# Chapter 5.

# RESULTS and DISCUSSION

## 5. RESULTS AND DISCUSSION

The present chapter is devoted to compilation of results of various studies undertaken. The results are also discussed wherever needed.

### 5.1) Plant material

Dried roots of *Aconitum ferox* and *Solanum indicum* as well as the dried fruits of *Piper nigrum* and *Piper longum* were authentified by NISCAIR, Delhi. The voucher specimens (No.Pharmacy/06-07/AF/PR), (No.Pharmacy/06-07/SI/PR), (No. Pharmacy/06-07/PN/PR) and (No. Pharmacy/06-07/PL/PR) respectively were submitted in the Pharmacy Department, The M.S. University of Baroda, Vadodara.

### 5.2) Standardization of the plant materials as per the WHO guidelines

#### *5.2.1) Macroscopic evaluation*

All the organoleptic features were found to match with that of the reported monographs.

#### *5.2.2) Microscopical and Histological analysis:*

Detailed anatomical features of the roots were studied. Transverse section of *Aconitum ferox* root is circular in outline and shows the following regions (Fig.5.1).

*Periderm:* In young root the epidermis is single layered with unicellular hairs where as in old roots the epidermis is replaced with periderm. Periderm is 6-8 layers, thick and is not clearly distinguished into phellem, phellogen and phelloderm.

*Cortex:* Followed by periderm is a homogenous cortex comprising of 8-10 layers of thin walled large parenchymatous cells. The cortical cells are devoid of any cellular inclusions.

*Endodermis:* A distinct, single layer of endodermis separates the cortical region from vascular region and shows the presence of casparian thickening.

Vascular bundle: It occupies more or less the central region and is separated into xylem and phloem by few layers of cambium. The xylem cylinder consists of patches of xylem included by parenchyma cells which are elongated thin walled

and closely packed. Within the xylem patch medullary rays of 2-4 layers extend from the primary xylem to up to the outer phloem region.

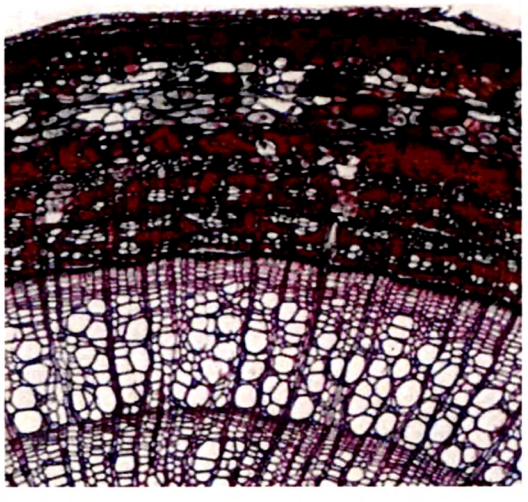
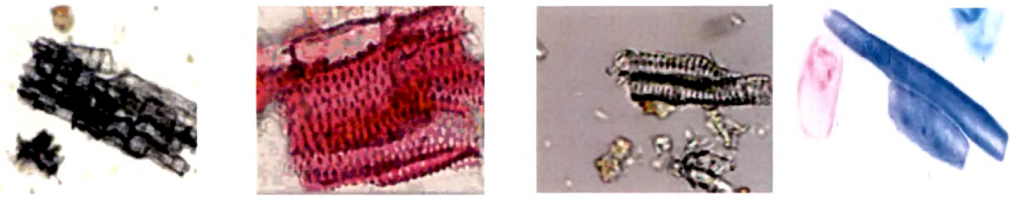


**Fig 5.1.Transverse section of *Aconitum ferox* roots.**

The powdered drug also showed the presence of similar microscopical stuctures as shown in Fig.5.2.



*(1)* *(2)* *(3)* *(4)*

**Fig 5.2. Diagnostic features for the powder microscopy of the roots of *Aconitum ferox*. (1) cork cells; (2) vessels with alternate pitting; (3) vessels with helical thickening; (4) Fibres.**

Transverse section of *Solanum indicum* root is circular in outline and shows the following regions (Fig.5.3).

*Cork*: It is composed of 5-15 layers of thin walled, tangentially elongated rectangular cells filled with yellowish brown content.

*Cork cambium:* It is single layered.

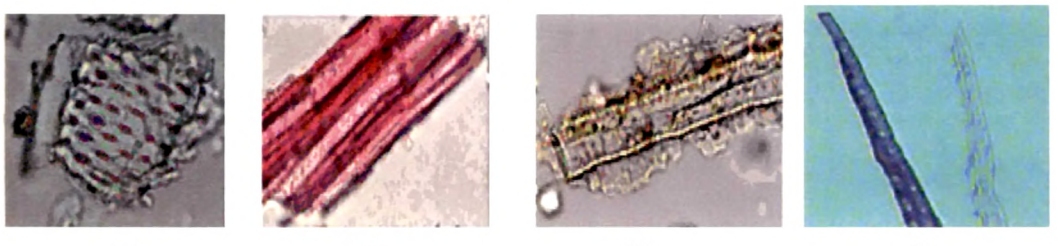
*Cortex*: It is composed of 5-9 layers of thin walled, oval and tengentially elongated cells, stone cells are present in singles or in groups of 2-5 or more in this region.

*Phloem:* It is composed of sieve elements, parenchyma and stone cells present in outer phloem region in singles or in groups of 2-5 , varying greatly in shape and in size, phloem rays elongated in inner phloem region and radially

elongated in outer phloem region, occassionally stone cells are also found in medullary rays, wood occupies bulk of root and composed of vessels, trachieds, fibres and xylem rays.

**Fig 5.3. Transverse section of *Solanum indicum* roots.**

The microscopic studies of powdered drug showed presence of similar structures as discussed above (Fig.5.4). The vessels occur singly or in groups of 2-5 with simple pits, xylem fibres moderately thick walled with simple pits and pointed ends found in abundence. Xylem parenchyma have simple pits or reticulate thickening, xylem rays uniseriate to biseriate, thick walled.



(1) (2) (3) (4)

**Fig.5.4. Diagnostic features for the powder microscopy of the roots of Solanum indicum (1) vessels with simple pitting; (2) tracheids; (3) xylem parenchyma (4) Fibres.**

The microscopic stuctures of the Piper species were in accordance with the reported details in the WHO monograph.

Thus from the microscopic studies, the crude drugs were authentified and studied. The microscopic characters are very important parameters for authentification to standardize the plant material before incorporation in the formulations.

### *5.2.3) Determination of ash:*

The Table 5.1 shows the ash values of all the four plant materials. All the values of total ash comply with the prescribed limits in the WHO monographs.

The water soluble components are higher in concentration as compared to the insoluble components.

**Table .5.1 Ash values of plant materials.(n=3)**

| Sample | Total ash (%) | Acid insoluble ash (%) | Water soluble ash (%) |
|---|---|---|---|
| *Aconitum ferox* | 1.08 ± 0.09 | 0.57 ± 0.086 | 0.62 ± 0.01 |
| *Solanum indicum* | 1.56 ± 0.12 | 0.59 ± 0.01 | 0.98 ± 0.02 |
| *Piper nigrum* | 0.76 ± 0.11 | 0.29 ± 0.02 | 0.47 ± 0.05 |
| *Piper longum* | 1.45 ± 0.07 | 0.51 ± 0.07 | 0.96 ± 0.1 |

The inorganic content of the plant materials are determined by the ash value. The acid insoluble ash is the total soil or silicieous matter present in the plants. Thus ash value of the plant material determines the raw material quality of the plant materials. The results showed that the ash values are within the prescribed limits.

### *5.2.4) Determination of Extractable matter*

Coarse powder of the dried plant materials was extracted with petroleum ether, methanol and water, separately, in a soxhlet extractor; the extract was concentrated and dried under vacuum. The extractive values of the plant materials in the three solvents have been given in Table 5.2.

**Table. 5.2 Extractive values of plant materials.(n=3)**

| Sample | Water soluble extractive value (%) | Alcohol soluble extractive value (%) | Ether soluble extractive value (%) |
|---|---|---|---|
| *Aconitum ferox* | 12.3 ± 0.52 | 15.9± 0.12 | 8.2 ± 0.2 |
| *Solanum indicum* | 17.9 ± 0.80 | 19.2± 0.34 | 6.3 ± 0.32 |
| *Piper nigrum* | 21.9 ± 0.89 | 18.3± 0.19 | 10.1± 0.19 |
| *Piper longum* | 20.67 ± 0.43 | 23.78± 0.48 | 11.2 ± 0.61 |

In the absence of the known active constituents, the different classes of compounds extracted in different solvents become important standardization

parameter. The extractive values in all the solvents of all the plant materials are in accordance with the WHO monographs.

### 5.2.5) Determination of water and volatile matter

The % loss on drying of the plant materials is given in Table 5.3. The values comply with those of the limits prescribed in the standard monographs.

**Table 5.3 % Loss on drying of plant materials.(n=3)**

| Sample | % Loss on drying |
|---|---|
| *Aconitum ferox* | 0.78 ± 0.08 |
| *Solanum indicum* | 1.03 ± 0.16 |
| *Piper nigrum* | 0.67 ± 0.21 |
| *Piper longum* | 0.83 ± 0.19 |

The quality of the plant material has to be maintained for which the degradation conditions have to be minimised. The moisture content should therefore be determined. In the present study, the % loss on drying of all the plant materials was found to be within the limits.

### 5.2.6) Determination of pesticides residue

The TLC studies were performed for detection of possible organochorinated pesticides and organophosphorus pesticides. The results of the analysis are shown in Table 5.4.

**Table 5.4 Analysis for Pesticide residues in plant materials.**

| Sample | Organochlorinated Pesticides | | Organophosphorus pesticides | |
|---|---|---|---|---|
| | A | B | C | D |
| *Aconitum ferox* | -ve | -ve | -ve | -ve |
| *Solanum indicum* | -ve | -ve | -ve | -ve |
| *Piper nigrum* | -ve | -ve | -ve | -ve |
| Piper longum | -ve | -ve | -ve | -ve |

-ve-Negative

A- Solvent system: DMF: Ether (4:6), Detecting Reagent: Tetrabromophenolphthalein.

B- Solvent system: Methylcyclohexane , Detecting Reagent: Tetrabromophenolphthalein.

C- Solvent system: DMF: Ether (4:6) , Detecting Reagent: 0.5 %Silver nitrate in water and acetone.

D- Solvent system: DMF: Ether (4:6) , Detecting Reagent: 0.5 %Silver nitrate in water and acetone.

The results of the tests for pesticide residues showed the absence of organochlorinated and organophosphorus pesticides. Pesticide residues are the important parameter for standardization of the plant materials as plant materials are generally prone to be contaminated with such substances and may prove to be harmful on long term usage.

### *5.2.7) Determination of Heavy metals*

Elemental analysis of plant materials was done by Atomic absorption spectrophotometer, where Lead, Cadmium, Mercury and Arsenic were found to be absent. (Table.5.5)

**Table- 5.5 Heavy metal analysis of plant materials.**

| ***Sample*** | ***Cadmium*** | ***Arsenic*** | ***Mercury*** | ***Lead*** |
|---|---|---|---|---|
| *Aconitum ferox* | -ve | -ve | -ve | -ve |
| *Solanum indicum* | -ve | -ve | -ve | -ve |
| *Piper nigrum* | -ve | -ve | -ve | -ve |
| *Piper longum* | -ve | -ve | -ve | -ve |

-ve-Negative

The determination of heavy metals in the plant material showed the absence of Cadmium, Arsenic, Mercury and Lead. The determination of these heavy metals is important for standardization because if their content is above the prescribed limit, the heavy metals prove to be toxic on long term usage.

### *5.2.8) Determination of microbial contamination*

The analysis of bio-burden present in the plant materials was performed and all the results were within the limits prescribed in the WHO guidelines (Table 5.6). The total microbial contamination in the plant materials should be determined for the quality control because these plant materials have to be taken internally and may prove to be harmful in a number of cases. The presence of microbial bioburden also leads to degradation of the active constituents which may lead to the loss of activity of the formulation.

**Table 5.6 Results of the tests performed for the microbial contamination in the plant materials.**

| **Parameters** | ***Aconitum ferox*** | ***Solanum indicum*** | ***Piper nigrum*** | ***Piper longum*** |
|---|---|---|---|---|
| Total bacterial count | > 10,000 cfu/gm | > 10,000 cfu/gm | > 10,000 cfu/gm | > 10,000 cfu/gm |
| *E.coli* | Absent | Absent | Absent | Absent |
| *Salmonella. Typhii* | Absent | Absent | Absent | Absent |
| *S,aureus* | Absent | Absent | Absent | Absent |
| *Pseudomonas aeruginosa* | Absent | Absent | Absent | Absent |
| Yeast and mould | Absent | Absent | Absent | Absent |

### *5.2.9) Qualitative Phytochemical Screening:*

The phytochemical screening of the plant materials was done by qualitative chemical tests and TLC studies.

Qualitative chemical tests were performed for Petroleum ether extract, Methanol extract and the Aqueous extract.

#### *(a) Petroleum Ether Extract*

The Petroleum ether extract of *Aconitum ferox* showed the presence of Phytosterols (Triterpenoids) and fats. While that of Solanum indicum showed the presence of Steroidal components. The Petroleum ether extract of *Piper nigrum* and *Piper longum* showed the presence of Steroidal components, phytosterols and fixed oils (Table 5.7).

**Table 5.7 Qualitative chemical tests of Petroleum ether extract of plant materials.**

| Sr. No. | Class of Compounds | *Aconitum ferox* | *Solanum indicum* | *Piper nigrum* | *Piper longum* |
|---|---|---|---|---|---|
| 1 | Carbohydrates | -ve | -ve | -ve | -ve |
| 2 | Alkaloids | -ve | -ve | -ve | -ve |
| 3 | Glycosides<br>Anthranol<br>Cardiac | -ve | -ve | -ve | -ve |
| 4 | Saponins<br>Saponins<br>Steroidal | -ve<br>-ve | -ve<br>+ve | -ve<br>+ve | -ve<br>+ve |
| 5 | Phytosterols<br>Free steroids<br>(Triterpenoids) | +ve<br>-ve | -ve<br>-ve | +ve<br>+ve | +ve<br>+ve |
| 6 | Phytosterols<br>Fixed oils and fats | +ve | -ve | +ve | +ve |
| 7 | Phenolics and tannins | -ve | -ve | -ve | -ve |
| 8 | Proteins and amino acids | -ve | -ve | -ve | -ve |

***b) Methanol extract***

The methanol extract of *Aconitum ferox* and *Solanum indicum* showed the presence of alkaloids, anthranol glycosides, saponins, phenolics and proteins. While that of extract of *Piper nigrum* and *Piper longum* showed the presence of alkaloids, saponins, phenolics, tannins, proteins and amino acids (Table 5.8).

**Table. 5.8 Qualitative chemical tests of Methanol extract of plant materials.**

| Sr. No. | Class of Compounds | *Aconitum ferox* | *Solanum indicum* | *Piper nigrum* | *Piper longum* |
|---|---|---|---|---|---|
| 1 | Carbohydrates | -ve | -ve | -ve | -ve |
| 2 | Alkaloids | +ve | +ve | +ve | +ve |
| 3 | Glycosides<br>Anthranol<br>Cardiac | +ve<br>-ve | +ve<br>-ve | -ve<br>-ve | -ve<br>-ve |
| 4 | Saponins<br>Saponins<br>Steroidal | +ve<br>-ve | -ve<br>-ve | +ve<br>-ve | +ve<br>-ve |
| 5 | Phytosterols<br>Triterpenoids | -ve | -ve | -ve | -ve |
| 6 | Phytosterols<br>Fixed oils and fats | -ve<br>-ve | -ve<br>-ve | -ve<br>-ve | -ve<br>-ve |
| 7 | Phenolics and tannins | +ve | +ve | +ve | +ve |
| 8 | Proteins and amino acids | +ve | +ve | +ve | +ve |

***c) Aqueous extract:***

The aqueous extract of *Aconitum ferox* and *Solanum indicum* showed the presence of carbohydrates, glycosides, saponins, proteins and amino acids. While that of extract of *Piper nigrum* and *Piper longum* showed the presence of carbohydrates, saponins, phenolics, tannins, proteins and amino acids.

**Table. 5.9. Qualitative chemical tests of Aqueous extract of plant materials.**

| Sr. No. | Class of Compounds | *Aconitum ferox* | *Solanum indicum* | *Piper nigrum* | *Piper longum* |
|---|---|---|---|---|---|
| 1 | Carbohydrates | +ve | +ve | +ve | +ve |
| 2 | Alkaloids | -ve | -ve | -ve | -ve |
| | Glycosides | | | | |
| 3 | Anthranol | +ve | +ve | -ve | -ve |
| | Cardiac | -ve | -ve | -ve | -ve |
| 4 | Saponins | | | | |
| | Saponins | +ve | -ve | +ve | +ve |
| | Steroidal | -ve | -ve | -ve | -ve |
| 5 | Phytosterols | | | | |
| | Free steroids | -ve | -ve | -ve | -ve |
| | (Triterpenoids) | -ve | -ve | -ve | -ve |
| | Phytosterols | | | | |
| 6 | Fixed oils and fats | -ve | -ve | -ve | -ve |
| 7 | Phenolics and tannins | -ve | -ve | +ve | +ve |
| 8 | Proteins and amino acids | +ve | +ve | +ve | +ve |

TLC studies were performed for the Petroleum ether extract, Methanol extract and Aqueous extracts.

**a) Petroleum ether extract:** The Petroleum ether extract of *Piper nigrum* and *Piper longum* showed the presence of essential oils (Table 5.10).

**Table 5.10 TLC studies of Petroleum ether extract of plant materials.**

| Class of Compound | *Aconitum ferox* | *Solanum indicum* | *Piper longum* | *Piper nigrum* | Inference |
|---|---|---|---|---|---|
| Anthraglycosides | No Spot | No Spot | No Spot | No Spot | Absent |
| Alkaloids | No Spot | No Spot | No Spot | No Spot | Absent |
| Arbutin like compounds | No Spot | No Spot | No Spot | No Spot | Absent |
| Bitter Principles | No Spot | No Spot | No Spot | No Spot | Absent |
| Saponins | No Spot | No Spot | No Spot | No Spot | Absent |
| Essential Oils | No Spot | No Spot | Purple spot | Purple spot | Present in PI PN. |

**b) Methanol extract:** Glycosides and alkaloids were found to be present in all the methanol extracts. The results along with the inferences have been shown in Table 5.11.

**Table 5.11 TLC studies of Methanol extract of plant materials.**

| Class of Compound | *Aconitum ferox* | *Solanum indicum* | *Piper longum* | *Piper nigrum* | Inference |
|---|---|---|---|---|---|
| Anthraglycosides | Blue | Dark blue | Blue | Blue | Present in all. |
| Alkaloids | Dark Orange | Dark Orange | Dark Orange | Dark Orange | Present in all |
| Arbutin like compounds | No Spot | No Spot | No Spot | No Spot | Absent |
| Bitter Principles | No Spot | No Spot | No Spot | No Spot | Absent |
| Saponins | No Spot | No Spot | No Spot | No Spot | Absent |
| Essential Oils | No Spot | No Spot | No Spot | No Spot | Absent |

**c) Aqueous extract:** Glycosides were found to be present in *Solanum indicum, Piper nigrum* and *Piper longum.* Alkaloids and saponins were found to be present in all the extracts. The results along with the inferences have been shown in Table 5.12.

**Table 5.12 TLC studies of Aqueous extract of plant materials.**

| Class of Compound | *Aconitum ferox* | *Solanum indicum* | *Piper longum* | *Piper nigrum* | Inference |
|---|---|---|---|---|---|
| Anthraglycosides | No Spot | Dark blue | Blue | Blue | Glycosides present in SI,PN,PI |
| Alkaloids | Dark Orange | Dark Orange | Dark Orange | Dark Orange | Present in all |
| Arbutin like compounds | No Spot | No Spot | No Spot | No Spot | Absent |
| Bitter Principles | No Spot | No Spot | No Spot | No Spot | Absent |
| Saponins | Purple | Purple | Purple | Purple | Present in all. |
| Essential Oils | No Spot | No Spot | No spot | No spot | Absent |

The qualitative chemical tests and the TLC studies showed similar results in all three extracts of all the plant materials. The different classes of compounds were found to be present in different extracts. The true standardization of these

plant materials is thus through the phytochemical screening. The tests show the presence of the vital chemical constituents and important constituents have to be selected and methods have to be developed for the analysis of such chemical constituents. Thus, the phytochemical analysis of the plant material is also an important standardization parameter.

## 5.3) Isolation of Marker Compounds:

### *5.3.1) Aconitum alkaloids:*

The yield of the alkaloid fraction from the tubers of *Aconitum ferox* was 3.8 %. The brown viscous mass was kept in a desicator until the moisture was removed completely and a constant weight was achieved. The fraction was weighed and a solution of 10 mg/ml in chloroform was prepared. Chromatographic study showed eight bands when developed in ethyl acetate: methanol (9:1). The preparative TLC of this solution was performed and the conditions were optimized as follows:

**Table 5.13 Optimized conditions for preparative TLC of Aconitum roots.**

| Parameters | Conditions |
|---|---|
| Size of the Glass plate | 7" X 3.5" |
| Stationary phase | Silica gel G |
| Air drying time for plate | Overnight |
| Activation time for plate | 30 min. at 110 °C |
| Application of Spot | 0.5 ml with marked capillary |
| Solvent system | Ethyl acetate: methanol (9:1) |
| Chamber saturation Time | 30 min |
| Height of solvent front | 12 cm |
| Rf of Aconitine | 0.45 |

The alkaloids in the fraction were separated on the preparative TLC plate. The reference standard aconitine purchased from Sigma Aldrich Pvt. Ltd. was used to determine the Rf value of aconitine. The band matching with the Rf value (~0.45) of standard aconitine was scrapped and centrifuged in chloroform at 3000 rpm for 15 minutes. The supernatant was collected and concentrated. The same procedure was repeated several times. About 5mg of a colourless crystals

were separated and further purified by recrystallization with methanol. This compound showed single spot on TLC using same solvent system which turned dark orange on spraying dragendorff's reagent. The melting point was found to be 203-205 °C.

### *5.3.2) Solanum alkaloids:*

Reported method was used for the isolation of Solanine from *Solanum indicum*. However, no precipitates were obtained on treatment of the alcohol extract with equal volume of 10% acetic acid and keeping the solution overnight at pH 10. Therefore, Solanine purchased from Sigma Aldrich Pvt. Ltd. was used as the reference standard for the standardization procedures.

### *5.3.3) Piper alkaloids:*

Pale yellow coloured crystals (yield~5.3 %) were obtained on recrystallization with acetone and hexane. Chromatographic study showed three bands when developed in Toluene: Ethyl acetate (9:1). The preparative TLC of this solution was performed and the conditions were optimized as follows:

**Table 5.14. Optimized conditions for preparative TLC of Piper species.**

| Parameters | Conditions |
|---|---|
| Size of the Glass plate | 7" X 3.5" |
| Stationary phase | Silica gel G |
| Air drying time for plate | Overnight |
| Activation time for plate | 30 min. at 110 °C |
| Application of Spot | 0.5 ml with marked capillary |
| Solvent system | Toluene: Ethyl acetate (9:1) |
| Chamber saturation Time | 10 min |
| Height of solvent front | 12 cm |
| Rf of Piperine | 0.22 |

The reference standard piperine purchased from Sigma Aldrich Pvt. Ltd. was used to determine the Rf value of piperine. The band matching with the Rf value (~0.22) of standard piperine was scrapped and centrifuged in methanol at 3000 rpm for 15 minutes. The supernatant was collected and concentrated. The same procedure was repeated several times. Pure needle shaped crystals (1.06 %) of

piperine (M.P. 230 °C) were obtained and further purified by recrystallization with acetone. This compound showed single spot on TLC using same solvent system which turned dark orange on spraying dragendorff's reagent.

## 5.4) Standardization of procedure for formulation preparation

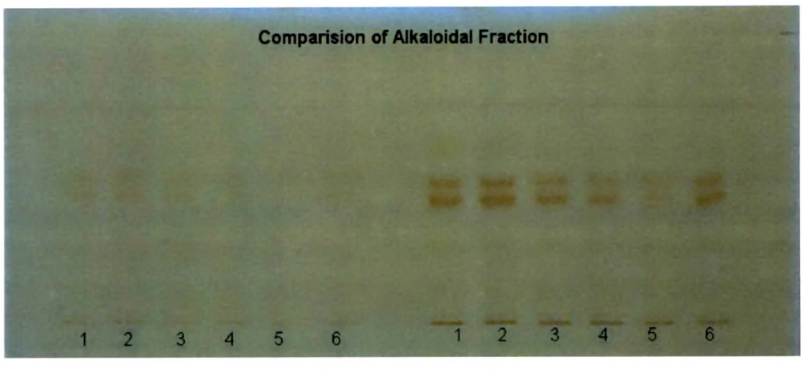
### *5.4.1) Aconitum alkaloids:*

#### *5.4.1.1) HPTLC studies.*

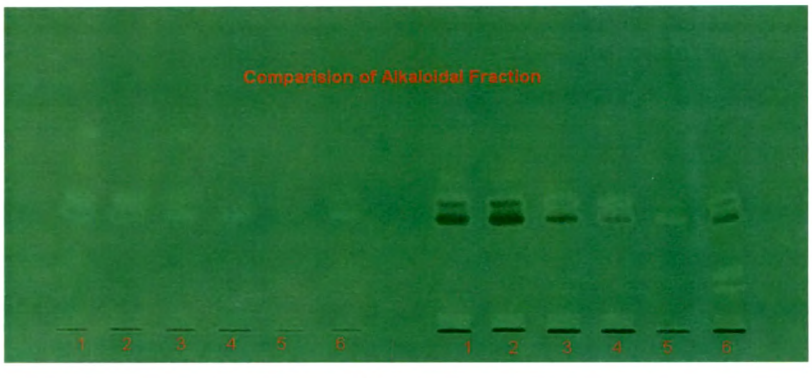
The contents of *Aconitum* alkaloids in different extracts of processed aconite roots and unprocessed aconite roots were compared using HPTLC method developed. The representative HPTLC chromatograms of the unprocessed aconite roots, the intermediate roots and the processed roots, are shown in Fig.5.5(a,b,c) and Fig. 5.6(a,b,c,d,e,f), respectively. The tracks from 1-6 showed that there was a gradual decrease in the concentration of two alkaloids, with increase in concentration of other alkaloids(Table 5.15). It has been reported[97] in earlier studies that the acetyl group at the 8-postion in the diester-type alkaloids is prone to hydrolysis in basic conditions into monoester alkaloids. The results demonstrated in the HPTLC studies also depict that some form of chemical degradation of the aconitum alkaloids has occurred. The degradation may thus be due to the hydrolysis of the ester bonds during the course of processing according to reported studies.

