered well. Then the Shodhita Hingula and Shodhita Vatsanabha were triturated well by adding sufficient quantity of triphala kwatha. Karpura was added in the end and triturated well. On drying the weight was noted. Pills were rolled out weighing 125 mg size (Figure 4).

Preparation of sample for HPTLC

2 g of crude drug Vatsanabha was weighed and taken in a round bottom flask. 25 ml of methanol was added to it. Then it was kept on a water bath and connected to a condenser and refluxed for 20 minutes. The extract was then filtered to a standard flask with a filter paper and made up to 50 ml by adding methanol. Like this the extract of Shodhita Vatsanabha and Swasanandam gutika was prepared. The thin layer chromatography was done to detect the presence of Aconitine and other alkaloids in the sample of Vatsanabha before and after shodhana

The standard Aconitine

5 mg of standard Aconitine for HPTLC analysis was purchased from Sigma Aldrich Company, Bangalore. The stock solution of standard was prepared by dissolving it in 10 ml of methanol.

HPTLC profile (High Performance Thin Layer Chromatography)

HPTLC system of CAMAG, Muttenz, Switzerland, Anchrom Enterprises (I) Pvt. Ltd, Mumbai, consisting of sample applicator (Linomat 5), Twin trough chamber with lid (10 x 10 cm, CAMAG, Switzerland), UV cabinet (Aetron, Mumbai) was used for the study.

The TLC was done prior to HPTLC in order to find out a solvent system suitable for separating Aconitine. Many solvent systems were tried on trial and error basis to achieve better separation and resolution. The mobile phase commonly called the solvent system selected was cyclohexane: ethyl acetate: di-ethylamine in the ratio 8:1:1. The presence of Aconitine was confirmed by spraying Dragendroff's reagent on the TLC plate which showed orange red spot on the plate.

Selection of plates

The plates were of two types: hand- made plates and pre-coated plates which were available commercially. For HPTLC purpose, plates in size of 20 × 20 cm with aluminium or polyester support were used. These plates were cut into required size and shape to suit particular analysis using general purpose scissors.

Activation of pre-coated plates

Freshly opened box of plates usually does not require activation. However, plates exposed to high humidity or kept on hand for long time may have to be activated by placing in oven at 110-120^{0}C for 30 minutes. Aluminium backed plates were activated by keeping between two glass plates.

Sample and standard preparation

The sample preparation procedure was done to dissolve the dosage form with complete recovery of intact compounds of interest and minimum of matrix with a suitable concentration of analytes for direct application on the HPTLC plate. The preparation method of extraction followed was same as that for TLC. The sample and reference substance were dissolved in the same solvent (cyclohexane: ethyl acetate: di-ethylamine in the ratio 8:1:1) to ensure comparable distribution at the starting zone.

Vial 1 - Raw Vatsanabha
Vial 2 – Shodhita Vatsanabha
Vial 3 – Swasanandam gutika
Vial 4 - Standard Aconitine

Application of sample

Sample application was the most critical step for obtaining good resolution for quantification by HPTLC. Automatic application device was used for this purpose. Usually application of 0.5-5 µl for HPTLC was recommended in keeping the size of starting zone down to the minimum 0.5-1 mm in the concentration range of 0.1-1 µg/µl. However, volume and concentration primarily depend on the component under analysis and their sensitivity to various detection techniques. If too much sample was applied, it may not be absorbed uniformly throughout the layer leading to over loading. As a result trailing of zones and poor resolution will

occur. Problem arising out of such overloading, when unavoidable can be overcome by applying the sample as a band. The only apparent disadvantage is that fewer samples can be accommodated on a given plate. So the samples were applied as a band in the plate using the automatic application device. The track, application position and application volume is shown in Table 1.

Development

Linear development was adopted since it is the most familiar technique in HPTLC. Hence the plate was placed vertically in the solvent system.

Steps in automated development chamber

Pre-drying: A short drying of the plate at sample application position

Humidity control was enabled and the time was set as 10 minutes.

Tank saturation: It was enabled with saturation pad and the time was set as 20 minutes for this step. Solvent system used was cyclohexane: ethylacetate: diethylamine. (8:1:1).

Development: Plate conditioning time was fixed as 5 minutes and the migration distance was 70 mm. Drying time was set as 5 minutes.