**Table 5.15. Results of the Finger printing analysis of different samples of Aconitum alkaloids.**

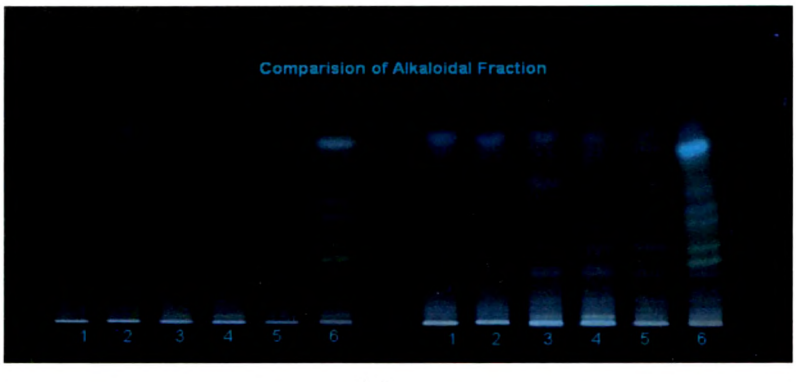
| Sample | Concentration µg per spot | Peak | Rf value | Height | Area | % Height | % Area |
|---|---|---|---|---|---|---|---|
| A-1 | 100 | AG-1 | 0.42 | 210.3 | 6295.1 | 44.77 | 41.28 |
| | 100 | AG-2 | 0.48 | 149.7 | 5618.0 | 31.86 | 38.58 |
| A-2 | 100 | AG-1 | 0.42 | 160.6 | 6092.4 | 41.12 | 40.21 |
| | 100 | AG-2 | 0.48 | 144.3 | 5294.0 | 29.26 | 32.79 |
| A-3 | 100 | AG-1 | 0.42 | 148.9 | 5748.1 | 30.00 | 35.40 |
| | 100 | AG-2 | 0.48 | 135.3 | 5070.5 | 26.25 | 33.28 |
| A-4 | 100 | AG-1 | 0.42 | 129.9 | 5309.5 | 24.88 | 28.74 |
| | 100 | AG-2 | 0.48 | 126.8 | 5142.2 | 24.01 | 32.98 |
| A-5 | 60 | AG-1 | 0.42 | 71.5 | 2048.1 | 20.30 | 25.22 |
| | 60 | AG-2 | 0.48 | 79.7 | 2296.7 | 21.04 | 29.14 |
| A-6 | 100 | AG-1 | 0.42 | 114.6 | 4974.6 | 16.39 | 22.06 |
| | 100 | AG-2 | 0.48 | 100.3 | 5325.6 | 14.89 | 24.72 |




(a)

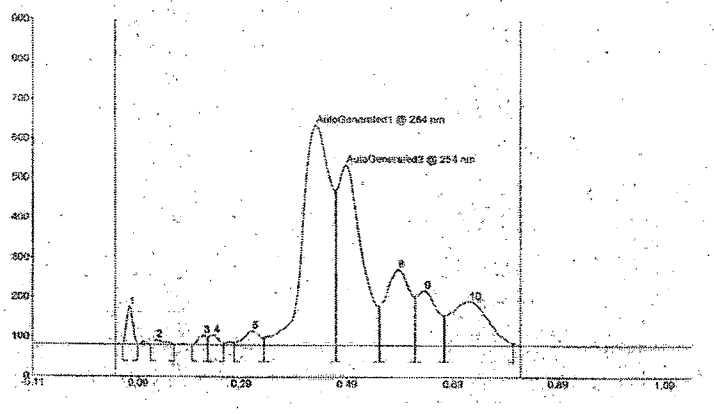



(b)




(c)

**Fig5.5. HPTLC Chromatograms of Aconitum alkaloids (a) Image at 235 nm, (b) Image at 254 nm and (c) Image at 366 nm.**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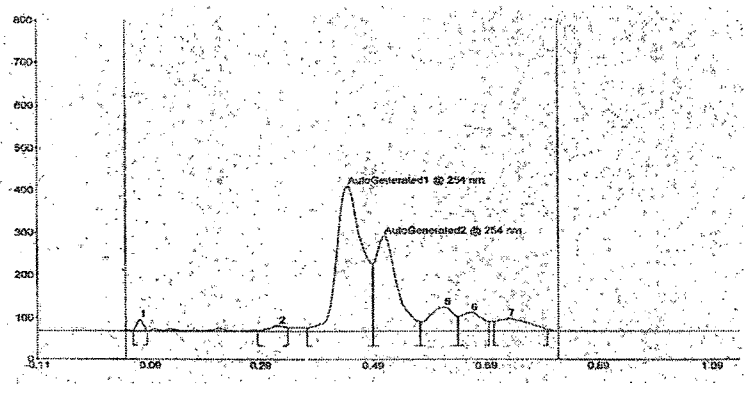
(a)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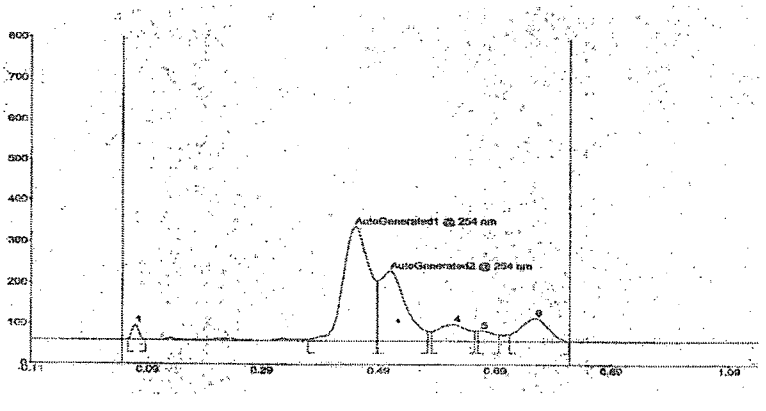
(b)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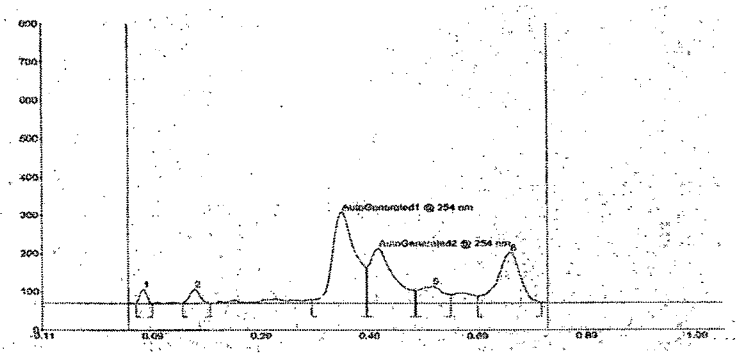
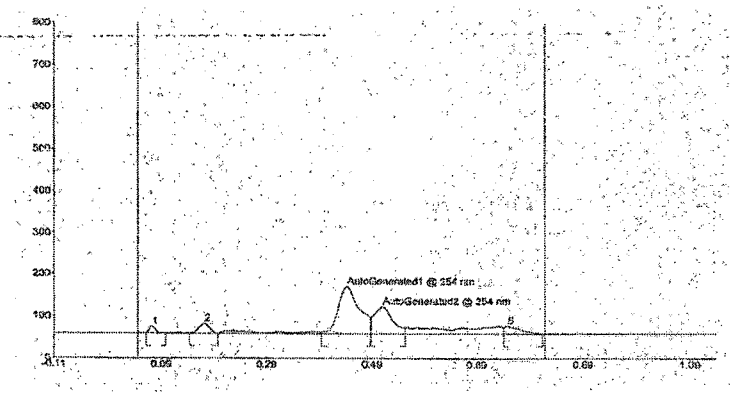
AutoGenerated2 @ 254 nm

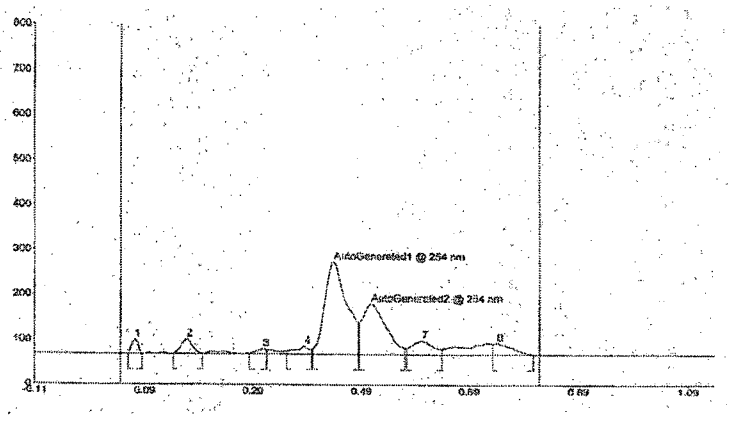
(c)




(d)




(e)




(f)

**Fig5.6 HPTLC Chromatograms of Alkaloid fractions A-1(a), A-2 (b), A-3 (c), A-4 (d), A-5 (e), A-6 (f).**

***5.4.1.2) IR studies:***

The alkaloid fraction of the raw materials, intermediate processed roots and final products were subjected to IR studies. The Peaks obtained in the six samples have been shown in Table 5.16. and the interpretation of the peaks is mentioned in Table 5.17. The IR spectra of all the samples have been depicted in Fig 5.7.

**Table 5.16. Peaks present in the IR spectra of alkaloid fractions**

| A1 | A2 | A3 | A4 | A5 | A6 |
|---|---|---|---|---|---|
| **Peaks, $cm^{-1}$** | | | | | |
| **3435** | **3436** | **3417** | **3420** | **3422** | **3372,2958** |
| 2929 | 2930 | 2930 | 2928 | 2929 | 2924 |
| **2819** | **2821** | **-------** | **-------** | **2851** | **2852** |
| **1720** | **1717** | **1713,1659** | **1710,1655** | **1708,1670** | **1676** |
| 1601 | 1601 | 1601 | 1601 | 1602 | 1597 |
| 1514 | 1515 | 1515 | 1515 | 1514 | 1514 |
| 1462 | 1463, 1419 | 1463,1455 | 1461 | 1459 | 1455 |
| 1368 | 1369 | 1372 | 1370 | 1376 | 1378 |
| **1294** | **1295** | **-------** | **--------** | **--------** | **------** |
| 1271 | 1271 | 1271 | 1271 | 1271 | 1271 |
| 1224 | 1224 | 1223 | 1224 | 1223 | 1223 |
| 1177 | 1177 | 1177 | 1177 | 1177 | 1176 |
| 1096 | 1097 | 1101 | 1101 | 1101 | 1101 |
| 1024 | 1024 | 1037 | 1037 | 1036 | 1036 |
| **985** | **985, 803** | **984, 878** | **984,935, 878** | **985,927, 874,** | **915** |
| 765 | 765 | 765 | 764 | 765 | 765 |
| | **689** | | | **611** | **612** |

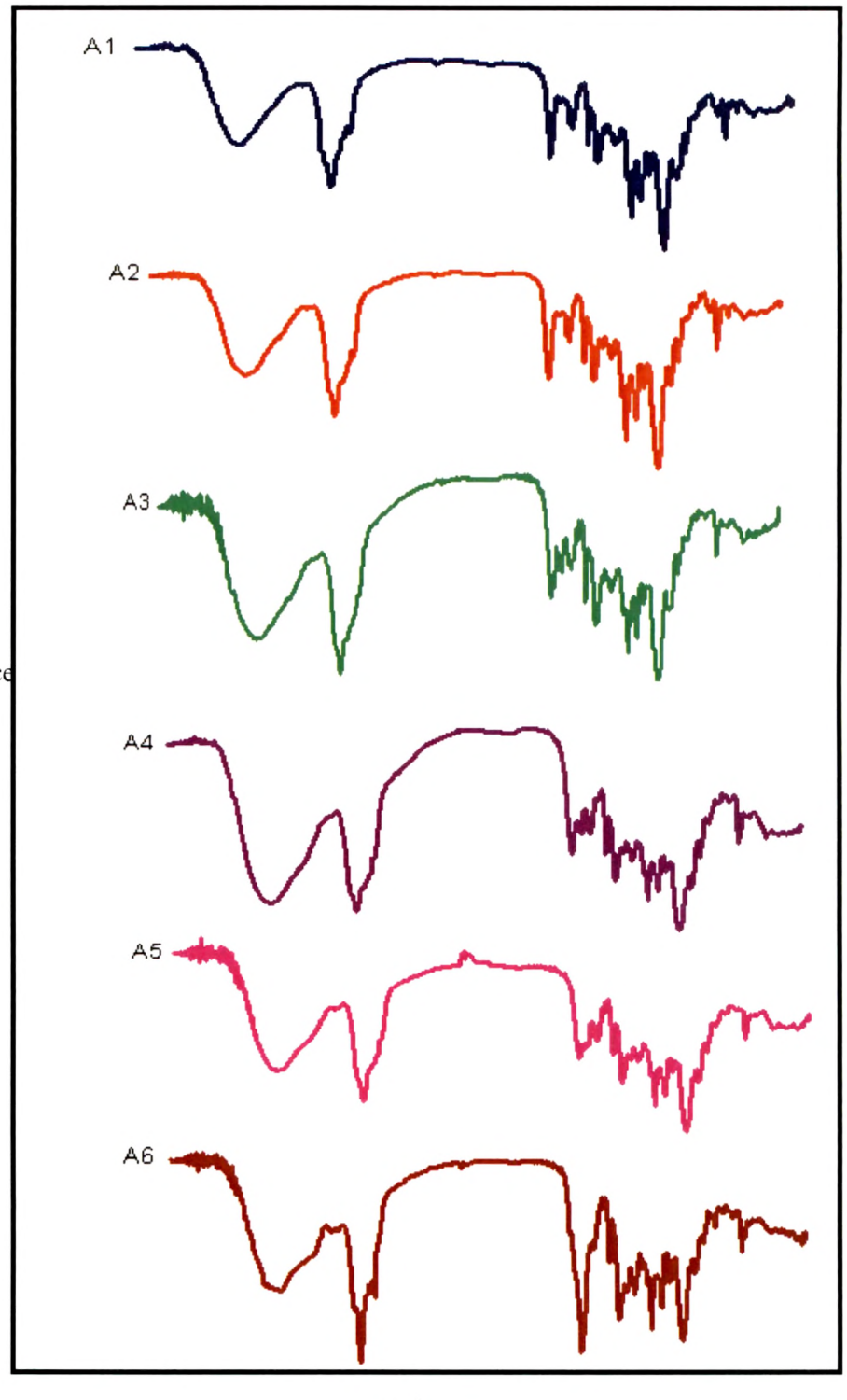
**Table. 5.17. The Functional goups depicted by the Peaks in IR Spectra.**

| Peak | Functional group |
|---|---|
| 3435 | O-H stretch |
| 2929 | Alkyl C-H stretch |
| 2819 | Alkyl C-H stretch |
| 1720 | C=O stretching of esters |
| 1676 | C=O stretching of Ketones. |
| 1601 | C=C stretching of ring |
| 1514 | N-H bending vibrations |
| 1462 | C-H Symmetrical bending vibrations of cycloalkanes |
| 1362 | C-H Assymmetrical bending vibrations of cycloalkanes |
| 1294 | C-O or O-H stretch |

| | |
|---|---|
| 1271 | Assymmetrical C-O-C stretch |
| 1224 | C-O stretch |
| 1177 | C-C(=O)-O saturated esters |
| 1096 | Symmetrical C-O-C stretch |
| 1024 | Aromatic ethers (O-$CH_2$) |
| 985 | Out of plane –C-H bend |
| 765 | Out of plane –C-H bend |

The peak at 1720 $cm^{-1}$ which denotes the C=O stretching of aromatic esters disappears gradualy till A-6. The peak has been replaced with a peak at 1676 $cm^{-1}$ depicting the conversion to a keto-group. The reduction in the frequency may be due to steric effect that reduce the co-planarity of the structure. The peak at 1294 $cm^{-1}$ is also lost which depicts the C-O or O-H stretch. Thus, there is an alteration in the ester group of the alkaloids.

**Fig. 5.7. IR Spectra of Aconitum Alkaloids**



***5.4.1.3) Inference:***

From the HPTLC and IR studies, it is evident that a number of alkaloids have been converted to other types of alkaloids. The results show that there were significant differences in alkaloid contents between the processed and unprocessed aconite roots, i.e., the processing appeared to have markedly changed the content of a number of alkaloids.

It has been reported in earlier studies[97] that the diester alkaloids of Aconitum alkaloids are prone to hydrolysis. The purification procedure may thus be responsible for the chemical degradation of the diester alkaloids. The study indicates that the traditional methods should be followed very carefully. It has been reported that the diester alkaloids are toxic as compared to the monoester alkaloids. The monoester alkaloids are significantly effective in anti-inflammation and analgesia in animals, and their analgesic potency is equivalent to that of processed aconite roots. Toxicological studies have demonstrated that the toxicity of diester alkaloids is almost the same with $LD_{50}$ values for mice per injection about 0.15 mg/kg body weight, while the the hydrolyzed monoester alkaloids show much lower toxicity. It has also been reported in the toxicological data that the monoester alkaloids have higher $LD_{50}$ values as compared to the diester alkaloids[98-99].

The processing of the aconitum alkaloids thus involve the hydrolysis of acetyl group which is depicted in the IR spectra. However, due to the presence of a number of alkaloids in the alkaloid fraction, the exact chemical changes cannot be predicted. The IR spectras may thus be used as reference for comparison of the raw materials as a quality control parameter. The changes in the processed roots can be checked using the standard IR spectras.

Therefore, if the safety and efficacy of medicines containing aconitum roots in clinical use is to be guaranteed, the contents of the alkaloids must be compared. Moreover, the clinical dosages of *Mahamrutyunjaya rasa* were normally high (from 250 mg/daily), thus the content of total toxic alkaloids administrated daily is high in patients. If limits are placed on the quantity of total toxic alkaloids in proprietary medicines, this would be helpful for safety treatment and also helpful for good manufacturing practices regarding quality control and assurance.

### 5.4.2) Sulphur

#### *5.4.2.1) Powder X-Ray Diffraction Analysis*

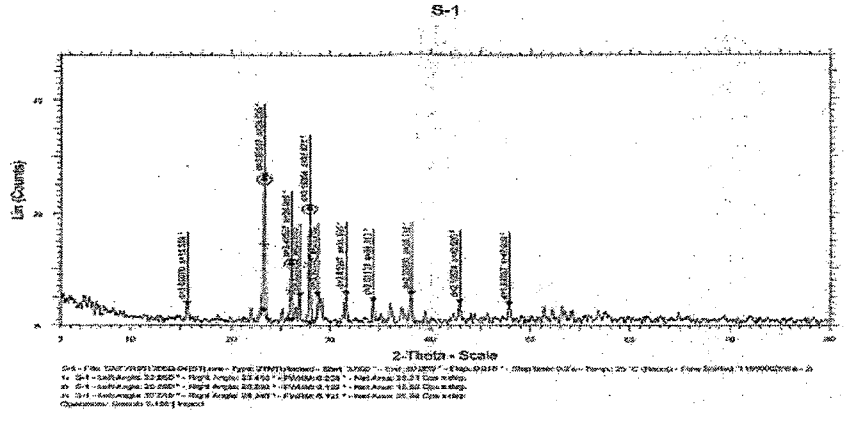
Fig 5.8(a,b,c,d,e) shows the powder XRD patterns of the raw material, intermediates and the final purified component. The d-spacing values of sulphur samples were compared with the reference standards of various allotropes of sulphur and the values coinciding were compared. The pattern of S-1 shows that the raw material *gandhaka* (sulphur) has a number of peaks coinciding with the reference orthorhombic sulphur in the Fddd space group. The diffraction pattern of S-2 shows that the number of peaks coinciding with that of orthorhombic sulphur increase depicting the increase in the orthorhombic form of sulphur crystals. The S-3 XRD pattern shows a decrease in the intensity of a number of peak. The pattern of S-4 shows the presence of only two major peaks which are found to be present in monoclinic type of sulphur crystal. The intensity of the two peaks is very high. Again in the S-5 sample the peaks are found to coincide with the orthorhombic sulphur with a decrease in the intensity as compared to S-4. However, the peaks are even sharper than those of the other samples and so reflect higher purity of the final product.

**Table.5.18. XRD data of samples and standard Sulphur.**

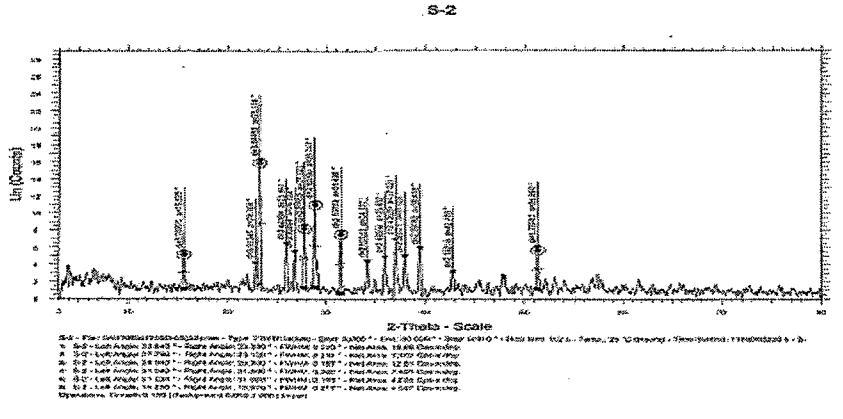
| S-1 d(Å) | Orthorhombic d(Å) |
|---|---|
| 3.825 (a=23.235) | 3.85 (a=23.083) |
| 3.198 (a=27.871) | 3.219 (a= 27.690) |
| 3.422 (a= 26.016) | 3.447 (a=25.826) |
| S-2 d(Å) | Orthorhombic d(Å) |
| 3.844 (a=23.114) | 3.85 (a=23.083) |
| 3.102 (a=28.757) | 3.113 (a=28.653) |
| 3.215 (a=27.721) | 3.219 (a=27.690) |
| 3.443 (a=25.851) | 3.447 (a=25.826) |
| 3.331 (a=26.743) | 3.336 (a=26.315) |
| 2.838 (a= 31.499) | 2.848 (a=31.385) |
| S-3 d(Å) | Orthorhombic d(Å) |
| 3.834 (a=23.178) | 3.85 (a=23.083) |
| 3.099 (a=28.77) | 3.084 (a=28.653) |

| | |
|---|---|
| 3.321 (a=26.823) | 3.336 (a=26.701) |
| S-4 d(Å) | Monoclinic d(Å) |
| 3.781 (a=23.505) | 3.803 (a=23.372) |
| 3.166 (a=28.156) | 3.168 (a=28.145) |
| S-5 d(Å) | Orthorhombic d(Å) |
| 3.853 (a=23.174) | 3.85 (a=23.083) |
| 3.084 (a=28.927) | 3.084 (a= 28.928) |
| 3.312 (a=26.891) | 3.336 (a= 26.701) |
| 3.205 (a=27.81) | 3.219 (a=27.690) |

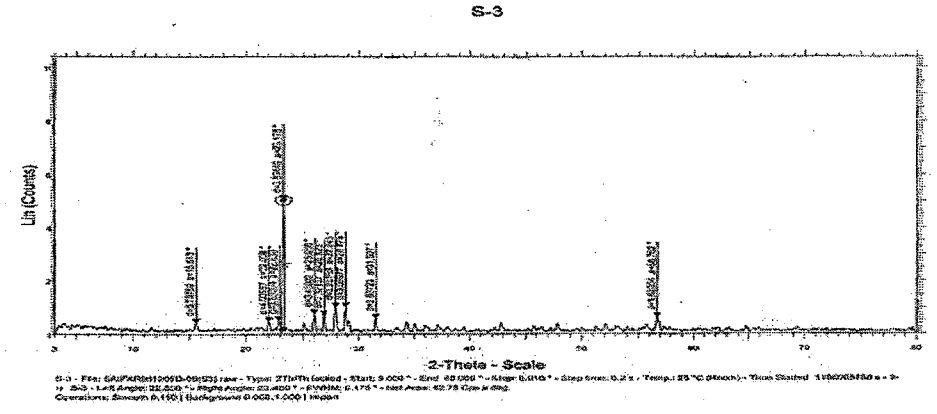
a= 2 Theta (deg.)



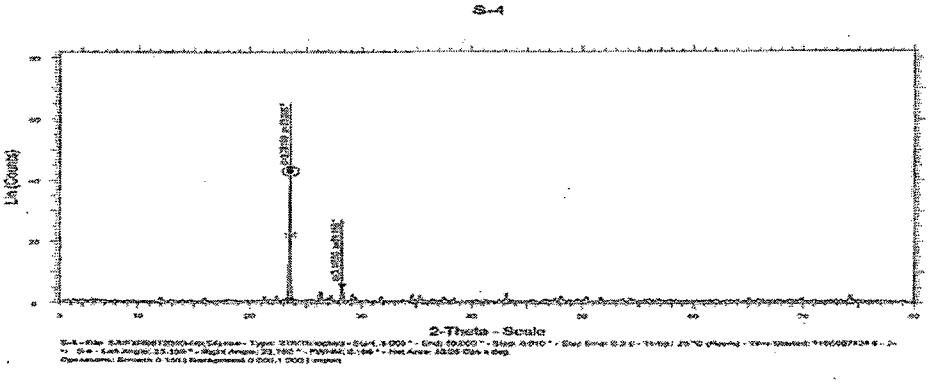

(a)



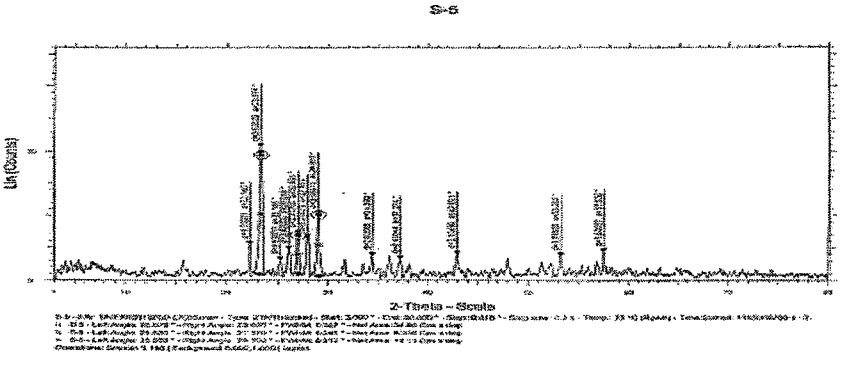

(b)




(c)




(d)




(e)

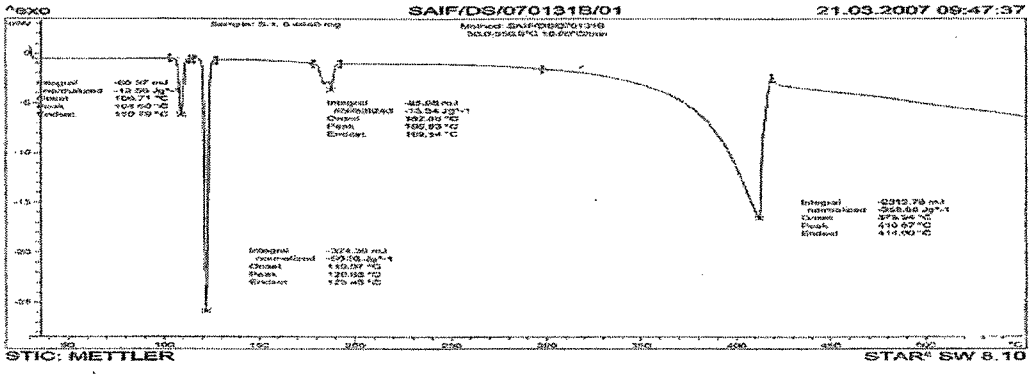
**Fig.5.8. X-ray diffraction spectra of S-1(a), S-2 (b), S-3 (c), S-4 (d), S-5 (e).**

***5.4.2.2) Thermal Studies***

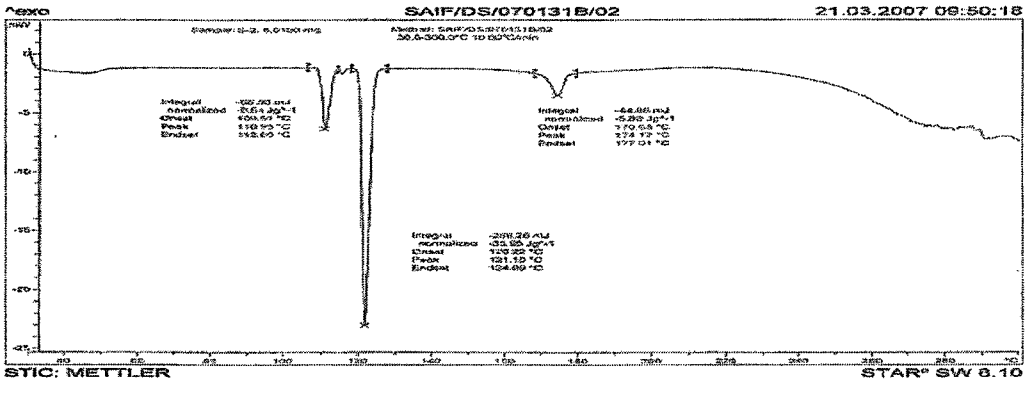
The samples of treated sulphur were analyzed by thermal analysis using Differential Scanning Calorimeter. The Differential thermograms of the raw materials, intermediates and the final products are shown in Fig.5.9. The Table 5.19 shows the peaks observed for each sample. All the sample thermograms showed two sharp peaks upto the temperature of 122°C. S-4 thermogram showed a peak at 75.97°C which may be due to the removal of the hydrates. The hydrates may have been formed during the washing of the sulphur with hot water. The peaks displayed for S-1, S-2 and S-5 are similar with changes in the number and sharpness of the peaks. A small endothermic peak was observed at 115.61°C in the S-5 sample which may be due to a different type of allotrope of sulphur formed during the heating procedure.