Detection

This step involved the scanning of the developed chromatographic plate using a scanner (Figure 5). Wavelength of 233 nm was used for scanning and the scanning speed was set as 20 mm/second.

RESULT

In the plate, a single band was visualised in the raw sample of Vatsanabha at a retention factor (Rf) of 0.21 while more than two bands were present in the sample after shodhana (Figure 6). Among the two bands, second band corresponds to Aconitine and the first band corresponds to some other compound which was not detected in raw Vatsanabha. In the baseline diagram, it was evident that there was the formation of a new peak in Shodhita Vatsanabha (Figure 7) which was not present in the baseline diagram of raw drug Vatsanabha (Figure 8). It may due to the formation of a new compound formed by the shodhana procedure and may be responsible for an enhanced therapeutic effect.

The baseline diagram (Figure 9) and peak diagram of standard Aconitine (Figure 10) was clearly depicted at an Rf 0.21 which proved that the compound tracked in samples under investigation was Aconitine. The band for Aconitine was not detected in Swasanandam gutika. There was a peak detected in the raw drug (Figure 11) and Shodhita Vatsanabha (Figure 12) which corresponds to the alkaloid Aconitine plotted as standard. On quantitative estimation (Table 2), the calculated area of the peak in the raw Vatsanabha sample was 1541.7AU (Area under curve) and 257.5AU in the peak of the Shodhita Vatsanabha sample. The quantity of Aconitine estimated was in the nanogram level in the extracts taken from 2 g of both raw Vatsanabha and Shodhita Vatsanabha.

Table 1: Track and application position of HPTLC

Track	Application position in millimetre (mm)	Application Volume in microlitre (µl)	Vial
1	15.0	6.0	1
2	33.8	6.0	2
3	52.6	6.0	3
4	71.4	6.0	4
5	90.2	2.0	4
6	109.0	4.0	4
7	127.8	6.0	4
8	146.6	8.0	4
9	165.4	10.0	4
10	184.2	12.0	4

Vial 1 - Methanolic extract of Raw Vatsanabha, Vial 2 - Methanolic extract of Shodhita Vatsanabha
Vial 3 - Methanolic extract of Swasanandam gutika, Vial 4 - Standard Aconitine

Table 2: Peak table

Track	Vial	Rf	Amount Fraction	Height	X (calc)	Area	X (calc)	Remark
1	1	0.21		57.87	< 900.00 ng	1541.71	< 900.00 ng	Sample: Out of permitted range
2	2	0.21		16.1	< 900.00 ng	257.54	< 900.00 ng	Sample: Out of permitted range
3	3	0.21						Sample: No peak detected
4	4	0.21		217.59	2.973µg	4580.14	3.140 µg	Sample:
5	4	0.21	1000 ng	89.66		1780.59		Std Level 1
6	4	0.21	2.000 µg	174.51		3392.93		Std Level 2
7	4	0.21	3.000 µg	235.99		4599.29		Std Level 3
8	4	0.21	4.000 µg	298.14		5968.93		Std Level 4
9	4	0.21	5.000 µg	313.48		6676.58		Std Level 5
10	4	0.22	6.000 µg	358.24		7527.26		Std Level 6

Aconitine level in Vatsanabha before and after shodhana and Swasanandam gutika, Aconitine was detectable only in Vatsanabha before shodhana and after shodhana. It was absent in SG sample (3), Sample 1 Vatsanabha before shodhana, Sample 2- Vatsanabha after shodhana

Figure 1: Vatsanabha

Figure 2: Shodhana process of Vatsanabha

Figure 3: Vatsanabha after shodhana

Figure 4: Swasanandam Gutika

Scan settings

Slit dimension :	4.00 x 0.30 mm, Micro
Optimize optical system for maximum :	Light
Scanning speed :	20 mm/s
Data resolution :	100 µm/step

Measurement	
Wavelength	233 nm
Lamp	D2 & W
Measurement type	Remission
Measurement mode	Absorption
Optical filter	Second order
Detector mode	Automatic
Y-position for 0 adjust	5.0 mm
Track # for 0 adjust	1
Track start for quick scan	Automatic
Track end for quick scan	Automatic
Track # for quick scan	Automatic
Analog offset	10 %
Sensitivity	Automatic