**Table.5.19. Peak values of Samples of Sulphur using DSC.**

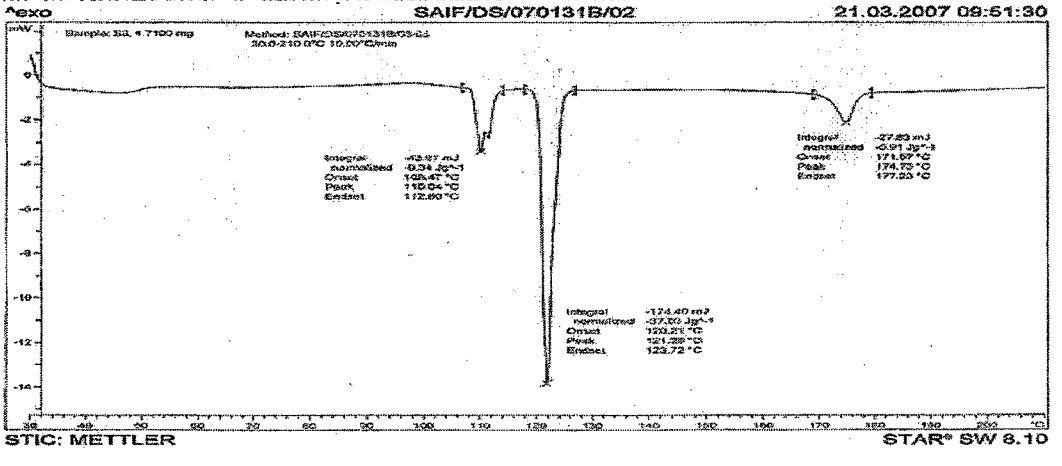
| S-1 | S-2 | S-3 | S-4 | S-5 |
|---|---|---|---|---|
| ---------- | ---------- | ---------- | 75.97°C | ---------- |
| 108.60°C | 110.93°C | 110.04°C | 108.49°C | 111.55°C |
| ---------- | ---------- | ---------- | ---------- | 115.61°C |
| 119.97°C | 121.10°C | 121.29°C | 121.01°C | 121.31°C |
| 186.83°C | 174.17°C | 174.73°C | 173.96°C | 174.74°C |
| 410.87°C | ---------- | ---------- | ---------- | ---------- |



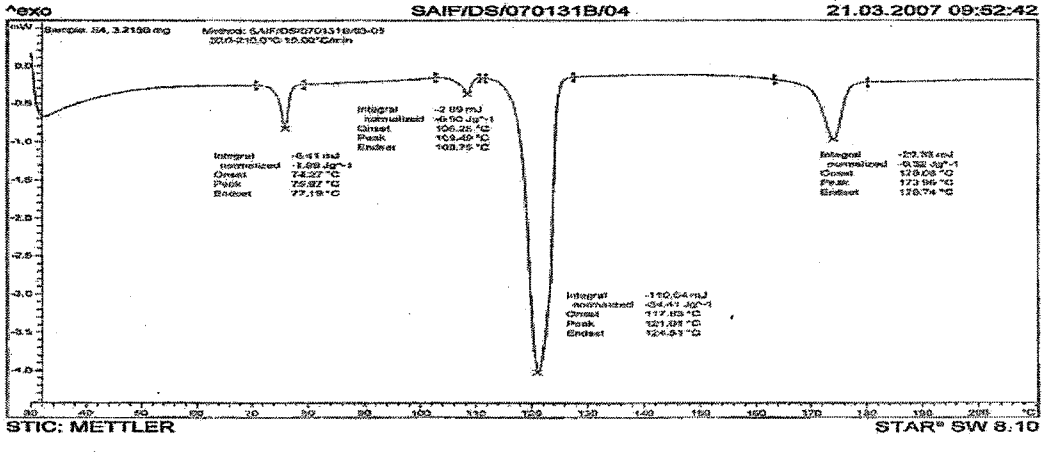

(a)


^exo
SAIF/DS/070131B/02
21.03.2007 09:50:18
STIC: METTLER
STAR° SW 8.10

(b)


^exo
SAIF/DS/070131B/02
21.03.2007 09:51:30
STIC: METTLER
STAR° SW 8.10

(c)


^exo
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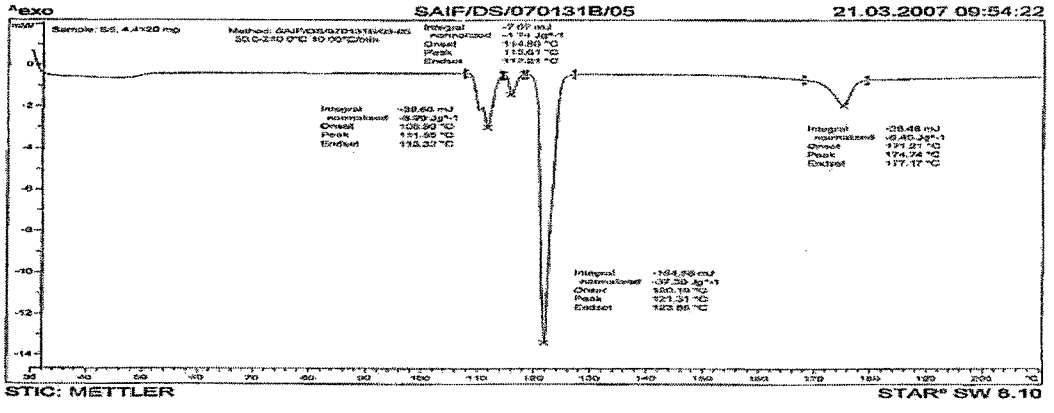
21.03.2007 09:52:42
STIC: METTLER
STAR° SW 8.10

(d)




(e)

**Fig. 5.9. Differential thermograms of Fig. X-ray diffraction pattern of S-1(a), S-2 (b), S-3 (c), S-4 (d), S-5 (e).**

***5.4.2.3) Inference***

Sulphur is one of the *chalcogenides*, or "ore-formers" which occupies VIA column of the periodic table. It is a non-metal with atomic number of 16 and atomic weight 32.07. It is the 15th element in order of abundance in the lithosphere, to the amount of 0.052%. Sulphur atoms aggregate, with each one sharing one of its electron pairs with the preceeding atoms, and form long chains. The long chains form rings due to the unstable end atom. The smallest ring with no strains consists of 8 atoms. It forms a crown-shaped structure with 4 atoms on top and 4 atoms on the bottom, rotated relatively by 45°C. In any sample of sulphur, thermal agitation is constantly breaking and reassembling chains, so they gradually assume the most stable state for the particular temperature. The yellow colour of sulphur is probably due to a small number of thermally-generated unterminated chains. The $S_8$ molecule is stable, and exists in solid, liquid and gaseous sulphur. Two crystalline forms compete, orthorhombic α-sulphur and monoclinic β-sulphur. Below 96°C, the orthorhombic form is more stable and the conversions between the two forms are slow. α-Sulphur and β-Sulphur melt to a straw-yellow liquid called λ-sulphur. If the liquid is cooled slowly, needle-like monoclinic crystals form. When the temperature falls below 96°C, these crystals slowly change to orthorhombic microcrystals, which is shown by the solution becoming cloudy. If the liquid is heated further, at about 200 °C it darkens to a reddish colour and becomes viscous. The $S_8$ rings are thermally broken, and recombine to form

long chains, called μ-sulphur. The dark colour is due to the free valences at the ends of the chains. If this dark sulphur is suddenly cooled by being poured into water, it becomes a rubbery mass of tangled chains. This was called amorphous sulphur, but is really a kind of glass. On standing, it eventually reverts back to $S_8$ and crystallizes.

Sulphur as is evident from XRD S-1, is largely of orthorhombic crystalline nature and is probably the mixture of α-Sulphur and small amounts of β-sulphur, displayed by two sharp endothermic peaks at about 109°C and 122°C in the differential thermogram. There is no significant difference between the DSC as well as XRD of S-1 and S-2, suggesting no interaction between sulphur and ghee. This also suggests amorphous nature of cow ghee, with absence of any additional peak in XRD pattern of sample S-2. XRD patterns of sample S-3 and S-4 display changes as compared to the raw material. However, DSC of S-3 is more or less same as that of S-2. Significant change is observed in DSC of S-4 wherein additional endothermic peak is observed at 76°C which may be due to the removal of the hydrates. The hydrates may have been formed during the washing of the sulphur with hot water. All endotherms in S-4 DSC are with lesser energy changes. As also seen in XRD patterns, the sample tends to display the presence of monoclinic sulphur.

The XRD and DSC patterns of S-5, display that the structure of S-4 reverts back to the $S_8$ orthorhombic sulphur. Further, the unwanted components are also reduced which may be observed by the sharpness of the peaks in the X-ray diffraction pattern of S-5.

Thus, from the above study it can be concluded that the processing of sulphur using the traditional methods brings about purification, reducing the toxic nature of sulphur. By impregnating with organic material, like ghee, sulphur is made homologous to the tissue cells and their toxicity is reduced and acceptability to the cell is increased. The above study can therefore be used as a quality control standard for the raw materials. The raw materials added in formulation should be screened by the above techniques and compared with the standard data in order to ascertain the quality of the sulphur added in preparation of *Mahamrutyunjaya rasa.*

### 5.4.3) Cinnabar:

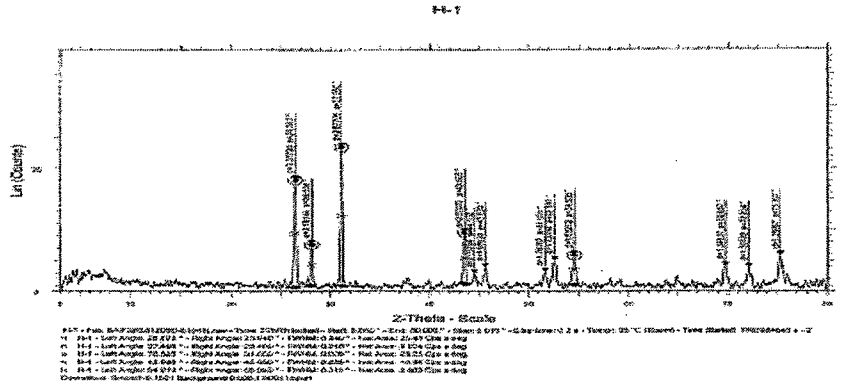
#### 5.4.3.1) Powder X-Ray Diffraction Analysis

Fig 5.10 shows the powder X-ray diffraction patterns of the raw materials, intermediates and the final product. The pattern of H-1 when compared with the reference XRD pattern shows that the raw material cinnabar is present alongwith few other components. In the intermediate sample, H-2 pattern, the peaks of the components other than cinnabar are reduced. Further in the final XRD pattern of H-3 the number of peaks are absent and the intensity of the peaks is increased. The d-spacing values of H-3 matched with the reference data showing high purity of cinnabar in the trigonal trapezohedral cystalline form.(Table 5.20).

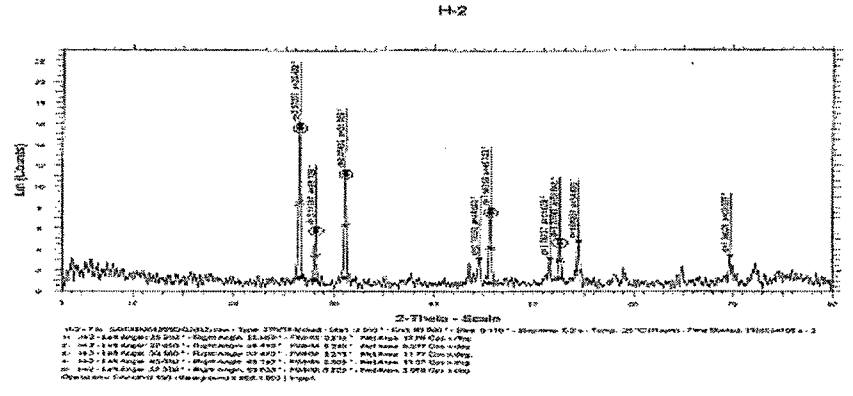
**Table 5.20. XRD data of samples and standard Cinnabar.**

| H-1 d(Å) | HgS d(Å) |
|---|---|
| 3.337 (a= 26.363) | 3.36 (a= 26.507) |
| 3.181 (a= 28.024) | 3.181 (a= 28.218) |
| 2.877 (a= 31.060) | 2.85 (a= 31.362) |
| 2.078 (a= 43.50) | 2.06 (a= 43.917) |
| 1.682 (a= 54.508) | 1.67 (a= 54.937) |
| H-2 d(A) | HgS d(A) |
| 3.370 (a=26.422) | 3.36 (a= 26.507) |
| 3.171 (a=28.109) | 3.181 (a= 28.218) |
| 2.874 (a=31.089) | 2.85 (a= 31.362) |
| 2.030 (a= 44.593) | 2.06 (a= 43.917) |
| 1.682 (a= 54.490) | 1.67 (a= 54.937) |
| H-3 d(A) | HgS d(A) |
| 3.358 (a= 26.517) | 3.36 (a= 26.507) |
| 3.162 (a= 28.199) | 3.181 (a= 28.218) |
| 2.862 (a= 31.224) | 2.85 (a= 31.362) |
| 2.071 (a= 43.665) | 2.06 (a= 43.917) |
| 1.677 (a= 54.677) | 1.67 (a= 54.937) |

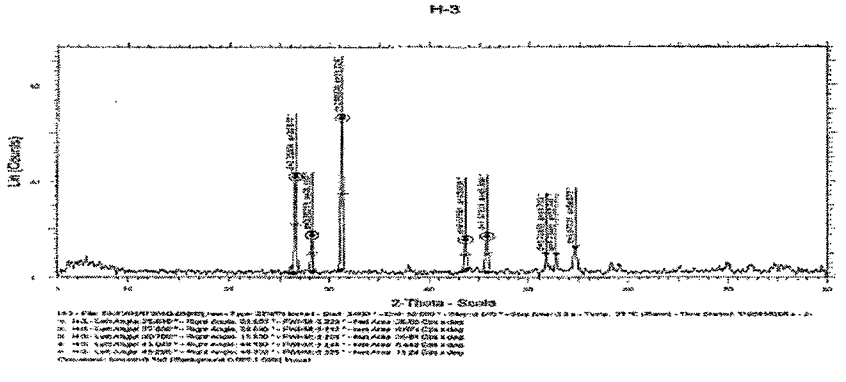
a- 2Theta (Deg)




**(a)**




**(b)**




**(c)**

**Fig.5.10. X-ray diffraction spectra of H-1 (a), H-2 (b), H-3 (c).**

***5.4.3.2) Inference***

Mercury occurs naturally in the metallic form and/or its sulfide ore form such as cinnabar (HgS). A small concentration of mercury is found throughout the lithosphere, the atmosphere, the hydrosphere and the biosphere. The speciation and transformation of mercury-sulphur systems is important in understanding the transport, toxicity and bioavailablity of mercury in the human body. Mercury is of great concern in the environment due to its toxicity and tendency to bioaccumulate and biomagnify. Mercury's toxicity and bioavailability are reduced in the form of cinnabar (HgS) because HgS is relatively insoluble and unavailable for methylation processes, which produce the most toxic forms of mercury. The dissolution of HgS, however, is significantly enhanced in the presence of dissolved organic matter.

*5.4.3.2.1) Removal of physical and chemical impurities*

In nature mercury is seldom available in its physically and chemically pure form. The contamination is natural and it takes place in the mine itself. Often contamination takes place knowingly or unknowingly during the process of extraction, storage, preservation and distribution. All this extraneous material are to be removed and mercury should be obtained in prescribed form for obtaining the desired therapeutic effect. Metals, even in their physically and chemically pure form might produce adverse effects because they are inorganic in nature and they are heterogeneous to the body tissue. By impregnating and triturating with organic material, like juices, decoctions of herbs etc., they are made homologous to the tissue cells and their toxicity is reduced and acceptability to the cell is increased. During this process certain organic and inorganic materials are added to mercury, which helps to increase its medicinal efficacy and safety. Metals used as such are heterogeneous to the body tissue and it will not be possible for them to get assimilated in the cell of the tissue to exercise their prescribed therapeutic effects. So it must be reduced to a fine state of division which would be homologous with the composition of the cells on which mercury has to act to produce the therapeutic effect. This fine state of metal is achieved by repeated trituration or pulverization.

The above study can therefore be used as a standardization tool. The raw material added in formulation should be screened by the above technique and

compared with the standard values in order to ascertain the quality standards of Cinnabar.

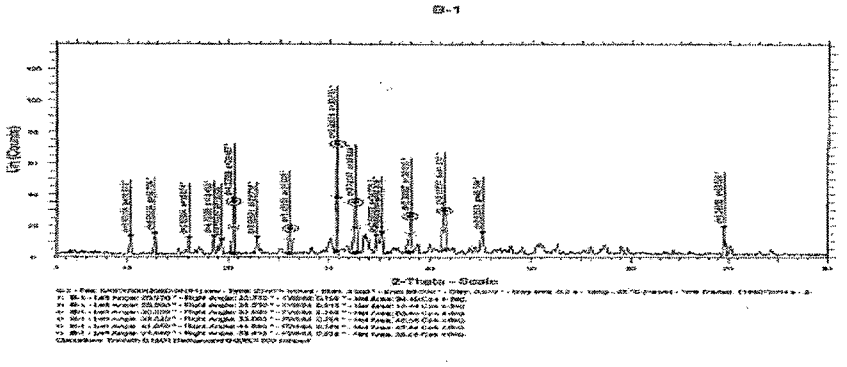
### *5.4.4) Sodium Metaborate:*

#### *5.4.4.1) Powder X-ray Diffraction Studies*

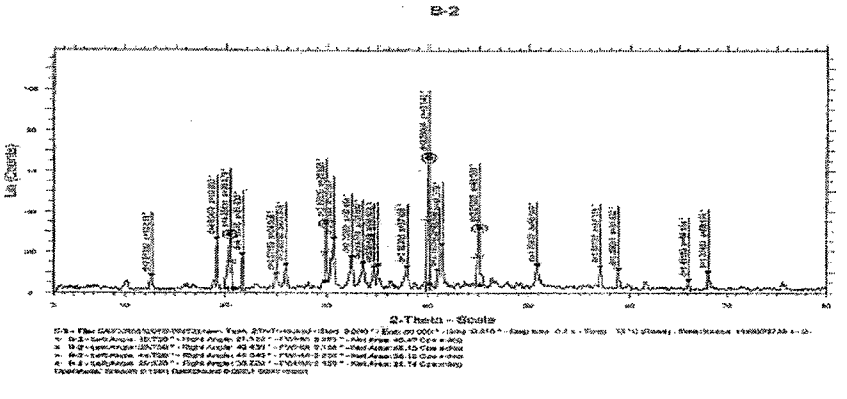
Fig.5.11 shows the powder X-ray diffraction patterns of the raw materials, intermediate and the final product. The XRD spectra of sodium metaborate depicted that there was a gradual loss in the sharpness and number of peaks from B-1 to B-4.

**Table.5.21. XRD data of samples of sodium metaborate.**

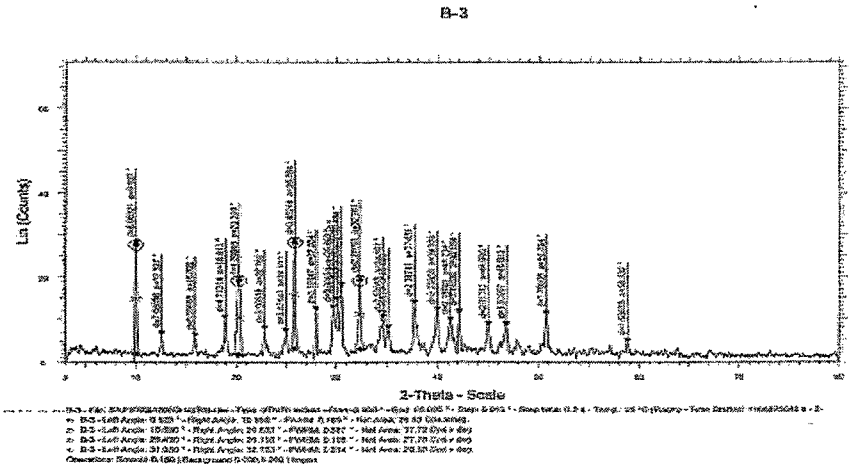
| B-1 d(A) | B-2 d(A) | B-3 d(A) | B-4 d(A) |
|---|---|---|---|
| 2.90 | 2.25 | 3.45 | 2.925 |
| 4.33 | 2.99 | 8.903 | 2.362 |
| 2.745 | 2.01 | 4.39 | 2.256 |



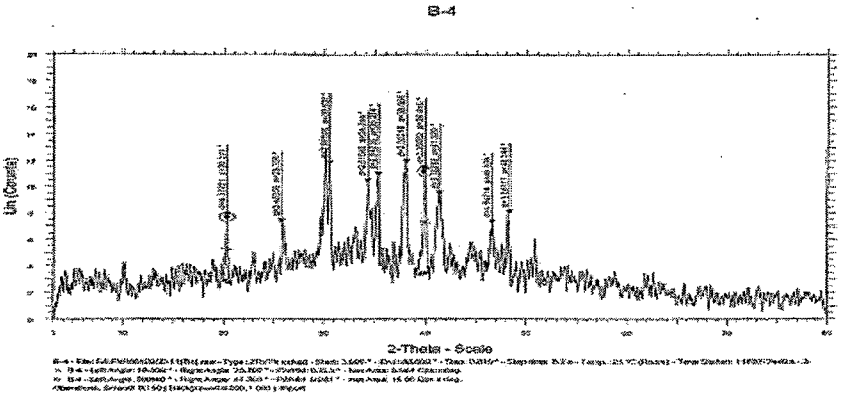
**(a)**



**(b)**




**(c)**




**(d)**

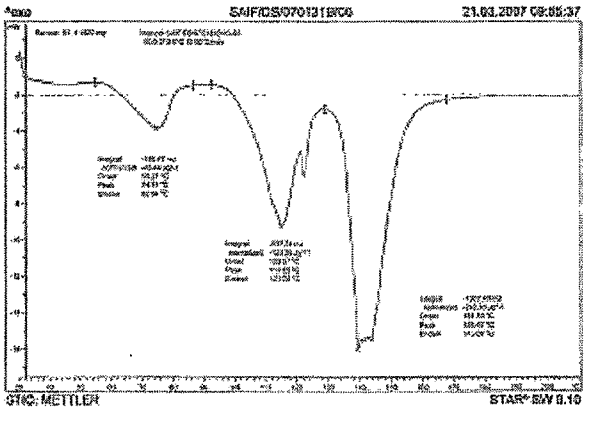
**Fig. 5.11. XRD spectra of B-1 (a), B-2 (b), B-3 (c), B-4 (d).**

***5.4.4.2) Thermal Studies***

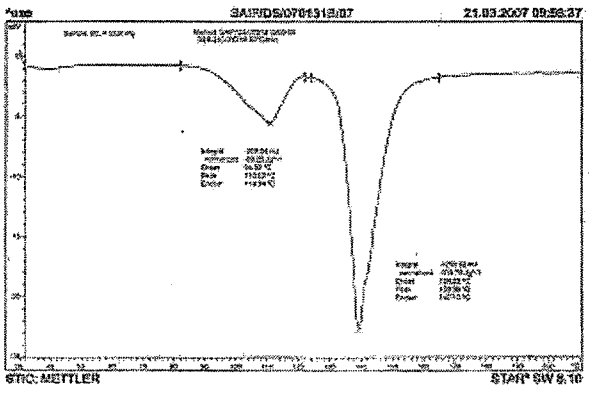
The Differential thermograms of B-1 show an extra peak at 74.71 °C which may be due to the presence of solvates of water, while the peak is missing in the differential thermogram of B-2. Further, the range of peak onset and endset is narrow in the B-3 thermogram as compared to B-1 and B-2. A gradual increase in the sharpness of an endothermic peak at 137 °C was observed. The peak at 110.62 °C of B-2 is slightly shifted to 104.22 °C in B-3, which may be due to some change in the crystal structure of the final product.

**Table 5.22. DSC Peaks of Sodium metaborate.**

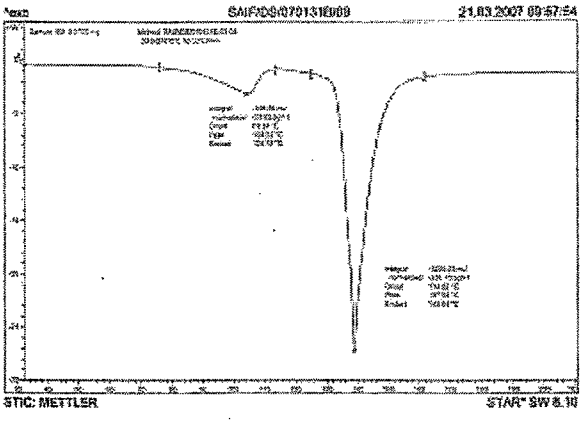
| B-1 | B-2 | B-2 |
|---|---|---|
| 74.71°C | --------- | --------- |
| 114.65 °C | 110.62 °C | 104.22 °C |
| 138.67 °C | 138.39 °C | 137.54 °C |



**(a)**



**(b)**




**(c)**

**Fig. 5.12. Differential thermograms of B-1 (a), B-2 (b), B-3 (c).**

***5.4.4.3) Inference:***

From the results of XRD and DSC studies, the actual physicochemical changes cannot be predicted due to absence XRD patterns of reference substances. However, marked changes are observed in the XRD and DSC patterns. The purity of the raw material can be ascertained from the DSC study as the sharpness of the endothermic peak at 137°C increased markedly even when low concentration of sample was analyzed. The above study can therefore be used as a standardization tool. The raw material added in formulation should be screened by the above technique and compared with the standard values in order to ascertain the quality standards of Sodium metaborate.

## 5.5) Physical Standardization of the three Formulations.

### *5.5.1) Determination of Physical properties as per IP*

Both the marketed tablets fulfilled the official tests for uniformity of weight. Other physiochemical properties such as, hardness, thickness, diameter, and friability results were determined and presented in Table 5.23.

**Table.5.23. Results of Official tests.**

| Sr. No. | Parameters | F2 | F3 |
|---|---|---|---|
| 1. | Uniformity of Weight (mg ± SD) | 63 ± 3.12 | 65 ± 4.67 |
| 2. | Disintegration test, minutes(n=10) | 13.5 ± 0.5 | 8 ± 0.2 |
| 3. | Hardness, kg/cm$^2$ (n=10) | 5.6 ± 0.01 | 4.7 ± 0.02 |
| 4. | Diameter, mm (n=10) | 5.1 ± 0.09 | 5 ± 0.2 |
| 5. | Thickness, mm (n=10) | 5.2 ± 0.1 | 4.4 ± 0.12 |
| 6. | Friability, % | 0.45-0.51 | 0.64-0.72 |

The results obtained for the offical tests showed that the physical properties of both the formulations were different. The disintegration time of FORM2 was very high as compared to FORM3. The hardness value was also high for FORM2. However, the friability test showed higher value in FORM3 formulation. The deviation in size was not observed in both the formulations. FORM2 was found to be very hard with slow rate of disintegration which would release the active ingredients slowly, where as FORM3 was found to be prone to breakage due to high friability. Thus, both the tablets did not comply with all the limits prescribed in IP.

### *5.5.1) Determination of Ash:*

The Table 5.24 shows the ash values of all the three formulations. The ash value is high due to presence of inorganic ingredients in the formulation.

**Table.5.24. Ash values of the three formulations(n=3).**

| Sample | Total ash | Acid insoluble ash | Water soluble ash |
|---|---|---|---|
| FORM -1 | 41.34 ± 1.87 | 32.98 ± 0.78 | 11.98 ± 1.76 |
| FORM-2 | 43.16 ± 1.34 | 31.89 ± 1.12 | 13.87 ± 2.09 |
| FORM-3 | 46.78 ± 0.97 | 36.78 ± 1.54 | 12.06 ± 1.78 |

The inorganic content in all the formulations was found to be very high due to presence of a number of minerals. The total ash and acid insoluble ash of FORM3 was much higher as compared to the two other formulations. The high values signify presence of higher quantities of inorganic contents which may prove to be toxic in nature. Thus, the ash value should be determined to perform the preliminary standardization.

### *5.5.2) Determination of extractable matter*

Coarse powder of the formulations was extracted with methanol and water in a soxhlet extractor; separately, the extract was concentrated and dried under vacuum. The extractive values of the formulations in the methanol and water have been given in Table 5.25.

**Table. 5.25 Extractive values of formulations(n=3).**

| Sample | Water soluble extractive value | Alcohol soluble extractive value |
|---|---|---|
| FORM -1 | 23.87 ± 1.34 | 24.35 ± 1.54 |
| FORM-2 | 21.67 ± 1.89 | 23.89 ± 1.89 |
| FORM-3 | 18.97 ± 1.78 | 19.08 ± 1.90 |

The extractive values of the formulations, FORM1 and FORM2 were found to be higher than that of FORM2 depicting the presence of phytochemicals in the particular solvents in higher quantity. The reason may be higher concentration of mineral components in FORM3.

### *5.5.3) Determination of water and volatile matter*

The % loss on drying of the formulations is given in Table 5.26. The values comply with those of the limits prescribed in the standard monographs.

Table. 5.26. % Loss on drying of formulations.(n=3)

| Sample | % Loss on Drying |
|---|---|
| FORM -1 | 2.13 ± 0.09 |
| FORM-2 | 1.87 ± 0.15 |
| FORM-3 | 1.78 ± 0.11 |

### *5.5.4) Determination of pesticide residues.*

The TLC studies were performed for detection of possible organochlorinated pesticides and organophosphorus pesticides. The results of the analysis are shown in Table 5.27.

Table 5.27. Analysis for Pesticide residues in formulations.

| Sample | Organochlorinated Pesticides | | Organophosphorus pesticides | |
|---|---|---|---|---|
| | A | B | C | D |
| FORM -1 | -ve | -ve | -ve | -ve |
| FORM-2 | +ve | -ve | -ve | -ve |
| FORM-3 | -ve | -ve | -ve | -ve |

A- Solvent system: DMF: Ether (4:6), Detecting Reagent: Tetrabromophenolphthalein.
B- Solvent system: Methylcyclohexane, Detecting Reagent: Tetrabromophenolphthalein .
C- Solvent system: DMF: Ether (4:6), Detecting Reagent: 0.5 % Silver nitrate in water an acetone.
D- Solvent system: DMF: Ether (4:6) , Detecting Reagent: 0.5 %Silver nitrate in water and acetone.

FORM2 was found to contain organochlorinated pesticides which may be due to the presence of pesticides in the raw materials. Thus, the results signify the importance of the tests for pesticides residues on the raw materials before incorporation in the formulation. It should become mandatory for the manufacturers to perform the tests for pesticides residues.

### *5.5.5) Determination of heavy metals.*

Elemental analysis of plant materials was done by Atomic absorption spectrophotometer, where Lead, Cadmium and Arsenic were found to be absent. Mercury was found be present as cinnabar is one of the ingredients of the formulation (Table.5.28)

**Table- 5.28. Heavy metal analysis of formulations.**

| *Sample* | *Cadmium* | *Arsenic* | *Lead* | *Mercury* |
|---|---|---|---|---|
| FORM -1 | -ve | -ve | -ve | +ve |
| FORM-2 | -ve | -ve | -ve | +ve |
| FORM-3 | -ve | -ve | -ve | +ve |

Heavy metals may be present in the raw materials if the preliminary tests have not been performed as per the WHO guidelines. It is an important test as heavy metals are toxic in nature.

### *5.5.6) Determination of microorganisms*

The analysis of bio-burden present in the formulations was performed and all the results were within the limits prescribed in the WHO guidelines (Table 5.29).

**Table 5.29. Results of the tests performed for the microbial contamination in the formulations.**

| *Parameters* | *Aconitum ferox* | *Solanum indicum* | *Piper nigrum* | *Piper longum* |
|---|---|---|---|---|
| Total bacterial count | > 10,000 cfu/gm | > 10,000 cfu/gm | > 10,000 cfu/gm | > 10,000 cfu/gm |
| *E.coli* | Absent | Absent | Absent | Absent |
| *Salmonella. Typhii* | Absent | Absent | Absent | Absent |
| *S,aureus* | Absent | Absent | Absent | Absent |
| *Pseudomonas aeruginosa* | Absent | Absent | Absent | Absent |
| Yeast and mould | Absent | Absent | Absent | Absent |

Microbial bioburden should be tested to determine the safety of the formulations. The natural organic components present in the formulation lead to presence of high microbial contamination. Thus, the test becomes important for the quality control of the formulations.

### *5.5.7) Qualitative Phytochemical Screening:*

The phytochemical screening of the formulations was done by qualitative chemical tests and TLC studies.Qualitative chemical tests were performed for Methanol extract of the formulations. The Methanol extract of FORM1, FORM2 and FORM3 showed the presence of alkaloids, anthranol glycosides, saponins, phenolics, tannins, proteins and amino acids (Table 5.30).

**5.30. Qualitative chemical tests of Methanol extract of formulations.**

| Sr.No. | Class of Compounds | FORM -1 | FORM-2 | FORM-3 |
|---|---|---|---|---|
| 1 | Carbohydrates | -ve | -ve | -ve |
| 2 | Alkaloids | +ve | +ve | +ve |
| | Glycosides | | | |
| 3 | Anthranol | +ve | +ve | +ve |
| | Cardiac | -ve | -ve | -ve |
| | Saponins | | | |
| | Saponins | +ve | +ve | +ve |
| | Steroidal | -ve | -ve | -ve |
| | Phytosterols | | | |
| 5 | Free steroids (Triterpenoids) | -ve | -ve | -ve |
| | Phytosterols | -ve | -ve | -ve |
| 6 | Fixed oils and fats | -ve | -ve | -ve |
| 7 | Phenolics and tannins | +ve | +ve | +ve |
| 8 | Proteins and amino acids | +ve | +ve | +ve |

*5.5.7.1) TLC studies of Methanol extract of formulations:*

Glycosides, alkaloids, saponins and essential oils were found to be present in all the methanol extracts. The results along with the inferences have been shown in Table 5.31.

**Table 5.31. TLC studies of Methanol extract of formulations.**

| CLASS OF COMPOUND | FORM -1 | FORM-2 | FORM-3 | Inference |
|---|---|---|---|---|
| Anthraglycosides | Black | Black | Black | Present |
| Alkaloids | Dark orange | Dark orange | Dark orange | Present |
| Arbutin like compounds | No spot | No spot | No spot | Absent |
| Bitter Principles | No spot | No spot | No spot | Absent |
| Saponins | Violet | Violet | Violet | Present |
| Essential Oils | Violet | Violet | Violet | Present |

The qualitative tests showed the presence of alkaloids, anthranol glycosides, saponins, phenolics, tannins, proteins, amino acids and essential oils. The qualitative tests are important to determine the presence of the active ingredients in the formulations. They form an important standardization parameter for the poly herbal formulations where a large number of ingredients are present.

## 5.6) Chemical Standardization of the three Formulations

### *5.6.1) Development of analytical methods for estimation of Aconitine in Mahamrutyunjaya rasa*

Three different methods, HPLC, HPTLC and Spectrofluorimetry were developed for the estimation of aconitine.

#### 5.6.1.1) HPLC Method.

##### *5.6.1.1.1) Optimization of chromatographic conditions*

The choice of experimental conditions was guided by the need to obtain chromatograms with better resolution within short analysis time, especially when numerous samples were to be analyzed. In the beginning, various mixtures of methanol-water-triethylamine, 0.1% trifluoroacetic acid-tetrahydrofuran and methanol-water-acetonitrile were used as mobile phase but separation was not satisfactory. Acetonitrile and ammonium hydrogen carbonate buffer was then chosen as mobile phase because the alkaloids could be separated much better in this condition. The detection wavelength was selected to be 223 nm depending on its maximum absorbance in the scan range of 200nm - 400nm.

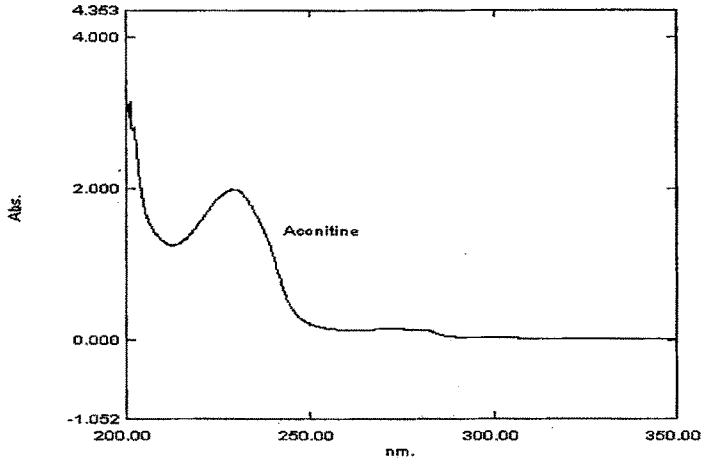



**Fig. 5.13 UV spectra of Aconitine (200µg/ml).**

***5.6.1.1.2) Effect of pH value in the mobile phase buffer on the retention time of HPLC.***

Traditional HPLC applications on silica-based columns were performed at low or mid-range pH values in the mobile phase buffer. But the aconitum alkaloids in the HPLC chromatograms exhibited low retention time in such mobile phase; therefore it was difficult to separate them from each other and from other compounds in the Ayurvedic formulation. An alternative method was employed to separate them by using a mobile-phase pH which was above its pKa. Under such conditions, aconitine was in its free base form because its positive charge was diminished, resulting in the improvement of the peak shape. The pH dependence of the retention time was investigated within a range of 7-8 pH values by adjusting with 1 % triethylamine, in which the ammonium bicarbonate concentration reached at 10 mM. This showed that along with increase of the pH values in the buffer, the retention time of HPLC was increasing due to the stronger interaction between this less-polar molecule and the hydrophobic bonded phase.

The results of the chromatographic analysis indicated that the pH value has distinct effects, on the resolution. At the pH value 7.5, all the components in the samples were baseline separated from each other with resolution values above 2. Therefore, the analysis was carried out at a pH range of 7.5-8.00, where the buffer solution works effectively.

Since alkaloids were to be analyzed in the sample solution, the ion-pair agents such as triethylamine was used to improve the separation of these components and to decrease in the tailing effect.

***5.6.1.1.3) Effect of mobile phase composition***

The effect of mobile phase composition (i.e. ratio of acetonitrile and ammonium bicarbonate buffer was studied at 65:35, 60:40 and 55:45 v/v levels) with pH and flow rate of 7.5 and 1.0 ml/min, respectively. The resolution was > 3 at 60:40, v/v level, which is one of the most desirable criteria. Also the retention time was shorter with satisfactory asymmetry values. An adequate theoretical plates (~14000) is indicative of a good column performance. The asymmetry was >1.12 at 55:45 v/v indicating the tailing of peaks and the resolution was <2 at 65:35, v/v.

***5.6.1.1.4) Effect of mobile phase flow rate***

The number of theoretical plates was highest at flow rate of 1 ml/min. The change in flow rate had no significant effect on resolution and asymmetry while retention time decreased as the flow rate increased. The other system suitability parameters also indicated optimum flow rate of 1 ml/min.

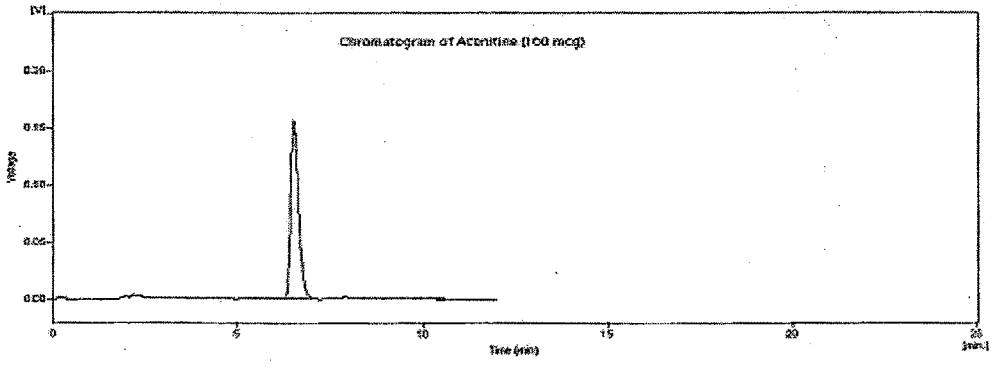



**Fig. 5.14. Representative HPLC chromatogram of standard Aconitine.**

***5.6.1.1.5) Extraction conditions***

Hydrochloric acid was chosen for dissolving the alkaloid which was reported to be stable in acidic solution. Ethyl acetate helped to remove the interferences in the acidic aqueous solution before the final extraction of the alkaloids by organic solvent after alkalization with 28% ammonia solution up to pH value of 10. The influences of the extraction solvents on the yield of the alkaloid were investigated. The chemical properties of the alkaloids were considered and

extraction in chloroform, ethyl acetate and diethyl ether were employed. The results showed that chloroform was better as more alkaloids were extracted with high recoveries. The above method was chosen in order to reduce the other interfering components and maximum extraction.

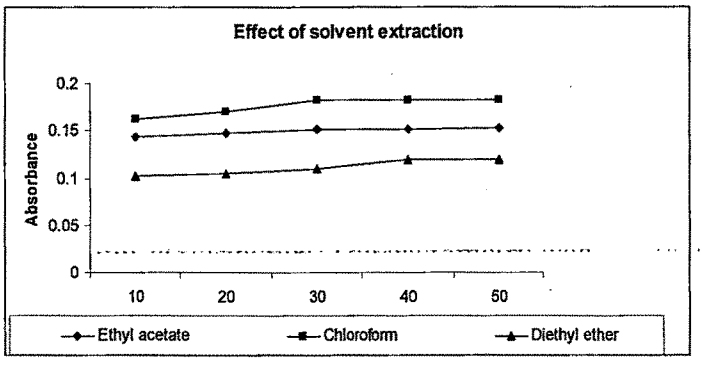



**Fig. 5.15 Effect of solvent used for extraction on the absorbance of the alkaloid fraction**

***5.6.1.1.6) Validation***

*a) Calibration curve (linearity)*

The calibration curves (n=3) constructed for aconitne was linear over the concentration range of 10-100 µg/ml. Peak area of the marker was plotted versus the concentration and linear regression analysis performed on the resultant curve. The coefficients of determination 0.9997 with % RSD values ranging from 0.5 to 2% across the concentration range studied were obtained following linear regression analysis (Table 5.32).

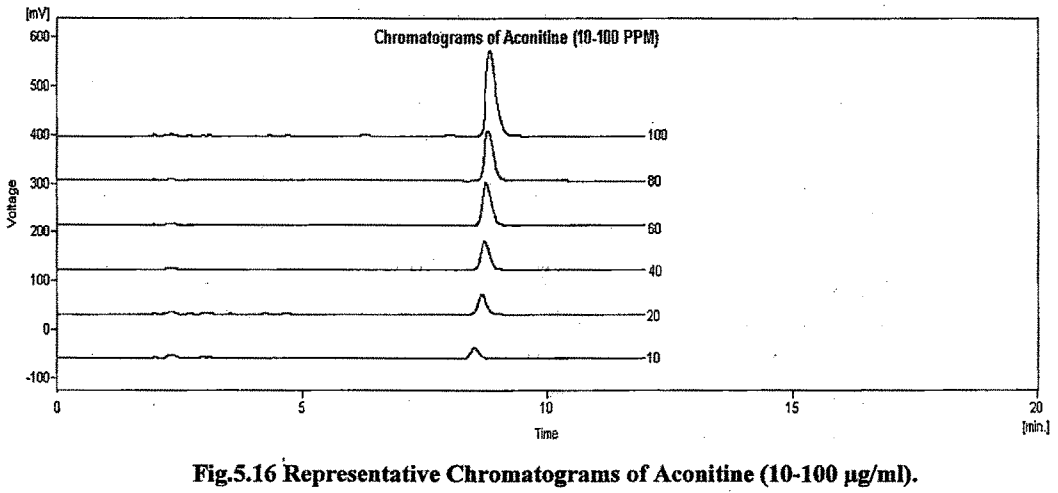



**Fig.5.16 Representative Chromatograms of Aconitine (10-100 µg/ml).**

**Table 5.32 Peak area and Peak Height of Aconitine.**

| Concentration (µg/ml) | Peak area (mV) | Peak Height (mV) |
|---|---|---|
| 10 | 162.098 | 13.432 |
| 20 | 335.361 | 24.957 |
| 40 | 508.901 | 36.318 |
| 60 | 760.093 | 54.989 |
| 80 | 1045.824 | 75.804 |
| 100 | 1301.389 | 93.654 |

**Fig5.17 Calibration Curve of Aconitine.**

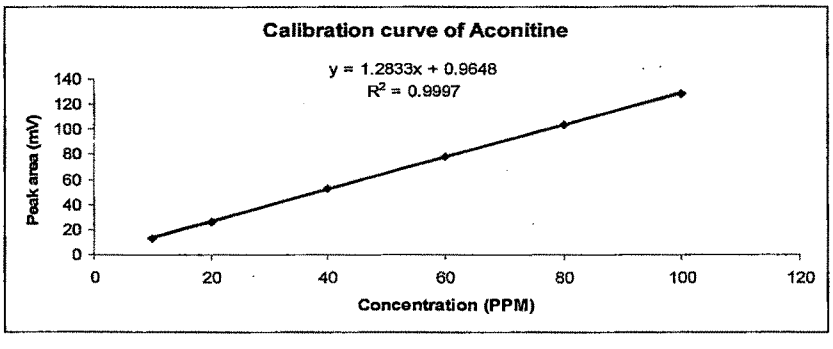



**Fig 5.33. Linear regression data for the calibration curves (n=3)**

| | |
|---|---|
| Retention Time, min | 6.82 ± 0.1 |
| Detection wavelength, nm | 223 nm |
| LOD, µg/ml | 0.85 |
| LOQ, µg/ml | 2.10 |
| Linearity Range | 10-100 µg/ml |
| Coefficient of Determination (Height) | 0.9994 |
| Coefficient of Determination (Area) | 0.9997 |
| Regression equation (Height) | Y= 0.0743 x + 20.791 |
| Regression equation (Area) | Y= 1.2833 x + 0.9648 |
| Slope (Height) | 0.0743±0.03 |
| Slope (Area) | 1.2833±0.56 |
| Intercept (Height) | 20.791± 1.65 |
| Intercept (Area) | 0.9648± 0.04 |

*b) Repeatability, precision and stability*

Injection repeatability- The calculated % RSDs of the peak areas was less than 2.0 % at each of the three concentration levels (Table 5.34).

Analysis repeatability- The % RSD values for analysis repeatability were less than 2.0 % both for retention time and peak area (Table 5.34).

**Table 5.34. Repeatability of the developed method (n=6)**

| **Aconitine** | **Retention time (min)** | | | | **Peak area (mVs)** | | | |
|---|---|---|---|---|---|---|---|---|
| **(µg/ml)** | **Injection Repeatability** | | **Analysis repeatability** | | **Injection Repeatability** | | **Analysis repeatability** | |
| | Mean | % RSD | Mean | % RSD | Mean | % RSD | Mean | % RSD |
| 10 | 6.83 | 0.18 | 6.83 | 0.24 | 160.28 | 1.08 | 155.86 | 1.66 |
| 40 | 6.84 | 0.65 | 6.83 | 0.74 | 508.72 | 1.61 | 511.53 | 1.03 |
| 100 | 6.83 | 0.73 | 6.82 | 0.63 | 1295.3 | 1.95 | 1310.2 | 1.94 |

Instrument precision- The precision result of the solution at medium concentration is presented in Table 5.35, and it was shown that the % RSD values of retention time were less than 1%, while the % RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision (Intra 4 h six injections, inter 6 days).

**Table. 5.35.Intra-day and inter-day precision of HPLC method (n=6)**

| **Amount** | **Intra-day precision** | | | | **Inter- day precision** | | | |
|---|---|---|---|---|---|---|---|---|
| **(µg/ml)** | **Mean area** | **S.D.** | **% RSD** | **S.E.** | **Mean area** | **S.D.** | **% RSD** | **S.E.** |
| 10 | 159.098 | 3.32 | 1.08 | 2.96 | 163.098 | 4.97 | 0.87 | 3.02 |
| 40 | 512.901 | 3.76 | 0.96 | 3.15 | 515.901 | 6.75 | 1.43 | 3.35 |
| 100 | 1303.389 | 10.19 | 1.67 | 4.28 | 1299.389 | 12.56 | 1.87 | 5.12 |

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (%RSD values of the retention time and peak area were both less than 3%).

*c) Limit of detection and limit of quantification*

The LOD and LOQ were found to be 0.85 and 2.1 µg/ml for Aconitine.

*d) Specificity*

Satisfactory results were obtained, indicating the high specificity of the proposed method for the determination of aconitine in the formulations. Good resolution and absence of interference was observed as shown in Fig.5.17.

*e) Robustness*

Table 5.36 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean values were between 98 % and 102 % of aconitine. Therefore, it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

**Table.5.36. Robustness of the method (n=6)**

| **Chromatographic change** | | **Recovery, % Components** |
|---|---|---|
| **Factor** | **Level** | **Aconitine** |
| **Acetonitrile brand** | | |
| Spectrochem | 1 | 99.45 ± 0.78 |
| Rankem | 2 | 98.56 ± 0.98 |
| Qualigens | 3 | 98.88 ± 0.23 |
| **Column Brand** | | |
| Hypersil | 1 | 101.5 ± 1.06 |
| Phenomenex | 2 | 100.28 ± 0.84 |
| **Detection Wavelength, nm** | | |
| 221 | -1 | 100.78 ± 0.78 |
| 223 | 0 | 99.58 ±0.65 |
| 225 | 1 | 98.34 ±1.89 |

*f) System suitability*

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table.5.37). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

**Table 5.37. System Suitability Parameters**

| Sr. No. | Parameter | Values |
|---|---|---|
| 1 | Retention time (min) | 6.84 |
| 2 | Area (mVs) | 335.36 |
| 3 | Capacity factor (k') | 3.32 |
| 4 | Separation factor (α) | ------ |
| 5 | Efficiency/length(t.p/m) | 142231 |
| 6 | HETP (mm) | 0.052 |
| 7 | Resolution (Rs) | 3.12 |
| 8 | Assymmetry (As) | 1.02 |

*g) Accuracy*

As shown in Table 5.38, the recovery of the investigated components ranged from 97 % to 102 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed method manifested the reliability and accuracy for the measurement of these components.

**Table 5.38 Recovery Test (n=3):**

| Excess drug added to the analyte(%) | Theoretical content (µg) | Recovery (%) | % RSD |
|---|---|---|---|
| 0 | 10.23 | 101.97 | 1.28 |
| 80 | 18.41 | 99.10 | 1.74 |
| 100 | 20.46 | 97.31 | 1.89 |
| 120 | 22.50 | 98.22 | 1.64 |

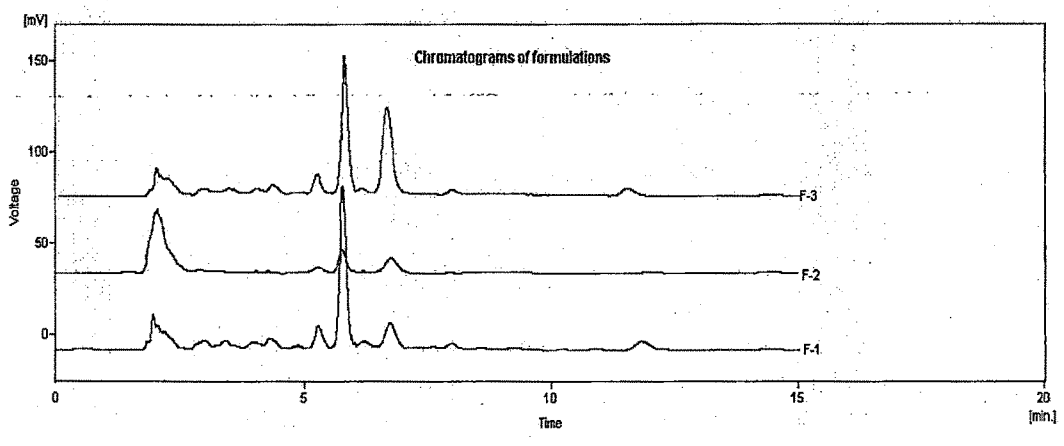
***5.6.1.1.7) Applicability of the developed method in formulations***

The developed HPLC method was applied to the determination of Aconitine in the Ayurvedic formulations (FORM1, FORM2, FORM3) and the results are presented in Table 5.39. It was observed that the content of Aconitine varied in the three formulations, with the %RSD values of higher than 10 %, which would significantly influence the quality stability because it is the target toxic component for the quality control of MHR tablets.

**Table. 5.39. Contents of the three alkaloids in three proprietary Ayurvedic medicines (n=3)**

| Formulation | Aconitine (µg) per tablet |
|---|---|
| Form-1 | 0.121 ± 0.02 |
| Form-2 | 0.14 ± 0.03 |
| Form-3 | 0.52 ± 0.03 |

**Fig. 5.18. Representative chromatogram of FORM-1 (F-1), FORM-2 (F-2) and FORM-3 (F-3).**




### *5.6.1.2) HPTLC Method*

#### *5.6.1.2.1) Development of the optimum mobile phase*

The TLC procedure was optimized with a view to develop an assay method for the estimation of Aconitine. Both the pure drug and the formulation extract solution were spotted on the TLC plates and run in different solvent systems. Initially, toluene–ethylcetate in varying ratios was tried. The mobile phase toluene-ethylacetate in the ratio (7.0: 2.0, v/v) gave good resolution but peak tailing was observed. Also, the typical peak nature was missing because the spot was slightly diffused. Addition of 1 ml of diethylamine to the above mobile phase improved the spot characteristics and increased the $R_f$ value to 0.48 ± 0.02 when densitometric scanning was performed at 223 nm. Finally, the mobile phase consisting of toluene-ethylacetate-diethylamine (7:2:1, v/v/v) gave a sharp and symmetrical peak. Resolution between spots of standard and formulation extract apeared better when TLC plates were pretreated with methanol and activated at 100 °C for 5 min. Well-defined spots (compact dense spots) were obtained when the chamber was saturated with the mobile phase for 30 mins at room temperature (Fig. 5.21). It was required to eliminate the

edge effect and to avoid unequal solvent evaporation losses from the developing plate that can lead to various types of random behavior usually resulting in generally lack of reproducibility in $R_f$ values.

***5.6.1.2.2)Validation of the method***

*a) Linearity*

With HPTLC, the analyte interacts with the layer surface of the stationary phase where scattering and absorption tend to take place, especially with high concentrations of analyte. Beer-Lambert law does not adequately describe this process, but the Kubelka Munk model, which relies on the idea that light, is travelling in all directions simultaneously within the precoated TLC plate. This is approximated as a flux of light travelling upwards and a flux travelling downwards at any depth in the plate. When this flux passes through a thin layer of material, some of it passes through, some of it is scattered backwards and some of it is absorbed. Calibration graph was found to be linear that is adherence of the system to Kubelka Munk theory, which was found over the concentration range of 200–1000 ng/spot ($r^2$ = 0.99988). Linearity was evaluated by determining the standard working solution containing 100 µg/ml of aconitine in triplicate. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data showed a good linear relationship over the concentration range of 200–1000 ng/spot. The linearity of calibration graphs and adherence of the system to Kubelka Munk theory were validated by high value of correlation coefficient and the SD for intercept value was less than 2. No significant difference was observed in the slopes of standard plots.

**Table 5.40. Linearity data of HPTLC analysis of aconitine.**

| Track | Vial | Rf | Amount (ng) | Height | X(calc) (ng) | Area | X(calc) (ng) | SampleID/ Remark |
|---|---|---|---|---|---|---|---|---|
| 2 | 2 | 0.47 | | 103.69 | 455.64 | 2322.48 | 425.34 | BD#80 µg |
| 3 | 3 | 0.48 | | 131.11 | 599.02 | 3050.17 | 578.58 | PN#80 µg |
| 4 | 4 | 0.49 | 200.00 | 49.85 | | 1191.88 | | |
| 5 | 4 | 0.49 | 400.00 | 91.68 | | 2188.53 | | |
| 6 | | | | | | | | |
| 7 | 4 | 0.47 | 600.00 | 132.60 | | 3193.12 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 8 | 4 | 0.47 | 800.00 | 165.14 | | 3996.88 | | |
| 9 | 4 | 0.48 | 1000.00 | 197.07 | | 4901.87 | | |
| 10 | 1 | 0.48 | | 115.73 | 517.24 | 2749.51 | 514.44 | LB# 80µg |
| 11 | 2 | 0.47 | | 105.30 | 463.77 | 2322.22 | 425.28 | BD# 80µg |

**Table 5.41 Regression analysis for calibration plots. (n=3)**

| | |
|---|---|
| Linearity Range | 200 ng/µL to 1000 ng/µL |
| Coefficient of Determination (Height) | 0.99988 |
| Coefficient of Determination (Area) | 0.99973 |
| Regression equation (Height) | y=0.184x+16.898 |
| Regression equation (Area) | y=4.6142x+325.96 |
| Slope (Height) | 0.184±0.02 |
| Slope (Area) | 4.6142±0.98 |
| Intercept (Height) | 16.898±1.23 |
| Intercept (Area) | 325.96±3.56 |

*b) Precision*

The repeatability of sample application and measurement of peak area were expressed in terms of % RSD and found to be 1.54 and 1.22 respectively for six replicate determinations. The % RSD for intra-day and inter-day precision of aconitine peak area at two different concentration levels 200, 1000 ng/spot are shown in Table.5.42 respectively.

**Table 5.42. Intra-day and inter-day precision of HPTLC method. (n=6)**

| **Amount (ng/spot)** | **Intra-day precision** | | |
|---|---|---|---|
| | **Mean area** | **S.D.** | **% RSD** |
| 200 | 1202.76 | 22.89 | 1.01 |
| 1000 | 4943.06 | 59.87 | 1.55 |
| **Amount (ng/spot)** | **Inter-day precision** | | |
| | **Mean area** | **S.D.** | **% RSD** |
| 200 | 1187.67 | 31.23 | 1.56 |
| 1000 | 4934.45 | 85.57 | 1.78 |

*c) Robustness of the method*

The standard deviation of peak areas was calculated for each parameter and % RSD was found to be less than 2%. The low values of % RSD as shown in Table 5.43 indicated robustness of the method.

**Table 5.43. Robustness of the method.**

| **Amount (ng/spot)** | **Mobile phase composition % RSD** | |
|---|---|---|
| | **Toluene: Ethyl Acetate: Diethyl amine (7:2:1 v/v)** | **Toluene: Ethyl Acetate: Diethyl amine (7.5:1.5:1 v/v)** |
| 200 | 1.56 | 1.54 |
| 1000 | 0.78 | 1.04 |

*d) LOD and LOQ*

The calibration plot in this study was plotted between amount of analyte versus average response (peak area) and the regression equation was obtained with a regression coefficient of 0.9982. Detection limit and quantification limit was calculated by the method as described earlier and found 25 ng and 50 ng respectively, which indicates the adequate sensitivity of the method.

*e) Specificity*

The peak purity of aconitine was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot i.e., $r^2$ = 0.9997 and $r^2$= 0.9998. Good correlation ($r^2$ = 0.9997) was also obtained between standard and sample spectra of aconitine.

*f) Accuracy*

The proposed method when used for extraction and subsequent estimation of aconitine from pharmaceutical dosage form after spiking with 80, 100 and 120% of additional drug afforded recovery of 97–102% as listed in Table 5.44.

**Table 5.44.Recovery studies. (n=3)**

| **Excess drug added to the analyte(%)** | **Theoretical content (ng)** | **Recovery (%)** | **% RSD** |
|---|---|---|---|
| 0 | 200 | 101.97 | 1.13 |
| 80 | 360 | 99.10 | 1.67 |
| 100 | 400 | 97.31 | 1.43 |
| 120 | 440 | 98.22 | 1.18 |

*g) Spot stability*

No decomposition was observed during spotting and development.

***5.6.1.2.3) Analysis of the marketed formulation***

A single spot at $R_f$ 0.48 was observed in the chromatogram of the drug samples extracted from formulation. There was no interference from the other components present in the tablet. The good performance of the method indicated the suitability of this method for routine analysis of aconitine in herbal dosage form.

**Table 5.45. Results of analysis:**

| Sr.No. | Formulation | Aconitine, µg per tablet |
|---|---|---|
| 1 | Form-1 | 0.11 ± 0.01 |
| 2 | Form-2 | 0.095 ± 0.01 |
| 3 | Form-3 | 0.47 ± 0.02 |

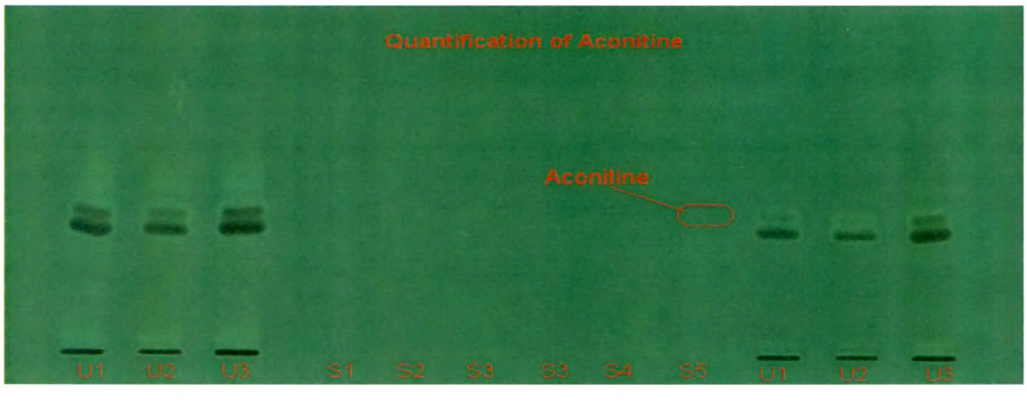



**Fig.5.19. Image of TLC plate before derivatization at 254nm.**

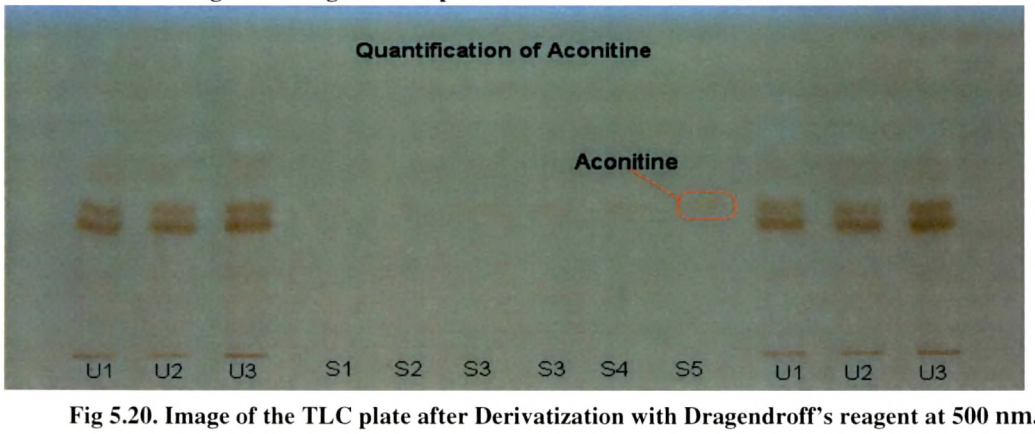



**Fig 5.20. Image of the TLC plate after Derivatization with Dragendroff's reagent at 500 nm.**

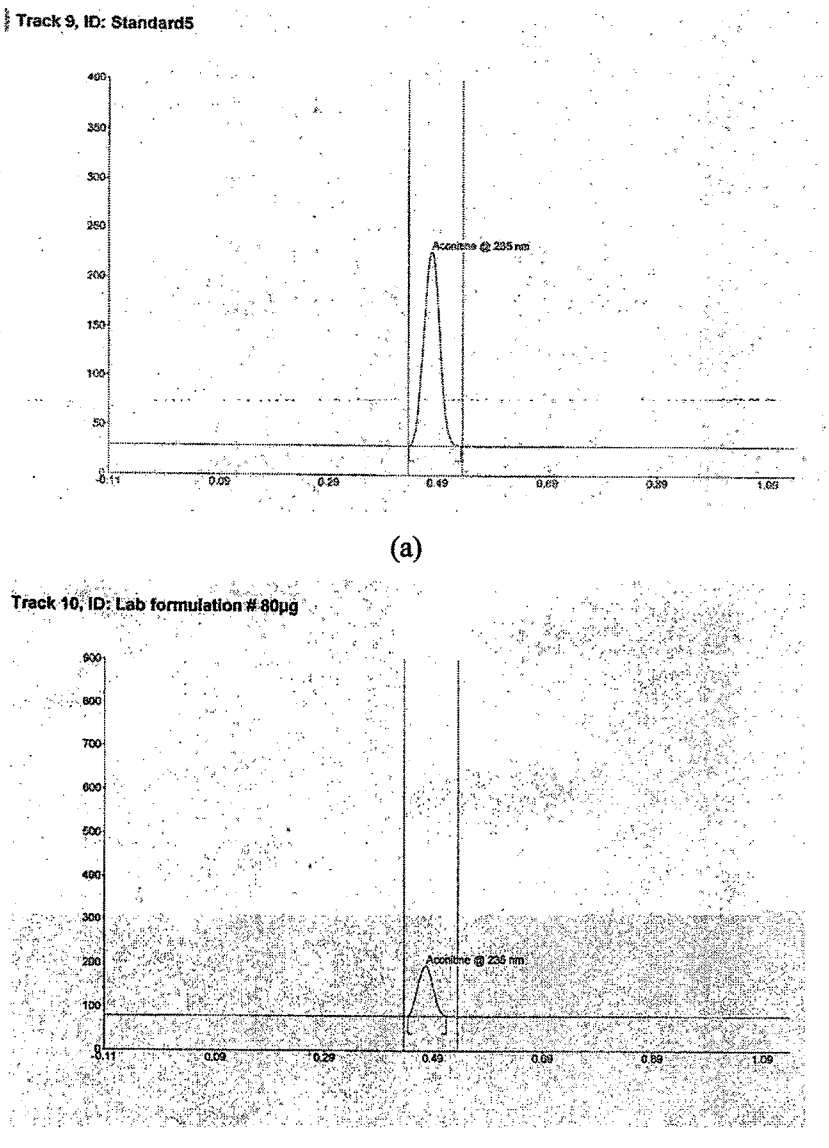



**Fig. 5.21. Representative HPTLC chromatograms of (a)Aconitine Standard and (b)Form-1.**

### 5.6.1.3) Spectrofluorimetric method.

#### *5.6.1.3.1) Excitation and emission spectra*

The standard aconitine was dissolved in methanol in the concentration range of 100-600 pg/ml. In this solution, the wavelengths of the maximum excitation and emission that belong to aconitine were at 270 nm and 505 nm respectively.