Figure 5: Settings of the scanner

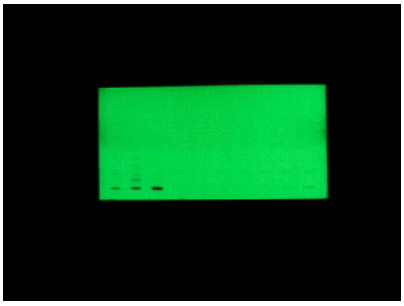

Figure 6: Plate visualised under UV

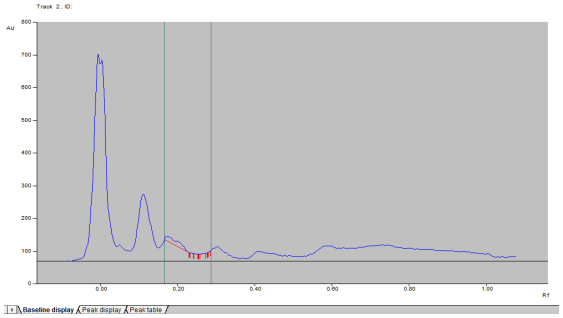

Figure 7: Baseline display of sample 2 – Shodhita Vatsanabha

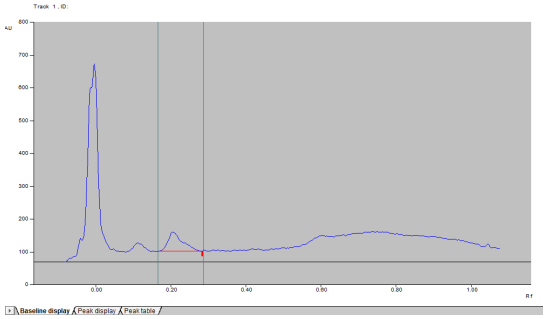

Figure 8: Baseline display of sample 1 – Raw Vatsanabha

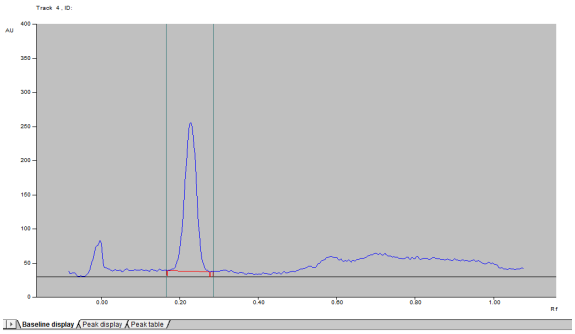

Figure 9: Baseline display of Standard Aconitine

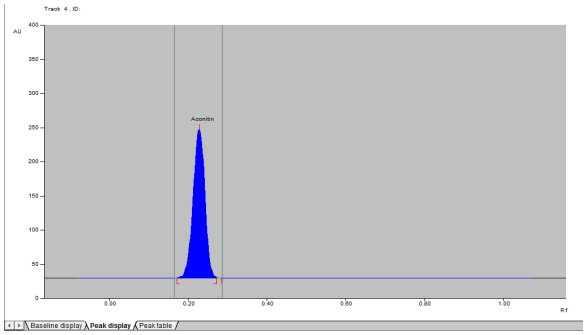

Figure 10: Peak display of standard Aconitine

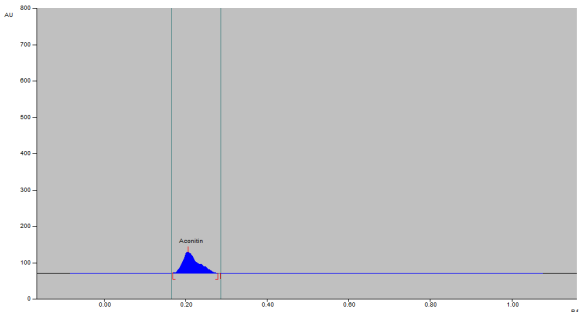

Figure 11: Peak display of sample 1 – Raw Vatsanabha

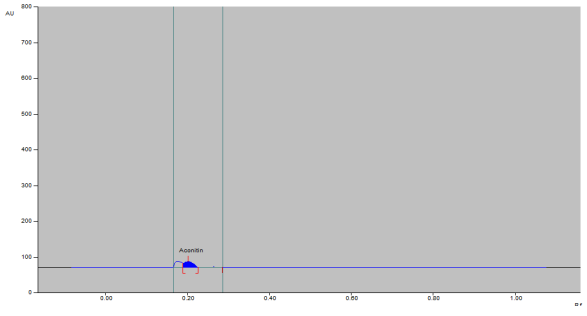

Figure 12: Peak display of sample 2 – Shodhita Vatsanabha

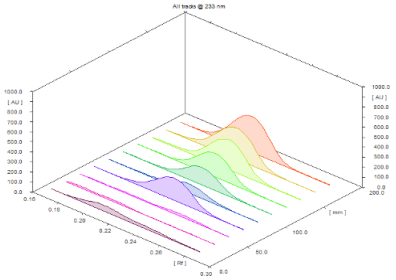

Figure 13: 3D Display of peaks

DISCUSSION

The study was done to evaluate the Aconitine content in Vatsanabha before shodhana and after shodhana and in Swasanandam gutika. Many of the Ayurvedic herbo-mineral drugs contain Vatsanabha and under certain circumstances, toxicity issues are occurring in some patients to whom those drugs are prescribed. Swasanandam gutika is widely used in clinical practice by practitioners in Kerala for respiratory ailments. The shodhana procedures are described in Ayurveda to reduce the toxicity of a drug. Like this in Traditional Chinese Medicine (TCM) also, various processing techniques like soaking and boiling are explained for the drug Aconite. Many of the research works in TCM suggest that the toxic alkaloids present in Aconite was hydrolysed to benzoyl aconine and aconine by steaming and boiling techniques.[8]

In this study the HPTLC clearly depicts the presence of Aconitine in the raw drug Vatsanabha. It was clearly understood from the calculated Area Under the Curve that the alkaloid content get reduced in Shodhita Vatsanabha due to purification method i.e. Dolayantra Swedana done in milk. The HPTLC baseline diagram depicts the presence of a compound which formed in Vatsanabha after shodhana which was not revealed in the baseline diagram before shodhana. The presence of Aconitine was not detected in the sample of Swasanandam gutika.