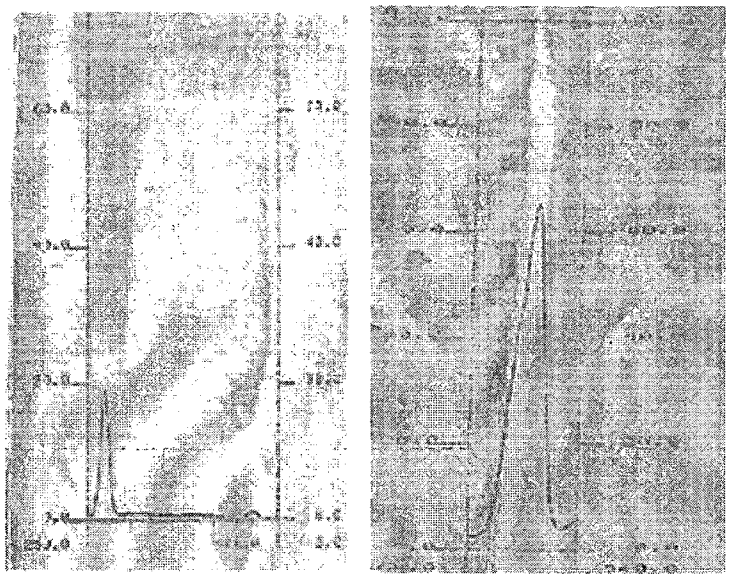


**Fig.5.22. Excitation of Aconitine at 270 nm and emission at 505 nm.**

***5.6.1.3.2) Effect of solvent***

The effect of addition of 10 mL of different solvents on the fluorescence of a standard solution of 100 pg/mL aconitine is outlined in Table 5.46. The maximum fluorescence intensity was obtained with methanol.

**Table 5.46. Effect of solvent on fluorescence intensity.**

| Sr.No. | Solvent | Fluorescence intensity |
|---|---|---|
| 1 | Methanol | 12.3 |
| 2 | Ethanol | 10.2 |
| 3 | Acetonitrile | 8.3 |
| 4 | Chloroform | 5.4 |
| 5 | Dichloromethane | 6.0 |
| 6 | Ethyl acetate | 6.4 |

***5.6.1.3.3) Method validation***

*a) Linearity and sensitivity*

The linear calibration graphs were obtained under the optimum experimental conditions. The analytical results obtained from this investigation are summarized in Table 5.47. The calibration data were fitted by least square treatment and the regression equations and a linear relationship was found

between fluorescence intensity and concentration in the ranges given in Table 2.42 LOD was and LOQ, respectively.

**5.47.Optical Characteristics and Analytical Data**

| **Parameters** | **Results** |
|---|---|
| Excitation wave length | 270 nm |
| Emission wave length | 505 nm |
| Scan Speed | Medium |
| Sensitivity | High |
| Abscissa scale | 2 |
| Ordinate scale | 3 |
| Linearity range (ng/mL) | 100-600 pg/ml |
| Regression equation (Ya) | Y = 0.1737x - 6.5333 |
| Slope (b) | 0.1737 |
| Intercept (a) | 6.5333 |
| Coefficient of determination (r2) | 0.9946 |
| Correlation coefficient (r) | 0.9972 |
| Limit of Detection | 12 pg/ml |
| Limit of Quantification | 39.6 pg/ml |
| Accuracy | > 98 % |

**Table 5.48. Data of Calibration Curve for Aconitine**

| **Concentration of Aconitine (pg/mL)** | **# Relative Fluorescence Intensity** | **% RSD** |
|---|---|---|
| 100 | 12.3 | 0.76 |
| 200 | 28 | 0.44 |
| 300 | 42.4 | 1.12 |
| 400 | 62.6 | 0.80 |
| 500 | 84.1 | 0.76 |
| 600 | 96.2 | 0.99 |

*b) Precision*

The precision of the method was tested with regard to both the intra-day and inter-day precision of the assay. The intra-day variability of the assay was determined by repeated analysis of three concentrations ($n$ = 6). Similarly, the inter-day variability of the assay was determined through replicate analysis of three concentrations ($n$ = 3), and the results are listed in Table 5.49. Both the intra-day and inter-day precision of the presented methods were fairly good (%R.S.D.≤0.02).

**Table 5.49.Intra-day and inter-day precision of spectrofluorimetric method. (n=6)**

| **Amount (pg/ml)** | **Intra-day precision** | | |
|---|---|---|---|
| | **Fluorescence Intensity** | **S.D.** | **% RSD** |
| 100 | 12.1 | 0.86 | 1.11 |
| 300 | 41.8 | 1.56 | 1.66 |
| 600 | 96.4 | 1.83 | 1.83 |
| | **Inter-day precision** | | |
| **Amount (pg/ml)** | **Fluorescence Intensity** | **S.D.** | **% RSD** |
| 10 | 11.9 | 1.32 | 1.14 |
| 300 | 42.8 | 1.47 | 1.54 |
| 600 | 96.9 | 1.76 | 1.42 |

*c) Recovery*

The proposed method when used for estimation of aconitine from pharmaceutical dosage form after spiking with 80, 100 and 120% of additional drug afforded recovery of 98–101% as listed in Table 5.50.

**Table 5.50. Data of sample analysis and recovery studies:**

| **Excess drug added to the analyte(%)** | **Theoretical content (pg/ml)** | **Recovery (%)** | **% RSD** |
|---|---|---|---|
| 0 | 170 | 98.64 | 1.08 |
| 80 | 255 | 98.12 | 1.66 |
| 100 | 340 | 98.33 | 1.93 |
| 120 | 425 | 98.74 | 1.53 |

*d) Robustness*

No significant difference could be observed in the results found out. The recovery obtained individually and the mean was between 98 % and 101 % aconitine. Therefore, it can be concluded that the method is consistent for solvent brand.

*e) Stability*

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (RSD values of the retention time and peak area were both less than 3%).

***5.6.1.3.4) Applicability of the developed method in formulations***

The developed method was applied to the determination of aconitine in the Ayurvedic formulations (FORM1, FORM2, FORM3) and the results are presented in Table 5.51. It was observed that the content of aconitine varied in the three formulations, with the %RSD values of higher than 10%, which would significantly influence the quality stability because it is one of the target toxic component for the quality control of *Mahamrutyunjaya rasa* tablets.

**Table. 5.51.Results of Analysis**

| Sr. No. | Formulation | Aconitine, µg per tablet |
|---|---|---|
| 1 | Form-1 | 0.116 |
| 2 | Form-2 | 0.13 |
| 3 | Form-3 | 0.47 |

### 5.6.1.4) Stability indicating method development for Aconitine in *Mahamrutyunjaya rasa.*

#### *5.6.1.4.1) Chromatographic separation*

A simple HPLC method was adopted for the determination of aconitine in *Mahamrutyunjaya rasa* formulation. To optimize the proposed HPLC method, all of the experimental conditions were investigated. For the choice of stationary phase, reversed-phase separation was preferred due to the drawbacks of the normal phase, eg., hydration of silica with water that can cause peak tailing. To optimize the mobile phase, different systems were tried for chromatographic separation of the components by combining homogenous design and solvent polarity optimization. The best resolution was achieved using a mobile phase consisting of acetonitrile - $KH_2PO_4$ buffer (10 mM, pH 8± 0.1) (50:50 v/v) for

aconitine, which gave good resolution and sensitivity at 223 nm with a flow rate of 1 ml/min.

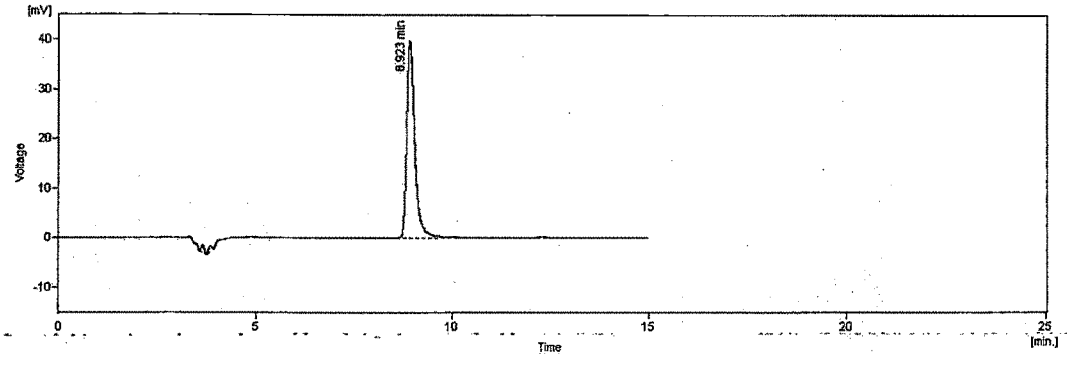



**Fig.5.24. Representative HPLC chromatohram of Aconitine.**

***5.6.1.4.2) Forced Degradation Studies***

Conditions used for forced degradation were attenuated to achieve degradation in the range of 20–80%. The following degradation behavior of drug was observed during the stress degradation studies.

*a) Acidic condition.* - The standard marker was subjected to heating in specified concentration of HCl for 12 h. The marker was found to be susceptible to acid degradation. A major single degradation product was seen in aconitine (Figure 5.26(a)) at 4.883 mins. The preparation of sample solutions for all the HPLC studies was performed by treating with 0.1 N HCl and then further extracting the alkaloids. Thus, the effect of HCl on aconitine with respect to time was performed. The degradation occurred in the samples after 8 hours when kept at room temperature as shown in the chromatogram Fig. 5.25.

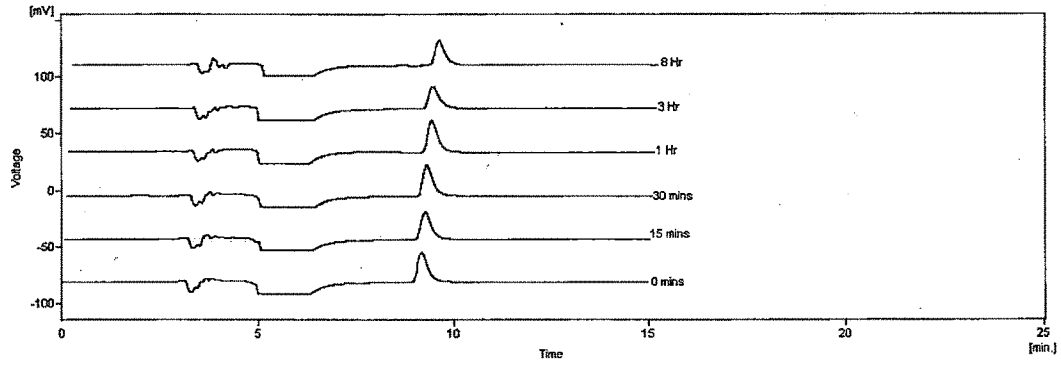



**Fig 5.25. Representative chromatogram aconitine in presence of 0.1 N HCl at different intervals of time.**

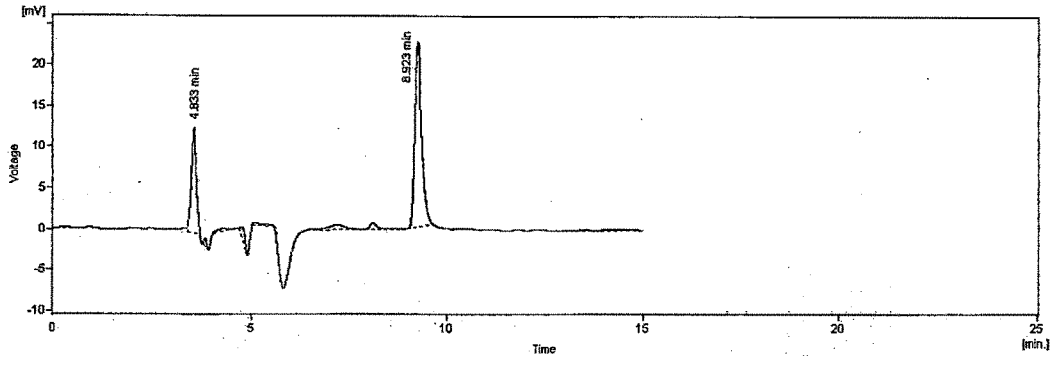



**Figure 5.26(a). Chromatographic separation of ACN (50 µg/mL) in stressed sample of the standard subjected to acid hydrolysis.**

*b) Degradation in alkali.* - Aconitine underwent alkali hydrolysis and the rate of hydrolysis was faster as compared to acidic condition. It took 3h for the drug to decompose by 20%. A major degradation peak for aconitine was found at 4.833 mins.(Figure 5.26(b)).

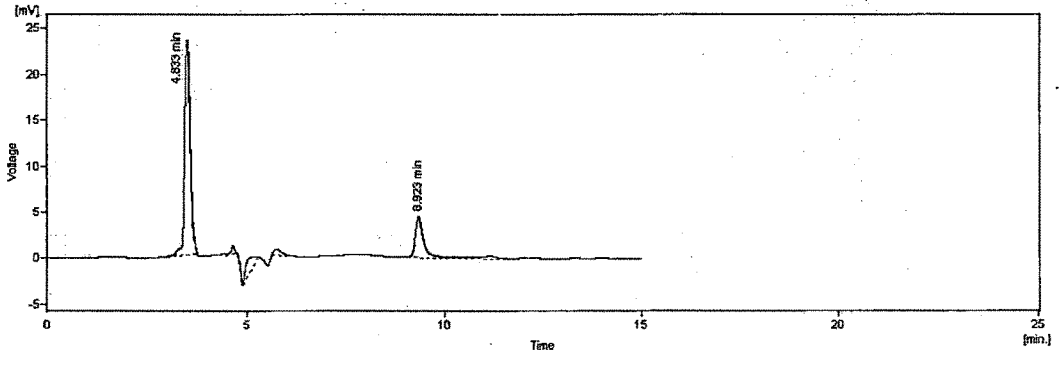



**Figure 5.26 (b). Chromatographic separation of ACN (50 µg/mL) in stressed sample of the standard subjected to alkali hydrolysis.**

*c) Neutral (water) condition* - It was observed that around 8–10% of the aconitine degraded on refluxing the drug in water at 80 °C for 24 h. Degradation peaks for aconitine were observed at retention times 4.833 and 7.314 min(Figure 5.26 (c)).

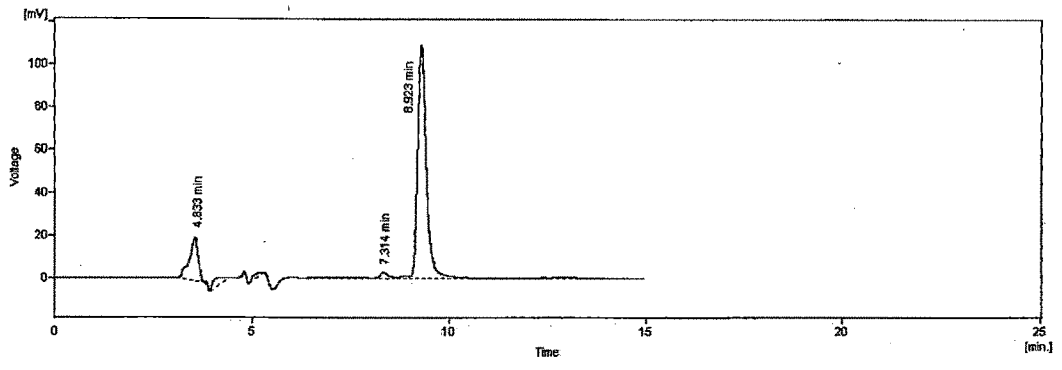



**Figure 5.26 (c). Chromatographic separation of ACN (50 µg/mL) in stressed sample of the standard subjected to water hydrolysis.**

*d) Oxidative conditions.* – Aconitine was found to be highly labile to oxidation in 0.5 % $H_2O_2$, when they were kept at room temperature for 24 h. The degradation of aconitine was found to be about 50-60 % in 0.5 % $H_2O_2$. The major degradation products of aconitine were at (RTs) 4.133, 4.528, 4.903 and 7.314mins, (Figure 5.26 (d)).

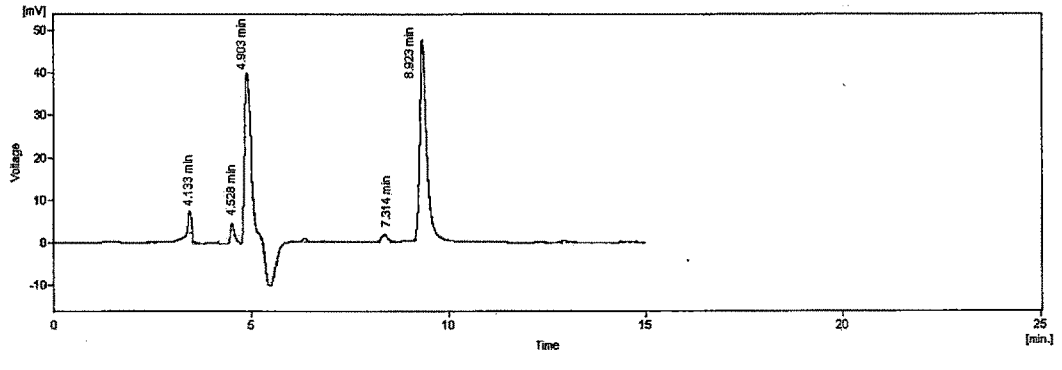



**Figure 5.26 (d). Chromatographic separation of ACN (50 µg/mL) in stressed sample of the standard subjected to oxidation.**

*e) Solid-state study (Thermal degradation).* – Aconitine was relatively stable when exposed to dry heat at 80°C for 48 h. The percent amount of aconitine remaining after 48 h of dry heat exposure was in the range of 90-92%. Degradation peak of aconitine was 4.833 mins (Figure 5.26 (e)).

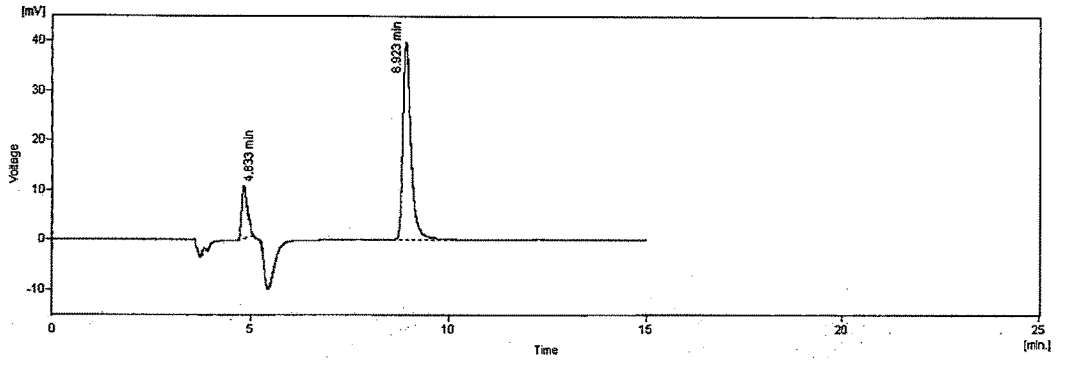



**Figure 5.26 (e). Chromatographic separation of ACN (50 µg/mL) in stressed sample of the standard subjected to thermal degradation.**

*f) Photolytic conditions* – Aconitine was found to be highly susceptible to photolytic degradation. Aconitine was found more susceptible to alkaline photolytic conditions (around 70-75 % degradation) in comparison to acidic and neutral conditions. A single large degradation peak of Aconitine was at 4.833 min (Figure 5.26 (f)).

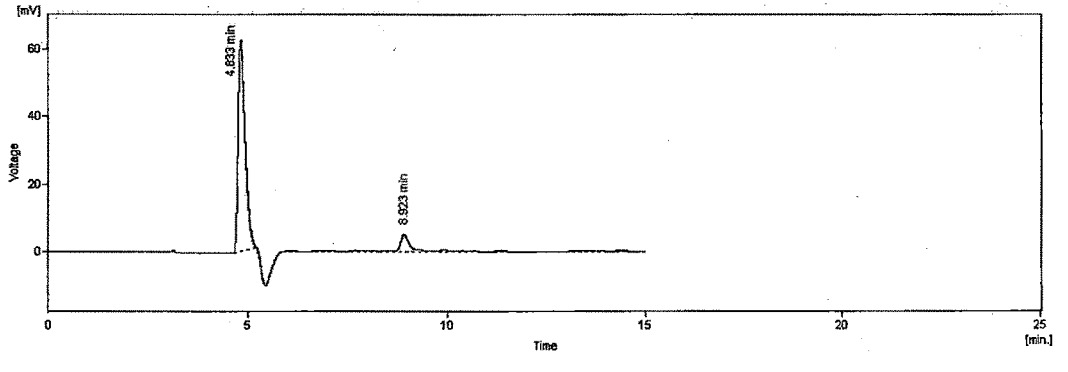



**Figure 5.26 (f). Chromatographic separation of ACN (50 µg/mL) in alkaline stressed sample of the standard subjected to photolytic degradation.**

*g) Humidity:* Aconitine was found to be susceptible to humid conditions. Around 40 % degradation was observed in aconitine after 7 days at (RTs) 4.833 and 7.314 mins.

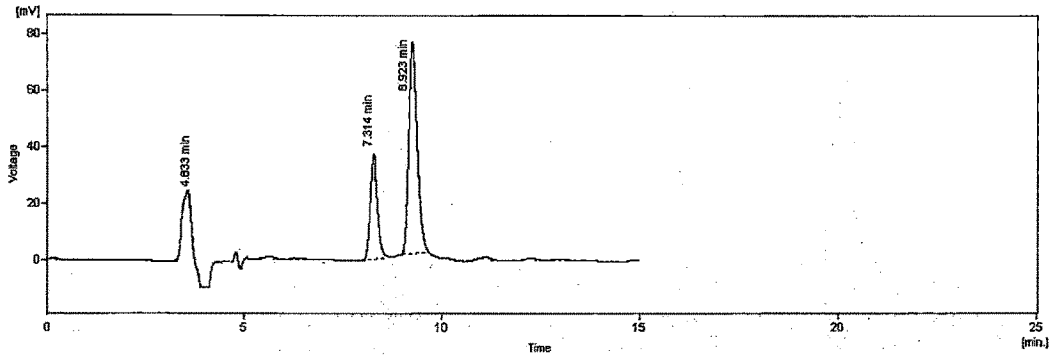



**Figure 5.26 (g). Chromatographic separation of ACN (50 µg/mL) in stressed sample of the standard subjected to 75% RH at 30°C.**

### *5.6.1.4.3) Establishment of a Stability-indicating Method for a Mixture of Stressed Solutions*

The mobile phase comprising of potassium dihydrogen phosphate (10 mM; pH 8.0±0.1 adjusted with triethylamine) – acetonitrile (50:50, v/v) was first used to analyze aconitine standard drug samples for the linearity study (Figure 5.27). These were then applied on stressed samples of individual drugs which in turn showed recognizable degradation (Figure 5.26(a)-(g)). The method was then successfully applied for the degradation studies of formulations (Figure 5.28).

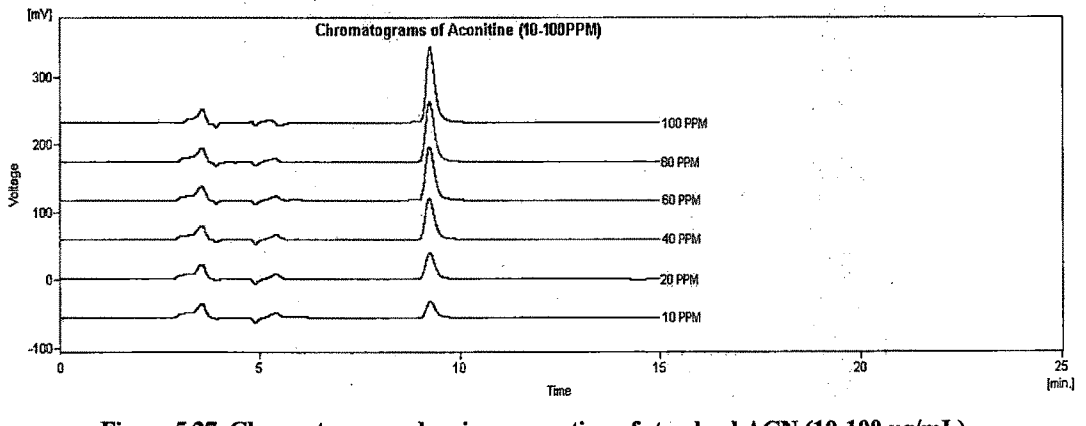



**Figure 5.27. Chromatograms showing separation of standard ACN (10-100 µg/mL).**

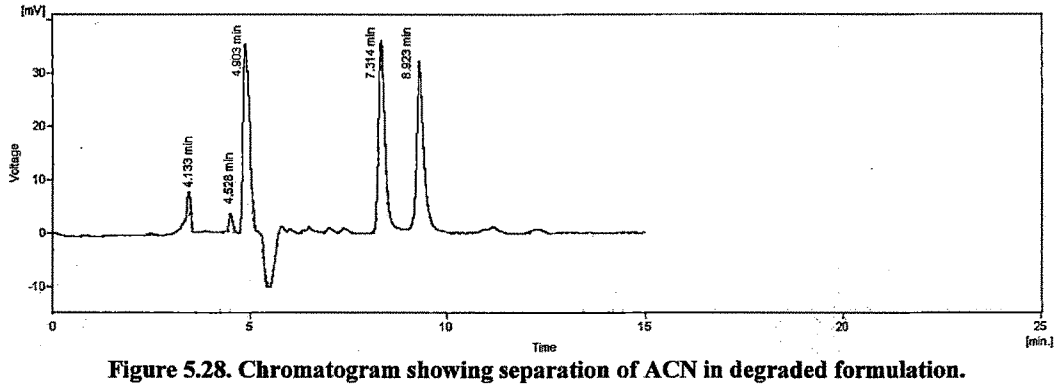



**Figure 5.28. Chromatogram showing separation of ACN in degraded formulation.**

***5.6.1.4.4) Validation***

The method developed for analysis of aconitine was validated for linearity, accuracy, precision, specificity, and quantification limits as per ICH guidelines.

*a) Calibration curve (linearity)*

Linear regression analysis confirms that the $r^2$ value for the drug was found to be >0.999, confirming the linear relationship between the concentration of the drug and area under the curve (Table.5.52). The calibration curve (n=3) constructed for the marker was linear over the concentration range of 10-100 µg/ml for each marker.

**Table 5.52. Linear regression data for the calibration curves (n=3)**

| **Parameter** | **Values** |
|---|---|
| Retention Time, min | 8.93 ± 0.2 |
| Detection wavelength, nm | 223 nm |
| LOD, µg/ml | 0.53 |
| LOQ, µg/ml | 1.749 |
| Linearity Range | 10-100 µg/ml |
| Correlation Coefficient (Height) | 0.999 |
| Correlation Coefficient (Area) | 0.9992 |
| Regression equation (Height) | Y= 0.4092x-2.5833 |
| Regression equation (Area) | Y=7.3768x-28.594 |

*b) Precision and stability*

The precision result of the solution at the three concentrations is presented in Table 5.53, and it was shown that the RSD values of retention time were less than 1%, while the RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision (Intra 4 h six injections, inter 6 days).