According to the research works done on processing of Aconite by TCM, after processing of the Aconitum tubers, it was found that the contents of diester-diterpenoid alkaloids were reduced. Monoester-diterpenoid alkaloids and amine-diterpenoid alkaloids were increased, suggesting that diester-diterpenoid alkaloids were transformed into monoester-diterpenoid alkaloids and amine-diterpenoid alkaloids. Further decocting the Aconitine in boiling water confirmed that the three alkaloids were progressively transformed. Pharmacological experiments with Aconitine, benzoylaconine and aconine in Sprague Dawley rats showed that the cardiac functions are enhanced by Aconitine at a dose of 0.01 mg/kg and aconine at a dose of 10 mg/kg. At the same time benzoylaconine at a dose of 2 mg/kg weakened the cardiac function. The effect of processing is attributed to the transformation of the most toxic diester-diterpenoid alkaloids into less toxic monoester-diterpenoid alkaloids and amine-diterpenoid alkaloids. Transformation in to new alkaloids after processing changes the pharmacological property of the drug and hence it plays synergistic and attenuated roles eventually.

In this study it can be assumed that the newly detected peak in Shodhita Vatsanabha may be the hydrolysed form of Aconitine. It has to be separated and subjected to further analytical studies.

CONCLUSION

From the study it was evident that the toxic alkaloid Aconitine present in the raw drug Vatsanabha was decreased on performing shodhana. The formulation Swasanandam gutika didn't exhibit the presence of Aconitine in HPTLC since alkaloid content was negligibly less to estimate. The toxicity allegations in Ayurvedic formulations due to the presence of Aconitine are uncorroborated, if the shodhana procedures were performed properly. Future research has to be done to evaluate the effect of other shodhana methods on the alkaloid level of Aconitine. Moreover further studies should be done to estimate the toxic alkaloid contents other than Aconitine.

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Cite this article as:

Sugeena P and Tharalakshmi S. A HPTLC Study of Vatsanabha and Swasanandam gutika to evaluate the Aconitine Content. Int. J. Res. Ayurveda Pharm. 2020;11(4):93-98 http://dx.doi.org/10.7897/2277-4343.110496

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