**Table 5.53 Intra-day and Inter-day precision of the developed method (n=6).**

| Aconitine µg/mL | Intra-day | | | | Inter-day | | | |
|---|---|---|---|---|---|---|---|---|
| | Retention Time | | Peak area | | Retention time | | Peak area | |
| | Mean | % RSD | Mean | % RSD | Mean | % RSD | Mean | % RSD |
| 10 | 8.93 | 0.22 | 65.721 | 1.25 | 8.92 | 0.19 | 62.67 | 1.25 |
| 40 | 8.92 | 0.18 | 261.23 | 0.73 | 8.93 | 0.47 | 265.2 | 1.17 |
| 100 | 8.93 | 0.26 | 741.25 | 0.58 | 8.93 | 0.21 | 738.4 | 1.88 |

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (RSD values of the retention time and peak area were both less than 3%).

*c) Limit of detection and limit of quantification*

The LOD and LOQ were found to be 0.53 and 1.749 µg/ml, respectively for aconitne.

*d) Specificity*

Specificity evaluation was carried out by analyzing aconitine. It was observed that the peak of the drug was well separated and was not being interfered by the degradation products and other components. Peak corresponding to the drug showed positive value for the minimum peak purity index over the entire range of integrated chromatographic peak thus indicating the purity of the peak (Table 5.54). Further, the peak corresponding to the drug obtained by the proposed method was seen to be pure (Figure 5.29). Thus, the method was confirmed to be specific for aconitine in the presence of the degradation products and other components.

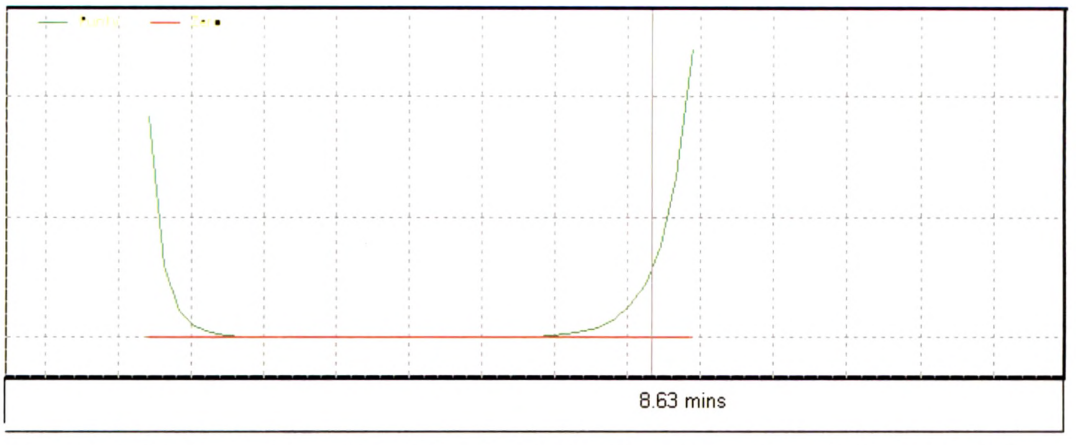



**Figure 5.29. Representative peak purity curve of ACN in peak purity analysis. The peak has a positive value of minimum peak purity index confirming the purity of the peak and specificity of the method developed in the presence of degradation products and other components.**

**Table 5.54. Peak purity data for the method.**

| **Parameter** | **ACONITINE** |
|---|---|
| Impurity | Not detected |
| Peak purity index | 1.0000 |
| Single point threshold | 0.999842 |
| Minimum peak purity index | 158 |

*e) Robustness*

Table 5.55 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recoveries obtained were between 98 % and 101 % for aconitine. Therefore, it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

**Table 5.55. Robustness of the method (n=6)**

| **Chromatographic change** | **Recovery, %** | |
|---|---|---|
| **Factor** | **Level** | **ACN** |
| **Acetonitrile Brand** | | |
| Spectrochem | 1 | 98.35 ± 0.27 |
| Rankem | 2 | 98.23 ± 0.62 |
| Qualigens | 3 | 100.31 ±0.94 |
| **Column Brand** | | |
| Hypersil | 1 | 100.19 ± 0.73 |

| | | |
|---|---|---|
| Phenomenex | 2 | 98.78 ± 0.64 |
| **Detection wavelength, nm** | | |
| λmax – 2 nm | -1 | 99.79 ± 1.34 |
| λmax | 0 | 99.97 ± 0.79 |
| λmax +2 nm | 1 | 98.72 ± 0.57 |

*f) System suitability*

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table 5.56). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

**Table 5.56 Results of system suitability parameters obtained from LC method.**

| Sr. No. | Parameter | Aconitine |
|---|---|---|
| 1 | Retention time (min) | 8.923 |
| 2 | Capacity factor (k') | 5.593 |
| 3 | Separation factor (α) | 1.377 |
| 4 | Efficiency/length(t.p/m) | 115239 |
| 5 | HETP (mm) | 0.049 |
| 6 | Resolution (Rs) | 5.34 |
| 7 | Assymmetry (As) | 1.08 |

*g) Accuracy*

As shown in Table 5.57, the recovery of the investigated components ranged from 98.17 % to 99.69 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed method manifested the reliability and accuracy for the measurement of the aconitine.

**Table 5.57 Recovery study of ACN and PIN added to the pre-analyzed samples using the proposed methods. (n=3)**

| Component | Quantity added % | Total Quantity Present | Amount Quantity Found | Recovery | % RSD |
|---|---|---|---|---|---|
| ACONITINE | 0 | 1.15 | 1.13 | 98.26 | 0.93 |
| | 80 | 2.07 | 2.03 | 98.06 | 0.81 |
| | 100 | 2.30 | 2.32 | 100.86 | 0.64 |
| | 120 | 2.53 | 2.50 | 98.81 | 0.91 |

***5.6.1.4.5) Applicability of the developed method in formulations***

The developed method was applied for the determination of Aconitine in *Mahamrutyunjaya rasa* formulation and the results are presented in Table 5.58. It was also observed that the content of aconitine varied in the three formulations and the content was found to be similar to the content obtained by above methods. The degradation profile of the marker and formulation was found to be similar. However, the formulation samples kept for degradation in humid conditions showed significantly higher rate of degradation. The reason being presence of biological components in the formulation. Thus, the determination of the active components in herbal tablets is important to study the effect of other components present in the formulation. The above method can therefore be successfully applied to improve the safety and quality control of the formulations available in the market. The WHO has emphasized the need to develop the quality control parameters for herbal products and in this connection the present method of estimation of Aconitine content of the formulation is very useful. The method is simple, precise and accurate method suitable for the routine analysis in pharmaceutical preparation.

**Table 5.58 Assay results of herbal dosage forms using the proposed LC method. (n=3)**

| Formulation | Clinical dosages(mg/day) | Aconitine (µg) per tablet |
|---|---|---|
| FORM -1 | 250 | 0.11 ± 0.01 |
| FORM-2 | 250 | 0.223 ± 0.01 |
| FORM-3 | 250 | 0.47 ± 0.02 |

## 5.6.2) Development of analytical methods for estimation of Solanine in *Mahamrutyunjaya rasa.*

### *5.6.2.1) HPLC Method*

#### *5.6.2.1.1) Optimization of chromatographic conditions*

Different parameters for chromatographic separation like different stationary phases (i.e C18 and C8 columns), pH and mobile phase composition were optimized to achieve maximum separation between solanine and other components present in the polyherbal formulation. An initial study was started with the selection of suitable HPLC column. A higher tailing factor was obtained while using C8 column than that of C18 column although both the columns offered good selectivity and sensitivity. Very sharp and symmetric peak of solanine was observed when a Phenomenex Luna $C_{18}$ (250 mm × 4.6 mm, 5 µm) column was employed. An attempt was made to determine the suitable pH of the mobile phase by trial and error method in the range of 3-8 pH. The pH 6 gave sufficient separation as well as symmetric peak shape. The final composition of the mobile phase was determined by varying the buffer: organic modifier ratio to give the best results. Several studies were carried out by decreasing the percentage of acetonitrile from 60-40%, till satisfactory resolution was obtained. A proportion of acetonitrile (40%) resulted in smoothening of baseline and complete separation of solanine from its degradation products. Finally, the mobile phase comprising of Tris buffer (10 mM; pH 6.0 adjusted with 1% Triethylamine) - acetonitrile (60: 40 v/v) showed good resolution, good peak symmetry, was used to formulation samples.

The detection wavelength was decided to be 218 nm from the UV scan in the range of 200 nm-400 nm.

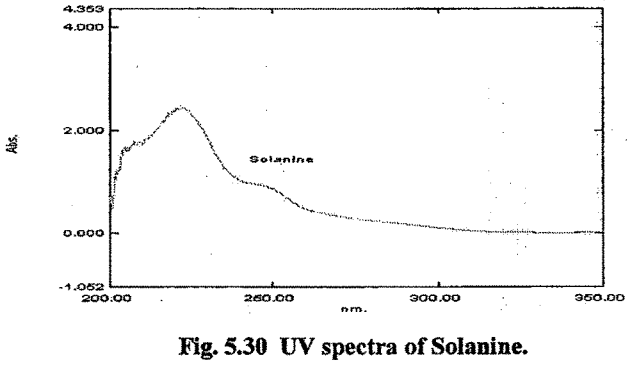



**Fig. 5.30 UV spectra of Solanine.**

*a) Effect of pH value in the mobile phase buffer on the retention time of HPLC.*

The pH dependence of the retention time was investigated with a range of 5-8 pH values by adjusting with 1 % triethylamine, in which the Tris Buffer reached a concentration at 10 mM. The peak at pH 6 showed a well separated symmetric peak.

The results of the chromatographic analysis indicated that the pH value has distinct effects, not only for the resolution of the alkaloids but also for separation of the alkaloids and other unknown compounds in the samples. At the pH value 6, all the components were baseline separated from each other with resolution values above 2. Therefore, the analysis was carried out at a pH range of 5.8-6.2, where the buffer solution works effectively.

Since alkaloids were to be analyzed in the sample solution, the ion-pair agents such as triethylamine was used to improve the separation of these components.

*b) Effect of mobile phase composition*

The effect of mobile phase composition (i.e. ratio of acetonitrile and Tris buffer was studied at 65:35, 60:40 and 55:45 v/v levels) with pH and flow rate of 6 and 1.0 ml/min respectively. The resolution was > 3 at 60:40, v/v level, which is one of the most desirable criteria. Also the retention time was shorter with satisfactory asymmetry values. An adequate theoretical plates (~51000) is indicative of a good column performance. All the other performance results were in acceptable limits.

*c) Effect of mobile phase flow rate*

The theoretical plates value was highest at flow rate of 1 ml/min. The change in flow rate had no significant effect on resolution and asymmetry while retention time decreased as the flow rate increased. The other system suitability parameters also indicated optimum flow rate of 1 ml/min.

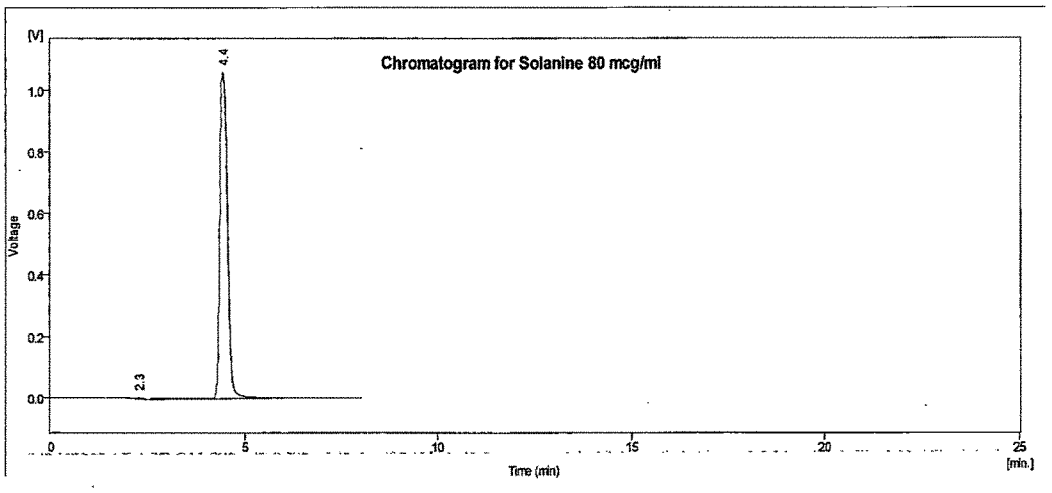



**Fig. 5.31. Representative HPLC Chromatograms of Solanine standard.**

***5.6.2.1.2) Extraction conditions***

The samples were treated with hydrochloric acid and extracted with ethyl acetate to remove the interferences in the acidic aqueous solution before the final extraction of the alkaloids by organic solvent after alkalization with 28% ammonia solution up to pH value of 10. The results showed that chloroform extracted alkaloids with high recoveries.

***5.6.2.1.3)Validation***

a) *Calibration curve (linearity)*

The calibration curves (n=3) constructed for solanine was linear over the concentration range of 1-100 µg/ml. Peak area of the marker was plotted versus the concentration and linear regression analysis performed on the resultant curve. The coefficients of determination 0.999 with % RSD values ranging from 0.5 to 2% across the concentration range studied were obtained following linear regression analysis (Table 5.60).

**Table 5.59. Peak area and Peak height of Solanine**

| Concentration(mcg/ml) | Peak area (mV) | Peak Height |
|---|---|---|
| **1** | 78.248 | 6.82 |
| **2.5** | 188.428 | 17.027 |
| **5** | 565.447 | 33.887 |
| **10** | 776.622 | 62.301 |
| **20** | 1843.05 | 138.189 |
| **40** | 3840.17 | 280.386 |
| **60** | 5577.941 | 423.276 |
| **80** | 7142.208 | 546.134 |
| **100** | 8877.587 | 684.719 |

**Fig 5.32. Calibration curve of solanine.**

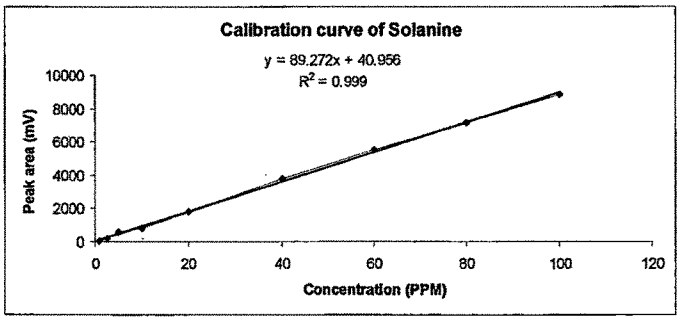



**Fig. 5.33. Representative chromatograms of solanine (10-100 µg/ml).**

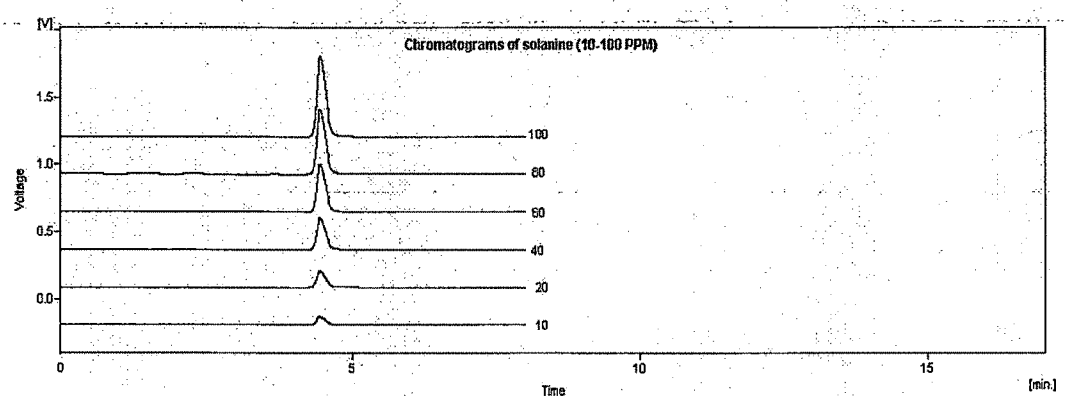



**Table 5.60. Linear regression data for the calibration curves (n=3)**

| | |
|---|---|
| Retention Time, min | 4.43 ± 0.1 |
| Detection wavelength, nm | 218 nm |
| LOD, µg/ml | 0.14 |
| LOQ, µg/ml | 0.345 |
| Linearity Range | 1-100 µg/ml |
| Coefficient of Determination (Height) | 0.9992 |
| Coefficient of Determination (Area) | 0.9996 |
| Regression equation (Height) | Y= 4.2316 x -0.0965 |
| Regression equation (Area) | Y= 89.272x+40.956 |
| Slope (Height) | 4.2316±0.13 |
| Slope (Area) | 89.272±1.176 |
| Intercept (Height) | 0.0965±0.05 |
| Intercept (Area) | 40.956±1.04 |

*b) Repeatability, precision and stability*

Injection repeatability- The calculated % RSDs of the peak areas was less than 2.0 % at each of the three concentration levels (Table 5.61).

Analysis repeatability- The RSD values for analysis repeatability were less than 2.0 % both for retention time and peak area (Table 5.61).

**Table 5.61 Repeatability of the developed method (n=6)**

| Solanine | Retention time (min) | | | | Peak area (mVs) | | | |
|---|---|---|---|---|---|---|---|---|
| (µg/ml) | Injection Repeatability | | Analysis repeatability | | Injection Repeatability | | Analysis repeatability | |
| | Mean | % RSD | Mean | % RSD | Mean | % RSD | Mean | % RSD |
| 10 | 4.43 | 0.28 | 4.42 | 0.14 | 79.08 | 1.08 | 80.13 | 1.66 |
| 40 | 4.41 | 0.34 | 4.43 | 0.22 | 3870.89 | 1.67 | 3865.82 | 1.25 |
| 100 | 4.42 | 0.27 | 4.43 | 0.29 | 8867.98 | 1.29 | 8860.15 | 1.73 |

Instrument precision- The precision result of the solution at medium concentration is presented in Table 5.62, and it was shown that the %RSD values of retention time were less than 1%, while the %RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision (Intra 4 h six injections, inter 6 days).

**Table 5.62. Intra-day and inter-day precision of HPLC method (n=6)**

| Amount | Intra-day precision | | | | Inter- day precision | | | |
|---|---|---|---|---|---|---|---|---|
| (µg/mL) | Mean area | S.D. | % RSD | S.E. | Mean area | S.D. | % RSD | S.E. |
| 1 | 79.08 | 2.12 | 1.98 | 2.34 | 80.67 | 1.76 | 1.93 | 2.32 |
| 40 | 3870.89 | 5.78 | 1.34 | 2.98 | 3886.12 | 6.78 | 1.90 | 4.09 |
| 100 | 8867.98 | 12.09 | 1.09 | 3.98 | 8872.31 | 15.65 | 1.98 | 5.13 |

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (RSD values of the retention time and peak area were both less than 3%).

*c) Limit of detection and limit of quantification*

The LOD and LOQ were found to be 0.14 and 0.345 µg/ml for Solanine.

*d) Specificity*

Satisfactory results were obtained, indicating the high specificity of the proposed method for the determination of solanine in the formulations. Good resolution and absence of interference was observed as shown in Fig.5.34.

*e) Robustness*

Table 5.63 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean were between 98 % and 101 % Solanine. Therefore, it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

**Table 5.63.Robustness of the method (n=3)**

| **Chromatographic change** | | **Recovery, %** |
|---|---|---|
| **Factor** | **Level** | |
| **Wavelength, nm.** | | |
| 215 | -1 | 98.73 ± 2.19 |
| 218 | 0 | 99.75 ± 1.27 |
| 221 | 1 | 99.76 ± 2.76 |
| **Column brand** | | |
| Phenomenex | 1 | 99.42± 0.84 |
| Hypersil | 2 | 98.31± 1.73 |
| **Acetonitrile Brand** | | |
| Rankem | 1 | 100.67 ± 1.23 |
| Spectrochem | 2 | 99.87 ± 1.45 |
| Qualigens | 3 | 98.43 ± 2.65 |

*f) System suitability*

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table. 5.64). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

**Table 5.64. System Suitability Parameters**

| Sr. No. | Parameter | Values |
|---|---|---|
| 1 | Retention time (min) | 4.43 |
| 2 | Area (mVs) | 565.44 |
| 3 | Capacity factor (k') | 3.08 |
| 4 | Separation factor (α) | ------ |
| 5 | Efficiency/length(t.p/m) | 514232 |
| 6 | HETP (mm) | 0.047 |
| 7 | Resolution (Rs) | 7.13 |
| 8 | Assymmetry (As) | 1.10 |

*g) Accuracy*

As shown in Table 5.65, the recovery of the investigated components ranged from 98 % to 101 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed method manifested the reliability and accuracy for the measurement of these components.

**Table 5.65. Recovery Test (n=3)**

| Excess drug added to the analyte(%) | Theoretical content (µg) | Recovery (%) | % RSD |
|---|---|---|---|
| 0 | 13.30 | 100.12 | 1.45 |
| 80 | 23.94 | 99.45 | 1.34 |
| 100 | 26.60 | 98.67 | 1.76 |
| 120 | 29.26 | 99.08 | 1.98 |

***5.6.2.1.4) Applicability of the developed method in formulations***

The developed HPLC method was applied to the determination of solanine in the Ayurvedic formulations (FORM1, FORM2, FORM3) and the results are presented in Table 5.66. It was observed that the content of solanine varied in the three formulations, with the %RSD values of higher than 5 %, which would significantly influence the quality stability because it is one of the target toxic component for the quality control of *Mahamrutyunjaya rasa* tablets.

**Table 5.66. Contents of the Solanine in three proprietary Ayurvedic medicines (n=3)**

| Sr. No. | Formulation | Solanine, µg per tablet |
|---|---|---|
| 1 | Form1 | 0.13 ± 0.01 |
| 2 | Form2 | 0.145 ± 0.02 |
| 3 | Form3 | 0.078 ± 0.01 |

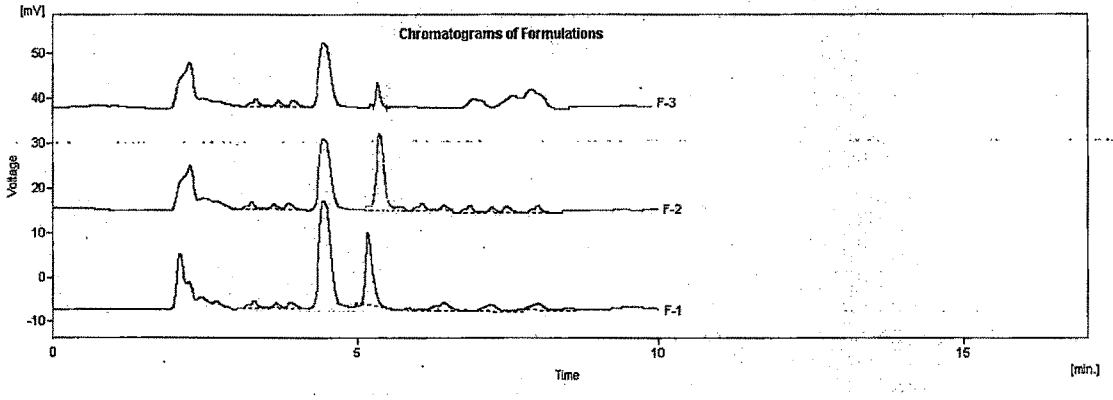



**Fig. 5.34. Representative chromatogram of FORM-1 (F-1), FORM-2 (F-2) and FORM-3 (F-3).**

### *5.6.2.2) HPTLC method*

#### *5.6.2.2.1)Development of the optimum mobile phase*

The TLC procedure was optimized with a view to develop an assay method for the estimation of Solanine. Both the pure drug and the formulation extract solution were spotted on the TLC plates and run in different solvent systems. Initially, Chloroform: Methanol in varying ratios was tried. The mobile phase Chloroform: Methanol (7:3, v/v) gave good resolution but $R_f$ value was less. Also, the typical peak nature was missing because the spot was slightly diffused. Addition of 0.5 ml of 28% ammonia solution to the above mobile phase improved the spot characteristics and increased the $R_f$ value to 0.05 ± 0.02 when densitometric scanning was performed at 500 nm after derivatisation with draggendorff's reagent. Finally, the mobile phase consisting of Chloroform: Methanol: Ammonia (7:3:0.5 v/v/v) gave a sharp and symmetrical peak. Resolution between spots of standard and other components appeared better when TLC plates (pretreated with methanol and activated at 100 °C for 5 min). Well-defined spots (compact dense spots) were obtained when the chamber was saturated with the mobile phase for 15 min at room temperature (Fig. 5.37).

***5.6.2.2.2) Validation of the method***

*a) Linearity*

Calibration graph was found to be linear that is adherence of the system to Kubelka Munk theory, which was found over the concentration range of 1600–4800 ng/spot ($r^2$=0.99932). Linearity was evaluated by determining the standard working solution containing 400 µg/ml of solanine in triplicate. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data showed a good linear relationship over the low concentration range of 1600–4800 ng/spot. The linearity of calibration graphs and adherence of the system to Kubelka Munk theory were validated by high value of correlation coefficient and the SD for intercept value was less than 2. No significant difference was observed in the slopes of standard plots.

**Table 5.67.Linearity data of solanine analysis by HPTLC.**

| Track | Vial | Rf | Amount (ng) | Height | X(calc) (ng) | Area | X(calc) (ng) | SampleID/ Remark |
|---|---|---|---|---|---|---|---|---|
| 2 | A1 | | | | | | | |
| 3 | A1 | 0.03 | | 147.34 | 2953 | 2133.41 | 3893 | LAB |
| 4 | A2 | 0.05 | | 11.30 | 736.98 | 104.45 | 745.98 | BD |
| 5 | A3 | 0.04 | | 15.07 | | 157.87 | | PUN |
| 6 | A4 | | | | | | | |
| 7 | A4 | 0.04 | 1600 | 79.82 | | 778.55 | | |
| 8 | A4 | 0.05 | 2400 | 126.78 | | 1358.79 | | |
| 9 | A4 | 0.05 | 3200 | 163.47 | | 1816.55 | | |
| 10 | A4 | 0.05 | 4000 | 198.74 | | 2265.29 | | |
| 11 | A1 | 0.05 | 4800 | 222.73 | | 2534.45 | | |
| 12 | | 0.04 | | 144.87 | 2898 | 2167.32 | 3954 | LAB |
| 13 | | 0.05 | | 12.36 | 741.78 | 117.85 | 750.56 | BD |
| 14 | | 0.05 | | 16.15 | 172.2 | 161.00 | 167.95 | PUN |

**Table 5.68. Regression analysis for calibration plots. (n=3)**

| Parameters | Values |
|---|---|
| Linearity Range | 1600 ng/µL to 4800 ng/µL |
| Coefficient of Determination (Height) | 0.99344 |
| Coefficient of Determination (Area) | 0.99242 |
| Regression equation (Height) | y=44.77x+ 15.14 |
| Regression equation (Area) | y=552.3x-16.6 |
| Slope (Height) | 44.77± 1.02 |
| Slope (Area) | 552.3± 2.23 |
| Intercept (Height) | 15.14± 0.83 |
| Intercept (Area) | 16.6 ± 0.21 |

*b) Precision*

The repeatability of sample application and measurement of peak area were expressed in terms of % RSD and found to be 1.84 and 1.67 respectively for six replicate determinations. The % RSD for intra-day and inter-day variation of solanine peak area at two different concentration levels 1600, 4800 ng/spot are shown in Table 5.69, respectively.

**Table 5.69. Intra-day and inter-day precision of HPTLC method. (n=6)**

| Amount (ng/spot) | Intra-day precision | | |
|---|---|---|---|
| | **Mean area** | **S.D.** | **% RSD** |
| 1600 | 758.97 | 24.98 | 1.05 |
| 4800 | 2541.88 | 76.83 | 1.65 |
| **Amount (ng/spot)** | **Inter-day precision** | | |
| | **Mean area** | **S.D.** | **% RSD** |
| 1600 | 760.32 | 38.52 | 1.23 |
| 4800 | 2545.40 | 82.66 | 1.57 |

*c) Robustness of the method*

The standard deviation of peak areas was calculated for each parameter and RSD was found to be less than 3%. The low values of % RSD as shown in Table 5.70 indicated robustness of the method.

**Table. 5.70.Robustness of the method.**

| Amount (ng/spot) | Mobile phase composition % RSD | |
|---|---|---|
| | **Chloroform :Methanol: Ammonia (7:3:0.5 v/v)** | **Chloroform :Methanol: Ammonia (7.5:2.5:0.5 v/v)** |
| 1600 | 1.76 | 1.94 |
| 4800 | 1.88 | 2.04 |

*d) LOD and LOQ*

The calibration plot in this study was plotted between amount of analyte versus average response (peak area) and the regression equation with a regression coefficient of 0.999 was obtained. Detection limit and quantification limit was calculated by the method as described earlier and found 50 ng and 100 ng respectively, which indicates the adequate sensitivity of the method.

*e) Specificity*

The peak purity of solanine was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot i.e., $r^2$ = 0.9991 and $r^2$ = 0.9988. Good correlation ($r^2$ = 0.9996) was also obtained between standard and sample spectra of solanine.

*f) Accuracy*

The proposed method when used for extraction and subsequent estimation of solanine from pharmaceutical dosage form after spiking with 80, 100 and 120% of additional drug afforded recovery of 98–100% as listed in Table 5.71.

**Table 5.71.Recovery studies. (n=3)**

| Excess drug added to the analyte(%) | Theoretical content (ng) | Recovery (%) | % RSD |
|---|---|---|---|
| 0 | 1600 | 99.45 | 1.63 |
| 80 | 2880 | 98.72 | 1.88 |
| 100 | 3200 | 98.34 | 2.23 |
| 120 | 3520 | 99.21 | 1.95 |

*g) Spot stability*

No decomposition was observed during spotting and development.

***5.6.2.2.3) Analysis of the marketed formulation***

A compact spot at $R_f$ 0.05 was observed in the chromatogram of the drug samples extracted from tablets. There was no interference from the other components present in the formulation. The good performance of the method indicated the suitability of this method for routine analysis of solanine in the herbal dosage form.

**Table. 5.72.Results of analysis:**

| Sr. No. | Formulation | Solanine, µg per tablet |
|---|---|---|
| 1 | Form-1 | 0.11± 0.02 |
| 2 | Form-2 | 0.158± 0.02 |
| 3 | Form-3 | 0.070± 0.01 |

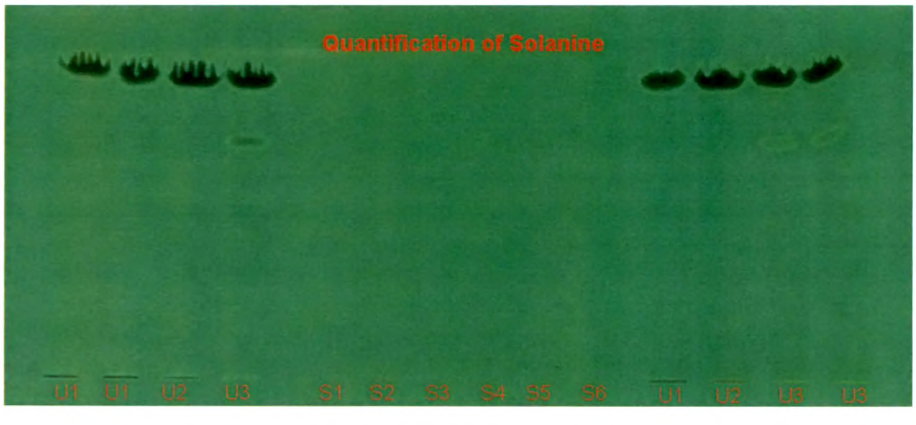



**Fig 5.35. Image of the TLC plate before derivatization at 254nm**

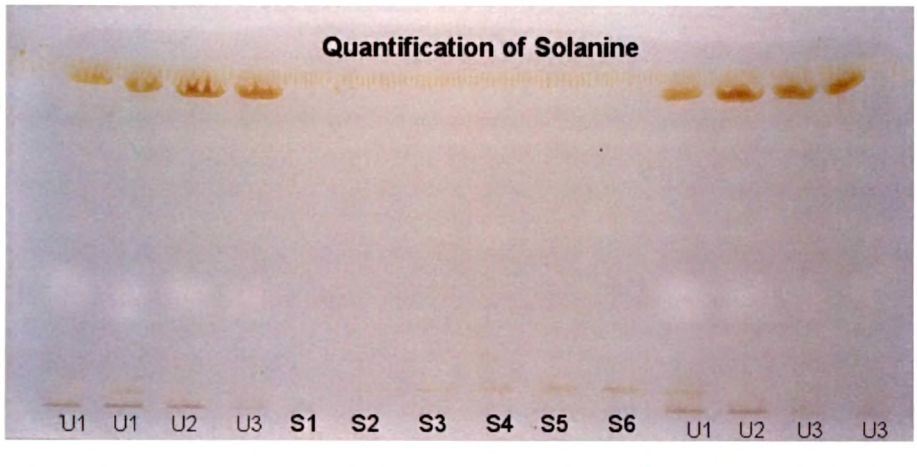
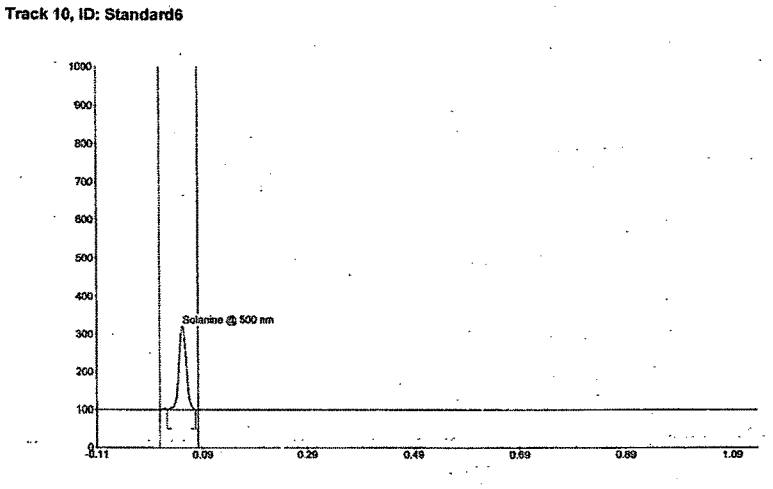
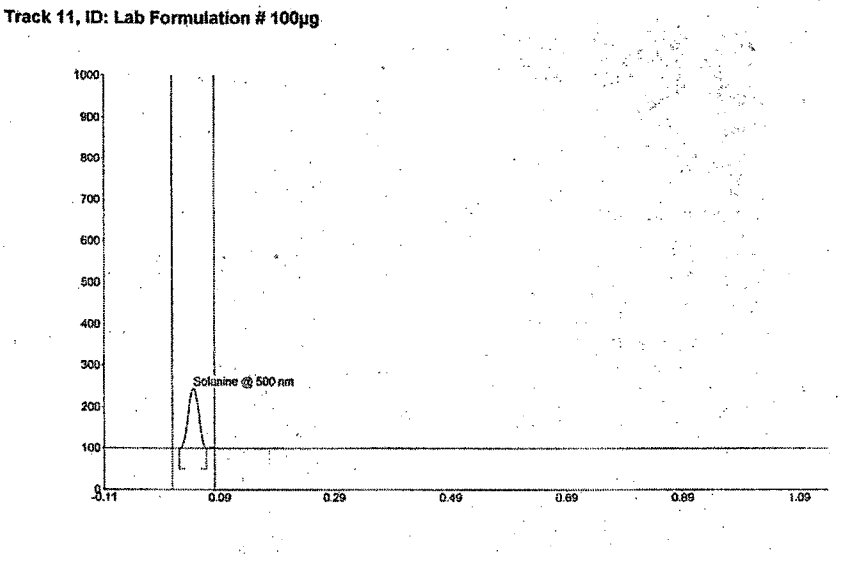



**Fig. 5.36. Image of the TLC plate after derivatization with Dragendroff at 500 nm.**




(a)




(b)

**Fig. 5.37. Representative HPTLC Chromatograms of (a)Solanine standard and (b)Form-1.**

**5.6.3) Development of analytical methods for estimation of Piperine in *Mahamrutyunjaya rasa.***

***5.6.3.1) HPTLC Method***

***5.6.3.1.1) Development of the optimum mobile phase***

The TLC procedure was optimized with a view to develop an assay method for the estimation of Piperine. Both the pure drug and the formulation extract solution were spotted on the TLC plates and run in different solvent systems. Initially, toluene–ethylacetate in varying ratios was tried. The mobile phase Toluene: Ethylacetate (7:3 v/v) gave good resolution and good spot characteristics and the $R_f$ value to 0.22 ± 0.01 when densitometric scanning was performed at 343 nm. Further, the post chromatographic derivatisation was performed using draggendorff's reagent and densitometric scanning was performed at 500 nm. Finally, the mobile phase consisting of toluene-ethylacetate (7:3, v/v) gave a sharp and symmetrical peak. Resolution between spots of standard and other components appeared better when TLC plates (pretreated with methanol and activated at 100 °C for 5 min). Well-defined spots (compact dense spots) were obtained when the chamber was saturated with the mobile phase for 10 mins at room temperature (Fig. 5.39).

***5.6.3.1.2) Validation of the method***

*a) Linearity*

Calibration graph was found to be linear that is adherence of the system to Kubelka Munk theory, which was found over the concentration range of 40–200 ng/spot ($r^2$ = 0.9998). Linearity was evaluated by determining the standard working solution containing 10 µg/ml of piperine in triplicate. Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficients. The regression data showed a good linear relationship over the low concentration range of 40–200 ng/spot. The linearity of calibration graphs and adherence of the system to Kubelka Munk theory were validated by high value of correlation coefficient and the SD for intercept value was less than 2. No significant difference was observed in the slopes of standard plots.

**Table. 5.73. Linearity data of Piperine analysis by HPTLC.**

| Track | Vial | Rf | Amount (ng) | Height | X(calc) (ng) | Area | X(calc) (ng) | Sample |
|---|---|---|---|---|---|---|---|---|
| 1 | A1 | 0.21 | | 223.78 | 56.50 | 4688.37 | 56.45 | |
| 2 | A2 | 0.22 | | 308.95 | 94.92 | 6780.94 | 96.53 | LAB |
| 3 | A3 | 0.22 | | 267.51 | 79.18 | 5999.11 | 80.59 | BD |
| 4 | A4 | 0.22 | 40 | 178.49 | | 3639.80 | | PUN |
| 5 | A4 | 0.22 | 80 | 284.36 | | 6138.98 | | |
| 6 | A4 | 0.22 | 120 | 348.68 | | 7697.70 | | |
| 7 | A4 | 0.22 | 120 | 354.98 | | 7822.99 | | |
| 8 | A4 | 0.22 | 160 | 405.43 | | 9199.76 | | |
| 9 | A4 | 0.22 | 200 | 439.35 | | 10274.72 | | |
| 10 | A1 | 0.22 | | 230.81 | 59.36 | 4760.66 | 57.71 | |
| 11 | A2 | 0.22 | | 322.08 | 101.82 | 6891.82 | 98.91 | LAB |
| 12 | A3 | 0.21 | | 287.30 | 84.23 | 6144.34 | 83.45 | BD |

**Table 5.74. Regression analysis for calibration plots. (n=3)**

| Parameter | Values |
|---|---|
| Linearity Range | 40 ng/µL to 200 ng/µL |
| Coefficient of Determination (Height) | 0.99916 |
| Coefficient of Determination (Area) | 0.99934 |
| Regression equation (Height) | Y=1.645x +139.6 |
| Regression equation (Area) | Y=42.58x + 2440 |
| Slope (Height) | 1.645± 0.52 |
| Slope (Area) | 42.58± 1.62 |
| Intercept (Height) | 139.6± 1.93 |
| Intercept (Area) | 2440± 3.22 |

*b) Precision*

The repeatability of sample application and measurement of peak area were expressed in terms of % RSD and found to be 1.38 and 0.94% respectively for six replicate determinations. The % RSD for intra-day and inter-day variation of piperine peak area at two different concentration levels 40, 200 ng/spot are shown in Table.5.75, respectively.

**Table 5.75. Intra-day and inter-day precision of HPTLC method. (n=6)**

| Amount (ng/spot) | Intra-day precision | | |
|---|---|---|---|
| | **Mean area** | **S.D.** | **% RSD** |
| 40 | 182.67 | 8.43 | 1.21 |
| 200 | 441.54 | 13.65 | 1.36 |
| **Amount (ng/spot)** | **Inter-day precision** | | |
| | **Mean area** | **S.D.** | **% RSD** |
| 40 | 177.32 | 10.30 | 1.08 |
| 200 | 180.54 | 14.64 | 1.55 |

*c) Robustness of the method*

The standard deviation of peak areas was calculated for each parameter and %RSD was found to be less than 2%. The low values of % RSD as shown in Table 5.76 indicated robustness of the method.

**Table. 5.76. Robustness of the method.**

| Amount (ng/spot) | Mobile phase composition % RSD | |
|---|---|---|
| | **Toluene: Ethyl Acetate: (7:3: v/v)** | **Toluene: Ethyl Acetate: (8:2 v/v)** |
| 40 | 1.32 | 1.05 |
| 200 | 1.55 | 1.64 |

*d) LOD and LOQ*

The calibration plot in this study was plotted between amount of analyte versus average response (peak area) and the regression coefficient was 0.999. Detection limit and quantification limit was calculated by the method as described earlier and found 10 ng and 20 ng respectively, which indicates the adequate sensitivity of the method.

*e) Specificity*

The peak purity of piperine was assessed by comparing the spectra at peak start, peak apex and peak end positions of the spot i.e., $r^2$ = 0.9991 and $r^2$ = 0.9993. Good correlation ($r^2$ = 0.9988) was also obtained between standard and sample spectra of piperine.

*f) Accuracy*

The proposed method when used for extraction and subsequent estimation of piperine from pharmaceutical dosage form after spiking with 80, 100 and 120% of additional drug afforded recovery of 99–101% as listed in Table 5.77.

**Table 5.77. Recovery studies. (n=3)**

| Excess drug added to the analyte(%) | Theoretical content (ng) | Recovery (%) | % RSD |
|---|---|---|---|
| 0 | 120 | 99.60 | 1.80 |
| 80 | 216 | 100.63 | 2.05 |
| 100 | 240 | 100.91 | 2.21 |
| 120 | 264 | 98.52 | 1.94 |

*g) Spot stability*

No decomposition was observed during spotting and development.

***5.6.3.1.4) Analysis of the marketed formulation***

A compact spot at $R_f$ 0.22 was observed in the chromatogram of the drug samples extracted from tablets. There was no interference from the other components present in the formulation. The good performance of the method indicated the suitability of this method for routine analysis of piperine in the herbal dosage form.

**Table 5.78. Results of analysis:**

| Sr.No. | Formulation | Piperine, µg per tablet |
|---|---|---|
| 1 | Form-1 | 0.45±0.06 |
| 2 | Form-2 | 0.56±0.05 |
| 3 | Form-3 | 0.41±0.03 |

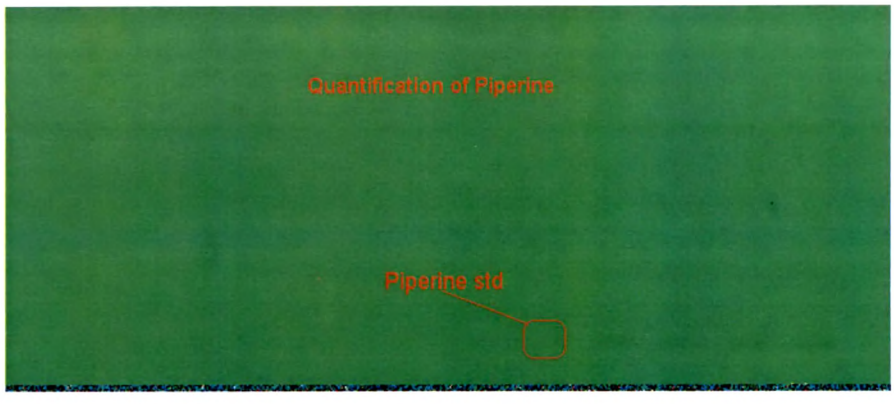



**Fig. 5.38. Image of TLC plate before derivatization at 254nm.**

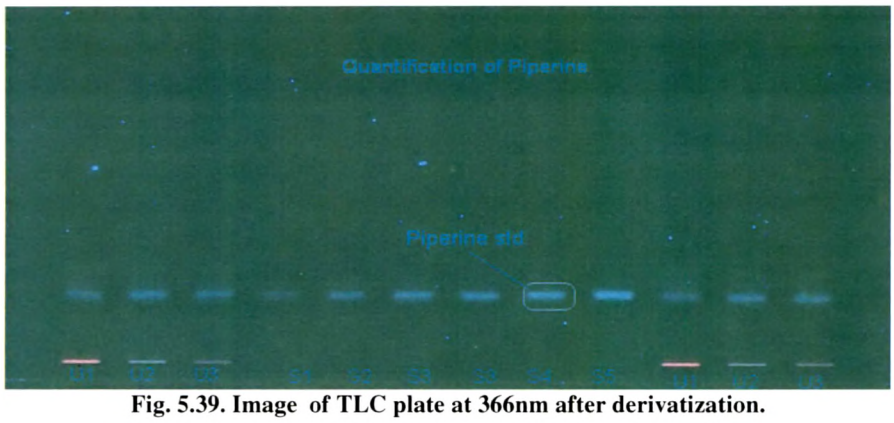
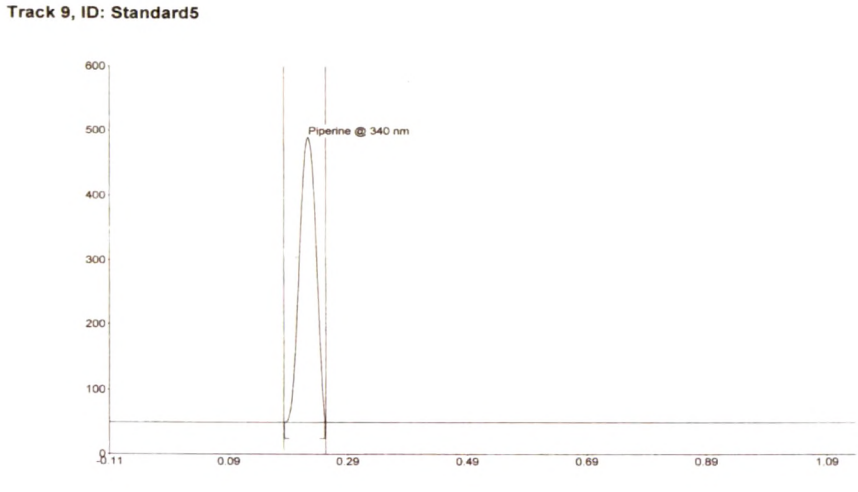
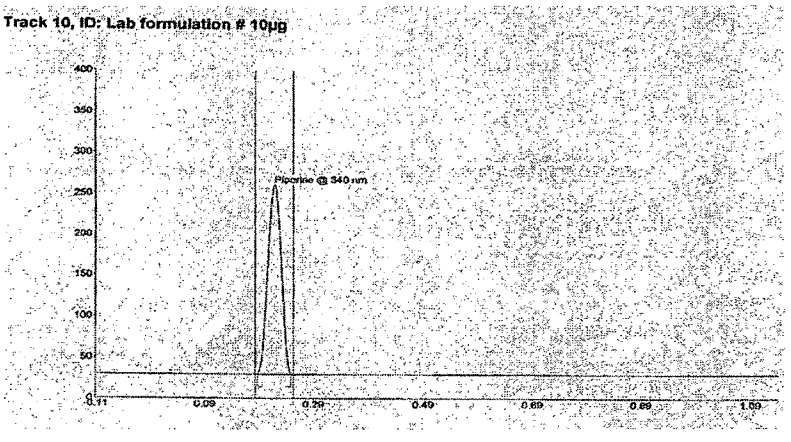



**Fig. 5.39. Image of TLC plate at 366nm after derivatization.**




(a)




(b)

**Fig. 5.40. Representative HPTLC Chromatograms of (a) Piperine standard and (b) Form-1.**

### 5.6.3.2) Stability indicating method development for Piperine in *Mahamrutyunjaya rasa.*

#### *5.6.3.2.1) Chromatographic separation*

A simple HPLC method was adopted for the determination of piperine in MHR formulation. To optimize the proposed HPLC method, all of the experimental conditions were investigated. For the choice of stationary phase, reversed-phase separation was preferred due to the drawbacks of the normal phase, eg., hydration of silica with water that can cause peak tailing. To optimize the mobile phase, different systems were tried for chromatographic separation of the components by combining homogenous design and solvent polarity optimization. The best resolution was achieved using a mobile phase consisting of acetonitrile - $KH_2PO_4$ buffer (10 mM, pH 7± 0.1) (35:65 v/v) for piperine, which gave good resolution and sensitivity.

#### *5.6.3.2.2) Forced Degradation Studies*

*a) Acidic condition.* - The individual markers were subjected to heating in specified concentration of HCl for 12 h. Piperine was found to be susceptible to acid degradation. About 20-25 % degradation was observed in piperine with a number of degradation peaks at retention times 4.71, 4.85, 4.95, 7.53, 8.02, 8.11, 8.23, 10.821 and 11.34 mins (Figure 5.41(a)). On further heating up to 24 h, there was significant rise in the proportion of the already degraded peaks of piperine.

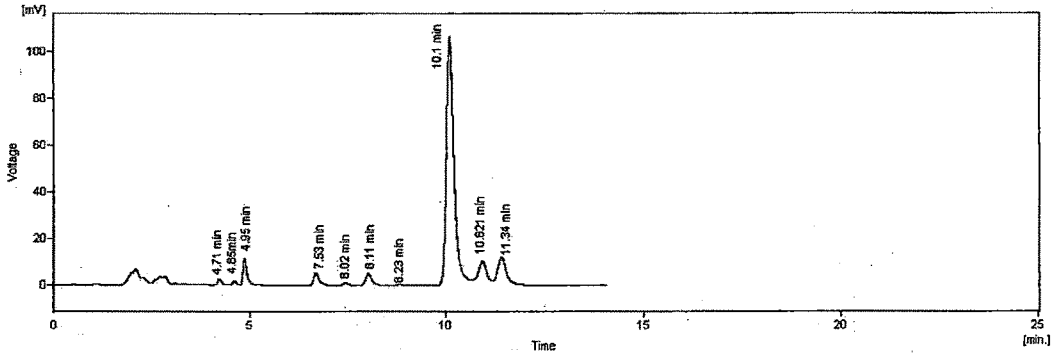



**Figure 5.41 (a). Chromatographic separation of PIN (75 µg/mL) in stressed sample of the standard subjected to acid hydrolysis.**

*Degradation in alkali.* - Piperine had similar degradation pattern as seen in acidic condition and it degraded to give a large degradation peak at retention time 4.045 mins and a number of small peaks at 7.17, 7.9, 10.95 and 11.345 mins (Figure 5.41 (b)).

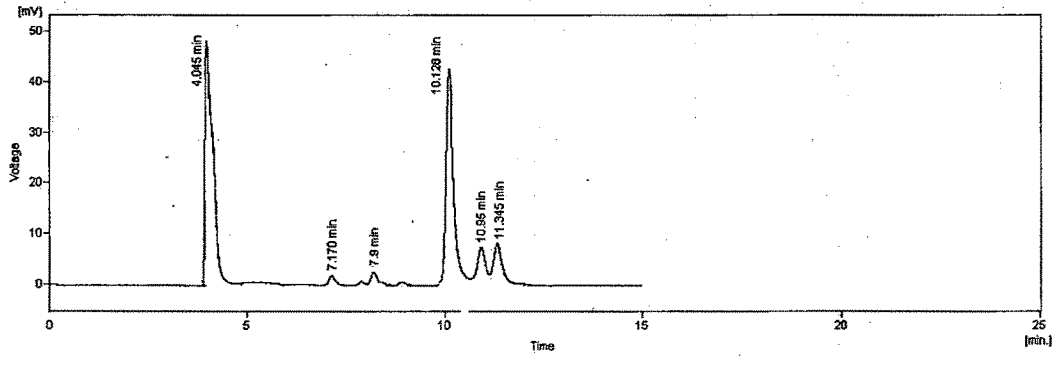



**Figure 5.41. (b). Chromatographic separation of PIN (75 µg/mL) in stressed sample of the standard subjected to alkali hydrolysis.**

*c) Neutral (water) condition* - It was observed that around 8–10% of the piperine degraded on refluxing the drug in water at 80 °C for 24 h. Degradation peaks for piperine were observed at retention times 7.53, 8.02, 8.13, 8.21, 10.821 and 11.34 mins (Figure 5.41(c)).

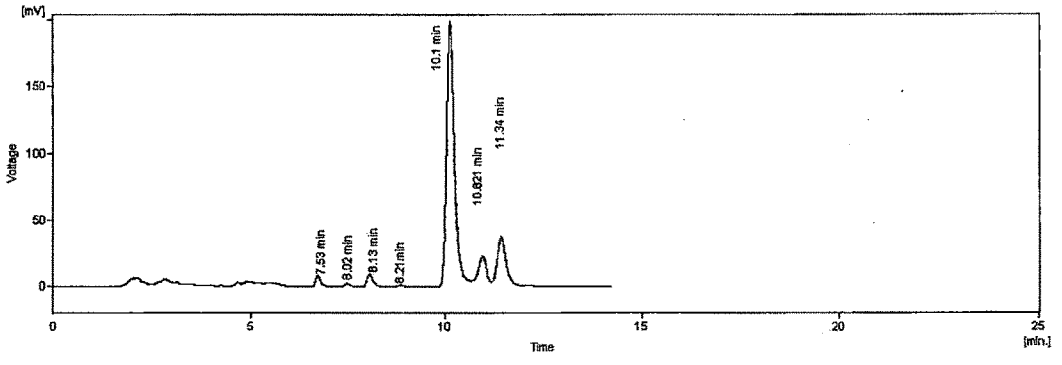



**Figure 5.41 (c). Chromatographic separation of PIN (75 µg/mL) in stressed sample of the standard subjected to water hydrolysis.**

*d) Oxidative conditions.* – Piperine was comparatively less labile as around 5-10% degradation was observed in the case of piperine in 3% $H_2O_2$. The major degradation products of piperine were at (RTs) 7.53 min, 10.821 and 11.345 min and a minute degradation was observed at 8.02 min (Figure 5.41 (d)).

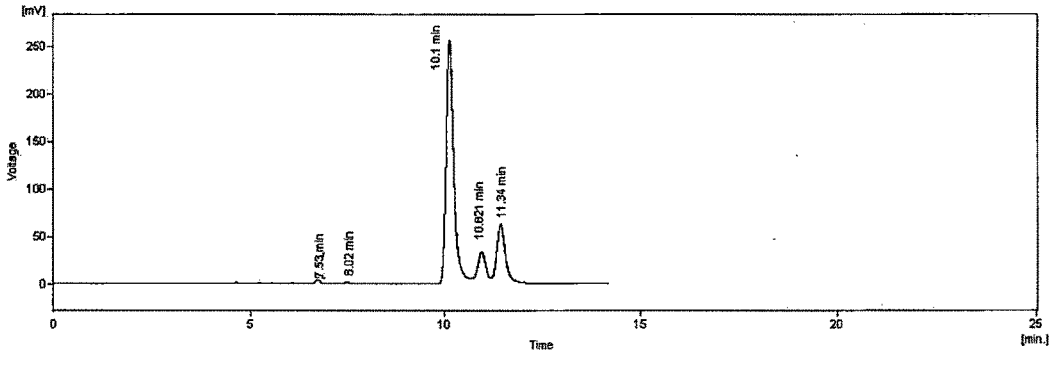



**Figure 5.41. (d). Chromatographic separation of PIN (75 µg/mL) in stressed sample of the standard subjected to oxidation.**

*e) Solid-state study (Thermal degradation).* - Piperine was found to be stable when exposed to dry heat at 80°C for 48 h. The percent amount of piperine remaining after 48 h of dry heat exposure was in the range of 90-92%. Degradation peak of piperine was observed at 7.53 min, 10.821 and 11.345 mins (Figure 5.41 (e)).

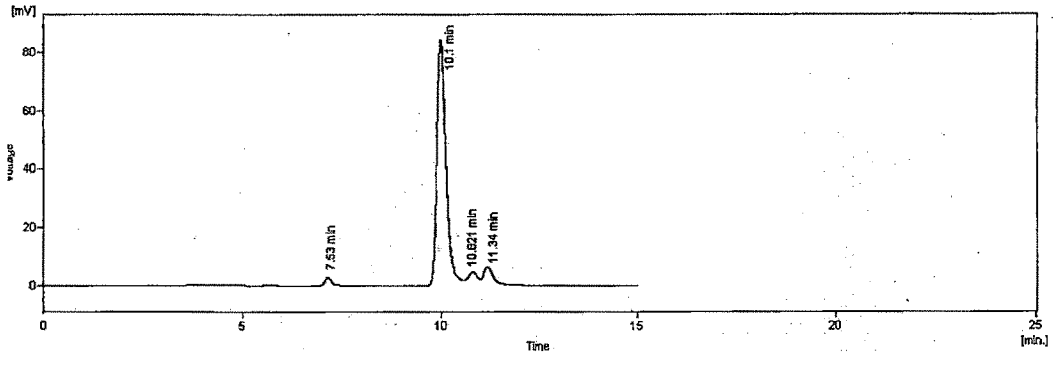



**Figure 5.41 (e). Chromatographic separation of PIN (75 µg/mL) in stressed sample of the standard subjected to thermal degradation.**

*f) Photolytic conditions* – Piperine was found to be highly susceptible to photolytic degradation. Piperine was also susceptible to alkaline photolytic condition (around 60% degradation). A number of degradation products were formed at retention times 4.732, 7.53, 8.02, 8.13, 10.821 and 11.345 mins (Figure 5.41 (f)).

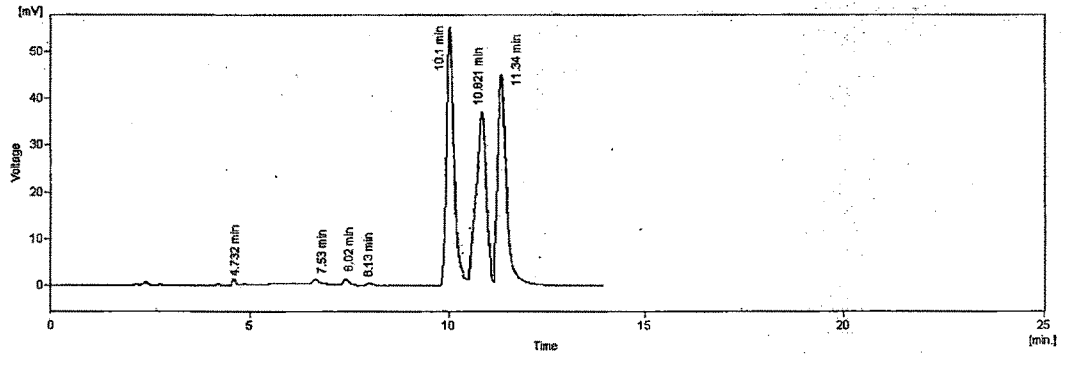



**Figure 5.41. (f). Chromatographic separation of PIN (75 µg/mL)in alkaline stressed sample of the standard subjected to photolytic degradation.**

*g) Humidity:* Piperine was found to be stable in humid conditions. Only three small peaks were observed of degradation products of piperine at retention times 7.53, 10.821 and 11.34 min (Figure 5.41 (g)).

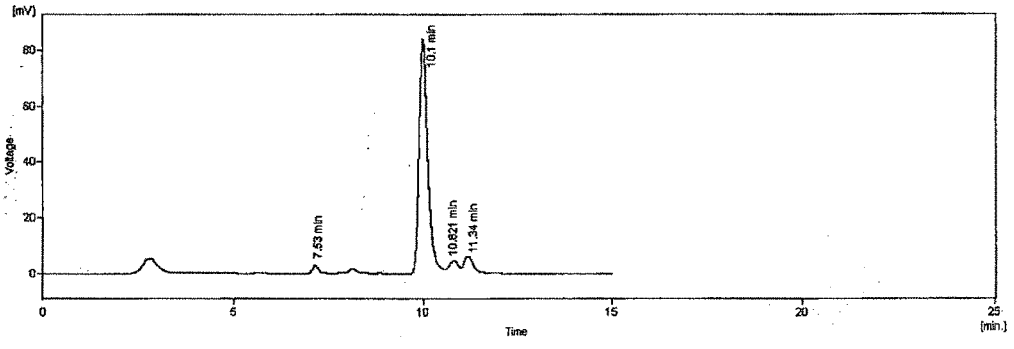



**Figure 5.41 (g). Chromatographic separation of PIN (75 µg/mL) in stressed sample of the standard subjected to 75% RH at 30°C.**

*h) Establishment of a Stability-indicating Method for a Mixture of Stressed Solutions*

The mobile phases comprising of potassium dihydrogen phosphate (10 mM; pH 7.0±0.1 adjusted with triethylamine) – acetonitrile (65:35, v/v) was first used to analyze piperine standard drug samples for the linearity study (Figure 5.42). These were then applied on stressed samples of individual drugs which in turn showed recognizable degradation (Figure 5.41(a)-(g). The method was then successfully applied for the degradation studies of formulations (Figure 5.44).

***5.6.3.2.3) Method Validation***

The methods developed for analysis of piperine was validated for linearity, accuracy, precision, specificity, and quantification limits as per ICH guidelines.

*a) Calibration curve (linearity)*

Linear regression analysis confirms that the $r^2$ values for piperine was to be >0.999, confirming the linear relationship between the concentration of the drugs and area under the curve (Table 5.79). The calibration curves (n=3) constructed for the piperine was linear over the concentration range of 10-100 µg/ml.

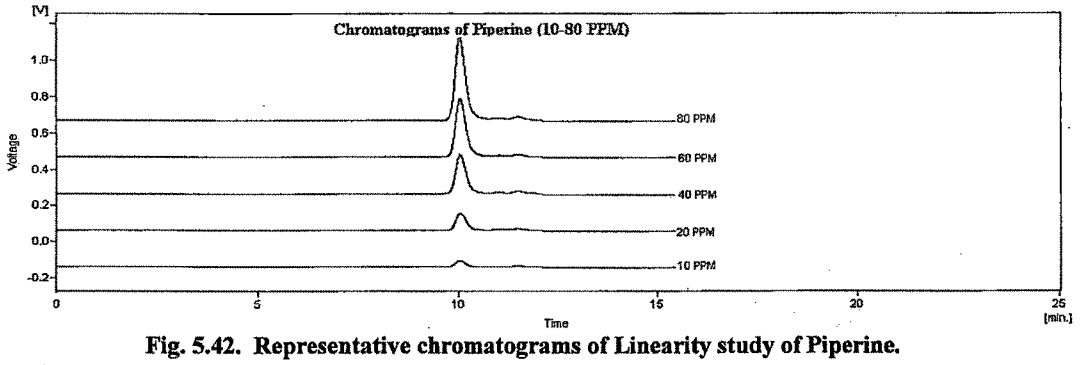



**Fig. 5.42. Representative chromatograms of Linearity study of Piperine.**

**Table 5.79 Linear regression data for the calibration curves (n=3)**

| **Parameter** | **Piperine** |
|---|---|
| Retention Time, min | 10.1 ± 0.2 |
| Detection wavelength, nm | 343 nm |
| LOD, µg/ml | 0.21 |
| LOQ, µg/ml | 0.693 |
| Linearity Range | 10-100 µg/ml |
| Correlation Coefficient (Height) | 0.9992 |
| Correlation Coefficient (Area) | 0.9996 |
| Regression equation (Height) | Y= 17.89x-140.11 |
| Regression equation (Area) | Y=1.0305x+2.4063 |

*b) Precision and stability*

The precision result of the solution at the three concentrations is presented in Table 5.80, and it was shown that the %RSD values of retention time were less than 1%, while the RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision (Intra 4 h six injections, inter 6 days).

**Table 5.80. Intra-day and Inter-day Precision study results.**

| Piperine, µg/ml | Intra-day | | | | Inter-day | | | |
|---|---|---|---|---|---|---|---|---|
| | Retention Time | | Peak area | | Retention time | | Peak area | |
| | Mean | % RSD | Mean | % RSD | Mean | % RSD | Mean | % RSD |
| 10 | 10.1 | 0.26 | 97.234 | 1.38 | 10.1 | 0.51 | 93.92 | 0.82 |
| 40 | 10.12 | 0.096 | 522.28 | 0.93 | 10.1 | 0.23 | 524.35 | 0.93 |
| 100 | 10.12 | 0.18 | 1774.11 | 1.28 | 10.12 | 0.28 | 1670.18 | 1.14 |

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (%RSD values of the retention time and peak area were both less than 3%).

*c) Limit of detection and limit of quantification*

The LOD and LOQ were found to be 0.21 and 0.693 µg/ml, respectively for piperine.

*d) Specificity*

Specificity evaluation was carried out by analyzing piperine. It was observed that the peak was well separated and was not being interfered by the degradation products and other components. Peak corresponding to piperine showed positive value for the minimum peak purity index over the entire range of integrated chromatographic peak thus indicating the purity of the peak. (Table 5.81). Further, peak of piperine obtained by the proposed method was seen to be pure (Figure 5.43). Thus, the method was confirmed to be specific for piperine in the presence of the degradation products and other components.

**Tabe 5.81. Peak Purity data of Piperine analysis.**

| Parameter | PIN |
|---|---|
| Impurity | Not detected |
| Peak purity index | 1.0000 |
| Single point threshold | 0.921019 |
| Minimum peak purity index | 78981 |

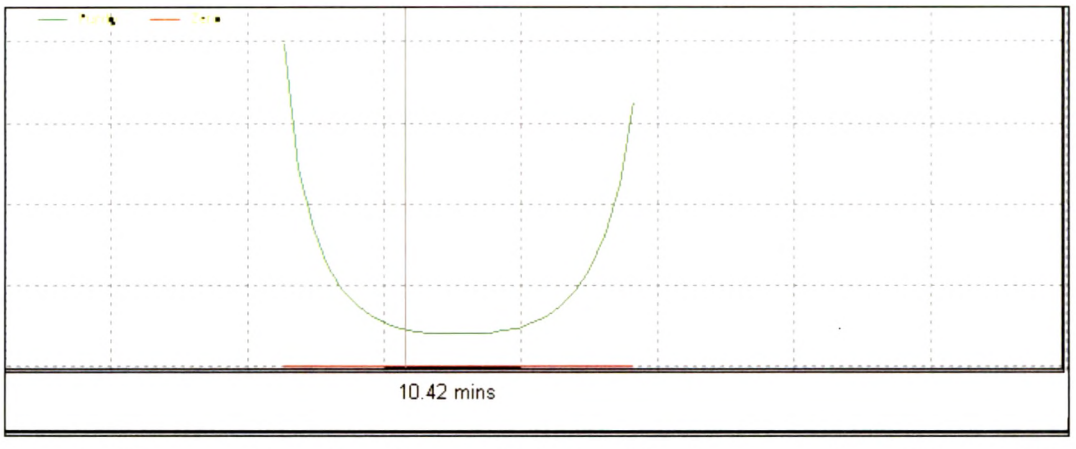



**Figure 5.43. Representative peak purity curve of PIN in peak purity analysis. The peak has a positive value of minimum peak purity index confirming the purity of the peak and specificity of the method developed in the presence of degradation products and other components.**

*e) Robustness*

Table 5.28 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean were between 98 % and 101 % for Piperine. Therefore, it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

**Table 5.82. Robustness of the method**

| **Chromatographic change** | **Recovery, %** | |
|---|---|---|
| **Factor** | **Level** | **Piperine** |
| **Acetonitrile Brand** | | |
| Spectrochem | 1 | 99.36 ± 0.56 |
| Rankem | 2 | 98.63 ± 0.67 |
| Qualigens | 3 | 99.62 ± 1.09 |
| **Column Brand** | | |
| Hypersil | 1 | 99.52 ± 0.53 |
| Phenomenex | 2 | 98.54 ± 0.54 |
| **Detection wavelength, nm** | | |
| λmax – 2 nm | -1 | 99.51 ± 1.98 |
| λmax | 0 | 99.98 ± 0.78 |
| λmax +2 nm | 1 | 98.11 ± 1.56 |

*f) System suitability*

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table 5.84). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

**Table 5.84. Results of system suitability test for piperine analysis.**

| Sr. No. | Parameter | Piperine |
|---|---|---|
| 1 | Retention time (min) | 10.1 |
| 2 | Capacity factor (k') | 7.703 |
| 3 | Separation factor (α) | 1.452 |
| 4 | Efficiency/length(t.p/m) | 147312 |
| 5 | HETP (mm) | 0.047 |
| 6 | Resolution (Rs) | 3.149 |
| 7 | Assymmetry (As) | 1.10 |

*g) Accuracy*

As shown in Table 5.85, the recovery of the investigated component ranged from 99 % to 100 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed method manifested the reliability and accuracy for the measurement of piperine.

**Table 5.85. Recovery test**

| Quantity of Piperine added % | Total Quantity Present | Amount Quantity Found | Recovery | % RSD |
|---|---|---|---|---|
| 0 | 43.72 | 43.53 | 99.56 | 0.32 |
| 80 | 78.69 | 78.44 | 99.68 | 1.02 |
| 100 | 87.44 | 86.91 | 99.39 | 0.93 |
| 120 | 96.184 | 95.94 | 99.74 | 1.33 |

***5.6.3.2.4) Applicability of the developed method in formulations***

The developed method was applied to the determination of Piperine in *Mahamrutyunjaya rasa* and the results are presented in Table 5.86. It was observed that the content of Piperine was comparable in the three formulations.

The degradation pattern in the formulations was also found to be similar to that of marker and the peak of the marker could be differentiated from the peaks of the other components as well as degradation products. The individual determination of this active component and their degradation pattern in herbal tablets could be successfully applied to improve the safety and quality control of the formulations available in the market. The WHO has emphasized the need to develop the quality control parameters for herbal products and in this connection the present method of estimation of Piperine content of the formulation is very useful. The method is simple, precise and accurate method suitable for the routine analysis in pharmaceutical preparations.

**Table 5.86 Results of analysis.**

| Formulation | Clinical dosages(mg/day) | Piperine (µg) per tablet |
|---|---|---|
| FORM -1 | 250 | 0.441±0.07 |
| FORM-2 | 250 | 0.572± 0.09 |
| FORM-3 | 250 | 0.40±0.05 |

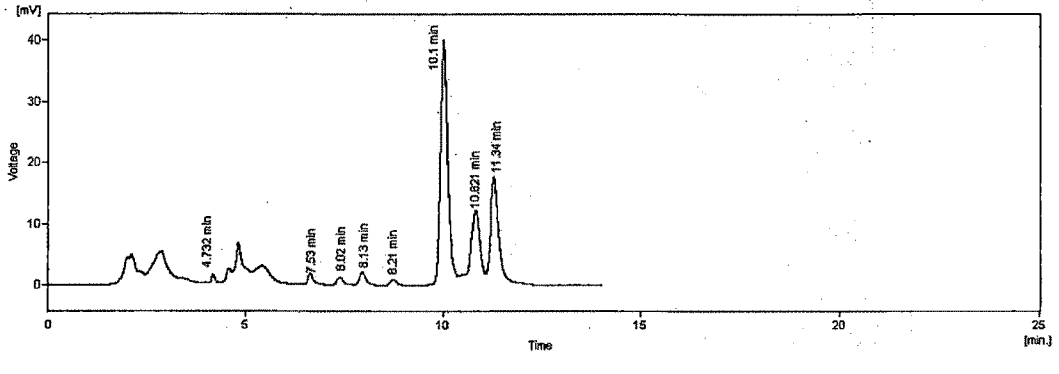



**Figure 5.44. Representative chromatogram showing separation of Piperine in degraded formulation.**

**5.6.4) Development of analytical method for simultaneous estimation of aconitine, solanine and piperine by HPLC in *Mahamrutyunjaya rasa*.**

***5.6.4.1) Chromatographic separation***

*a) Effect of pH value in the mobile phase buffer on the retention time of HPLC.*

Traditional HPLC applications on silica-based columns were performed at low or mid-range pH values in the mobile phase buffer. The alkaloids to be analyzed exhibited low retention time in such mobile phase; therefore it was difficult to separate them from each other and from other compounds in the Ayurvedic formulation. An alternative method was employed to separate them by using a mobile-phase pH which was above their pKa. The peak shape was improved as under such conditions, they were in their free base forms because their positive charges were diminished. The pH dependence of the retention time was investigated with a range of 7-8 pH values by adjusting with 1 % triethylamine. The results of the chromatographic analysis indicated that the pH value has distinct effects, not only for the resolution of the three alkaloids but also for separation of the alkaloids and other unknown compounds in the samples. At the pH value 7.5, all the three alkaloids were baseline separated from each other with resolution values above 2. Therefore, the analysis was carried out at a pH range of 7.5-8.00, where the buffer solution works effectively.

Since alkaloids were to be analyzed in the sample solution, the ion-pair agents such as triethylamine was used to improve the separation of these components.

*b) Effect of mobile phase composition*

The effect of mobile phase composition (i.e. ratio of acetonitrile, $KH_2PO_4$ buffer and methanol was studied at 65:15:15, 60:25:15 and 55:35:15 v/v/v levels) with pH and flow rate of 1.0 ml/min. The resolution was > 3 at 60:25:15, v/v/v level, which is one of the most desirable criteria. Also the retention time was shorter with satisfactory asymmetry values. An adequate theoretical plates (~14000) is indicative of a good column performance. As can be seen from Table 5.86, the asymmetry was >1.12 at 55:35:15 v/v/v which indicates tailing of the peaks and the resolution was <2 at 60:15:15, v/v/v. Other chromatographic parameters at different composition of mobile phase are also listed in Table 5.86.

**Table 5.86 Effect of mobile phase pH, composition, and flow rate on various chromatographic parameters.**

| Variable | Value | Retention time | | | W@50% | | | Assymmetry | | | Sep. F | | HETP | | | Cap.F | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SON | PIN | ACN | SON | PIN | ACN | SON | PIN | ACN | SON | PIN | SON | PIN | ACN | SON | PIN | ACN |
| pH | 7.0 | 4.3 | 5.8 | 7.6 | 0.170 | 0.21 | 0.24 | 0.97 | 0.91 | 0.92 | 1.40 | 1.32 | 0.05 | 0.052 | 0.050 | 3.88 | 5.03 | 7.56 |
| | 7.5 | 4.5 | 6.0 | 7.9 | 0.147 | 0.18 | 0.23 | 0.99 | 0.96 | 0.98 | 1.41 | 1.37 | 0.05 | 0.049 | 0.047 | 3.95 | 5.59 | 7.70 |
| | 8.0 | 4.6 | 6.1 | 8.0 | 0.160 | 0.20 | 0.21 | 0.99 | 0.96 | 1.02 | 1.54 | 1.42 | 0.05 | 0.049 | 0.048 | 4.02 | 5.72 | 7.93 |
| M.P. | 65:15:15 | 4.2 | 5.7 | 7.5 | 0.163 | 0.19 | 0.24 | 0.99 | 0.97 | 1.02 | 1.43 | 1.38 | 0.04 | 0.047 | 0.046 | 3.89 | 4.91 | 7.62 |
| Ratio | 60:25:15 | 4.5 | 6.0 | 7.9 | 0.147 | 0.18 | 0.22 | 0.99 | 0.96 | 0.98 | 1.41 | 1.37 | 0.05 | 0.049 | 0.047 | 3.95 | 5.59 | 7.70 |
| v/v/v | 55:35:15 | 5.0 | 6.4 | 8.3 | 0.172 | 0.24 | 0.27 | 0.91 | 0.92 | 0.91 | 1.40 | 1.20 | 0.05 | 0.052 | 0.051 | 4.12 | 5.87 | 8.15 |
| Flow rate | 0.8 | 5.3 | 7.1 | 9.1 | 0.163 | 0.23 | 0.25 | 0.87 | 0.89 | 0.89 | 1.43 | 1.39 | 0.05 | 0.049 | 0.047 | 4.23 | 6.02 | 8.88 |
| (ml/min) | 1.0 | 4.5 | 6.1 | 7.9 | 0.147 | 0.18 | 0.23 | 0.99 | 0.96 | 0.98 | 1.41 | 1.37 | 0.05 | 0.049 | 0.047 | 3.95 | 5.59 | 7.70 |
| | 1.2 | 3.7 | 5.2 | 6.9 | 0.135 | 0.17 | 0.20 | 1.12 | 1.12 | 1.18 | 1.33 | 1.31 | 0.54 | 0.55 | 0.053 | 3.72 | 5.23 | 6.89 |

*c) Effect of mobile phase flow rate*

The theoretical plates value was highest at flow rate of 1 ml/min. The change in flow rate had no significant effect on resolution and asymmetry while retention time decreased as the flow rate increased. The other system suitability parameters also indicated optimum flow rate of 1 ml/min.

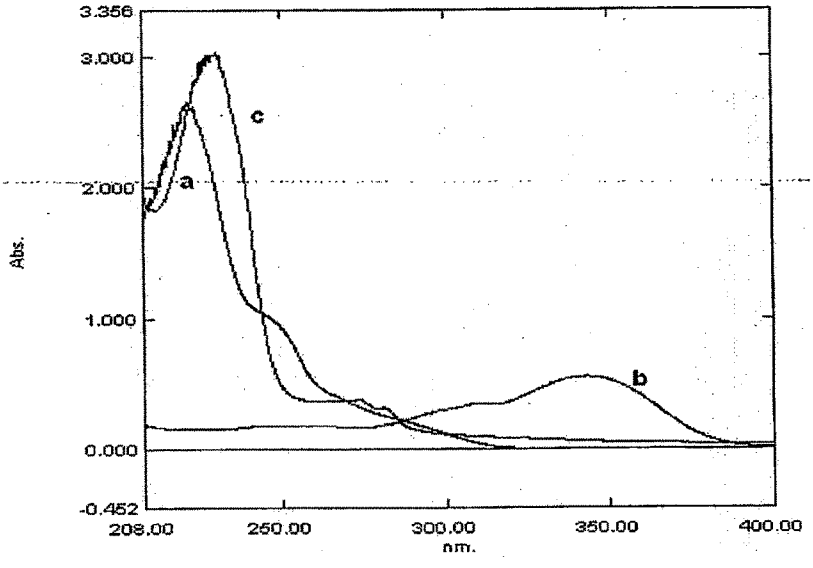



**Fig.5.45. UV spectra of a-ACN (λmax-223nm), b-SON (λmax-218nm) and c-PIN (λmax-343nm).**

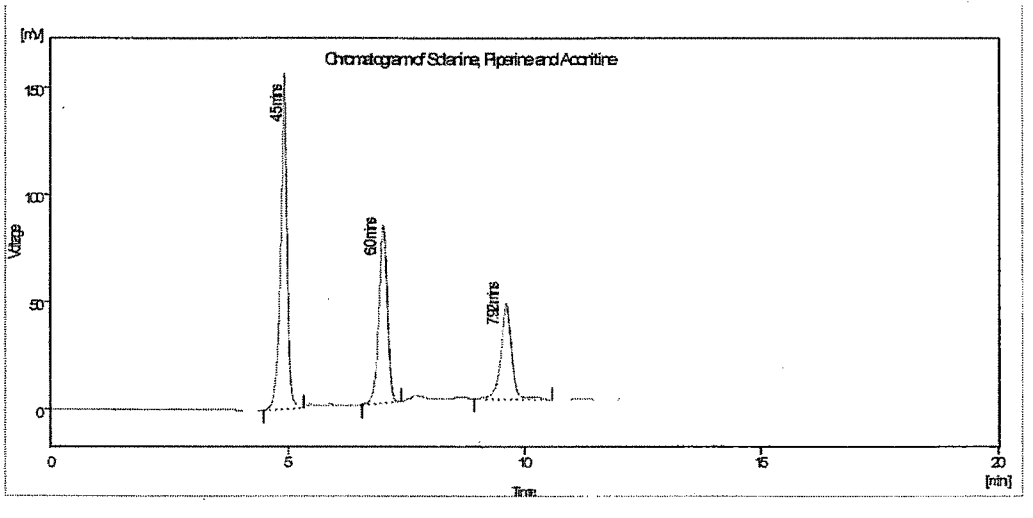



**Fig. 5.46 Representative Chromatogram of SON, PIN and ACN.**

***5.6.4.2) Extraction conditions***

The 0.1 N HCl was used to dissolve the alkaloids and ethyl acetate helped to remove the interferences in the aqueous solution. The chemical properties of the alkaloids were considered and extraction in chloroform, ethyl acetate and diethyl ether were employed. The results showed that chloroform was better as more alkaloids were extracted with high recoveries.

***5.6.4.3) Validation***

a) *Calibration curve (linearity)*

The calibration curves (n=3) constructed for the markers were linear over the concentration range of 10-100 µg/ml for each marker. Peak areas of the markers were plotted versus the concentration and linear regression analysis performed on the resultant curve. The coefficients of determination 0.999, 0.9942, 0.9989 for solanine, piperine and aconitine respectively with % RSD values ranging from 0.5 to 2% across the concentration range studied were obtained following linear regression analysis (Table 5.87).

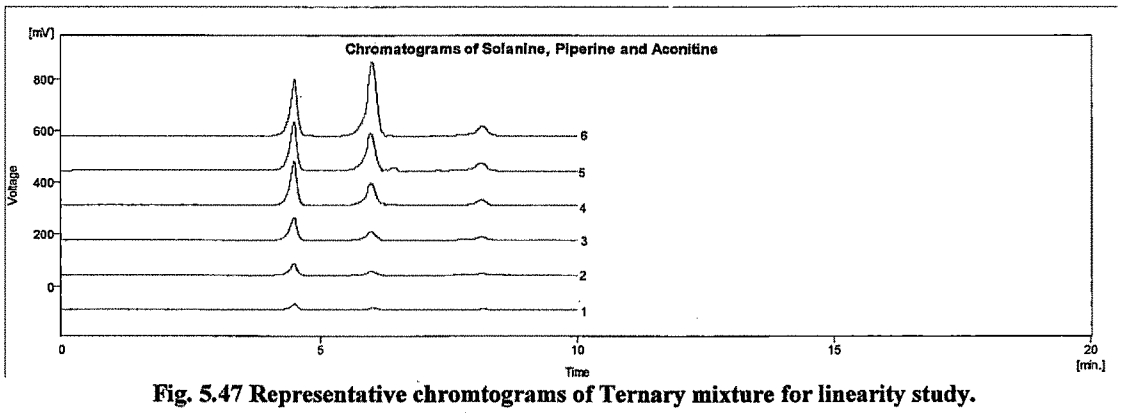



**Fig. 5.47 Representative chromtograms of Ternary mixture for linearity study.**

**Table 5.87 Linear regression data for the calibration curves (n=3).**

| Parameter | SOLANINE | PIPERINE | ACONITINE |
|---|---|---|---|
| Retention Time, min | 4.5 ± 0.1 | 6.0 ± 0.2 | 7.92 ± 0.2 |
| Detection wavelength | 223 nm | 343 nm | 223 nm |
| LOD, µg/mL | 0.14 | 0.10 | 0.23 |
| LOQ, µg/mL | 0.462 | 0.333 | 0.759 |
| Linearity Range, µg/ml | 10-100 | 10-100 | 10-100 |
| Correlation Coefficient | 0.997 | 0.996 | 0.997 |

| | | | |
|---|---|---|---|
| (Height) | | | |
| Correlation Coefficient (Area) | 0.999 | 0.9942 | 0.9989 |
| Regression equation (Height) | Y=3.0159x-1.0653 | Y= 17.89x-140.11 | Y= 0.4092x-2.5833 |
| Regression equation (Area) | Y=32.593x +23.715 | Y=1.0305x+2.4063 | Y=7.3768x-28.594 |
| Slope (Height) | 3.0159 | 17.89 | 0.4092 |
| Slope (Area) | 32.593 | 1.0305 | 7.3768 |
| Intercept (Height) | 1.0653 | 140.11 | 2.5833 |
| Intercept (Area) | 23.715 | 2.4063 | 28.594 |

*b) Repeatability, precision and stability*

Injection repeatability- The calculated % RSDs of the peak areas for all three compounds was less than 2 % at each of the three concentration levels (Table 5.88).

Analysis repeatability- The %RSD values for analysis repeatability were less than 2.0 % both for retention time and peak area (Table 5.88).

**Table 5.88. Repeatability of the developed method (n=6)**

| **Components** | **Retention time (min)** | | | | **Peak area (mVs)** | | | |
|---|---|---|---|---|---|---|---|---|
| | **Injection Repeatability** | | **Analysis repeatability** | | **Injection Repeatability** | | **Analysis repeatability** | |
| | **Mean** | **% RSD** | **Mean** | **% RSD** | **Mean** | **% RSD** | **Mean** | **% RSD** |
| SON | 4.5 | 0.23 | 4.5 | 0.40 | 1224.9 | 0.95 | 1230.4 | 0.98 |
| PIN | 6.0 | 0.30 | 6.0 | 0.32 | 508.67 | 0.53 | 513.22 | 1.05 |
| ACN | 7.92 | 0.18 | 7.92 | 0.24 | 237.89 | 1.08 | 230.54 | 1.66 |

Instrument precision- The precision result of the solution at medium concentration is presented in Table 5.89, and it was shown that the %RSD values of retention time were less than 1%, while the %RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision(Intra 4 h six injections, inter 6 days).

**Table 5.89. Intra-day and Inter-day precision of the developed method (n=6)**

| Component | Intra-day | | | | Inter-day | | | |
|---|---|---|---|---|---|---|---|---|
| | Retention Time | | Peak area | | Retention Time | | Peak area | |
| | Mean | % RSD | Mean | % RSD | Mean | % RSD | Mean | % RSD |
| Solanine | | | | | | | | |
| 10 | 4.51 | 0.09 | 342.87 | 1.67 | 4.50 | 0.14 | 339.78 | 1.78 |
| 40 | 4.52 | 0.08 | 1218.8 | 1.80 | 4.51 | 0.23 | 1227.67 | 1.67 |
| 100 | 4.51 | 0.11 | 3239.9 | 1.78 | 4.50 | 0.27 | 3248.67 | 1.98 |
| Piperine | | | | | | | | |
| 10 | 6.1 | 0.12 | 91.234 | 1.96 | 6.0 | 0.16 | 89.76 | 0.93 |
| 40 | 6.0 | 0.07 | 512.67 | 1.02 | 6.0 | 0.22 | 520.89 | 0.97 |
| 100 | 6.0 | 0.08 | 1674.9 | 1.6 | 6.1 | 0.21 | 1668.78 | 1.08 |
| Aconitine | | | | | | | | |
| 10 | 7.91 | 0.15 | 60.987 | 1.65 | 7.90 | 0.29 | 58.67 | 1.20 |
| 40 | 7.92 | 0.24 | 244.89 | 0.87 | 7.92 | 0.34 | 248.56 | 1.78 |
| 100 | 7.92 | 0.22 | 720.79 | 0.78 | 7.92 | 0.33 | 719.65 | 1.67 |

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (%RSD values of the retention time and peak area were both less than 3%).

*c) Limit of detection and limit of quantification*

The LOD and LOQ were found to be 0.14 and 0.462 µg/ml, respectively for Solanine, 0.10 and 0.333 µg/ml, respectively for Piperine and 0.23 and 0.759 µg/ml for Aconitine.

*d) Specificity*

Satisfactory results were obtained, indicating the high specificity of the proposed method for the determination of the markers in ternary mixture and formulations. No interferences were observed as shown in overlaid chromatograms of standard solution containing all 3 compounds. Good resolution and absence of interferences between the drugs determined are shown in Fig.5.47.

*e) Robustness*

Table 5.90 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these

parameters. The recovery obtained individually and the mean were between 98 % and 102 % for solanine and aconitine and 97 % and 101 % for piperine. Therefore it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

**Table 5.90. Robustness of the method (n=6)**

| **Chromatographic change** | | **Recovery, % Components** | | |
|---|---|---|---|---|
| **Factor** | **Level** | **Solanine** | **Piperine** | **Aconitine** |
| **Acetonitrile brand** | | | | |
| Spectrochem | 1 | 99.45 ± 0.78 | 98.56 ± 0.56 | 99.67 ± 0.34 |
| Rankem | 2 | 98.56 ± 0.98 | 98.76 ± 0.67 | 98.56 ± 0.87 |
| Qualigens | 3 | 98.88 ± 0.23 | 99.12 ± 1.09 | 100.34 ±0.84 |
| **Column Brand** | | | | |
| Hypersil | 1 | 101.5 ± 1.06 | 97.82 ± 0.63 | 101.19 ± 0.93 |
| Phenomenex | 2 | 100.28 ± 0.84 | 98.54 ± 0.54 | 100.78 ± 0.74 |
| **Detection Wavelength** | | | | |
| λmax – 2 nm | -1 | 100.78 ± 0.78 | 100.89 ± 1.98 | 100.67 ± 1.23 |
| λmax | 0 | 99.58 ±0.65 | 99.78 ± 0.78 | 99.78 ± 0.79 |
| λmax +2 nm | 1 | 98.34 ±1.89 | 98.11 ± 1.56 | 98.67 ± 0.67 |

*f) System suitability*

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table 5.91). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

**Table 5.91. System Suitability Parameters**

| Sr. No. | Parameter | Solanine | Piperine | Aconitine |
|---|---|---|---|---|
| 1 | Retention time (min) | 4.5 | 6.0 | 7.92 |
| 2 | Area (mVs) | 340.62 | 88.67 | 58.97 |
| 3 | Capacity factor (k') | 3.956 | 5.593 | 7.703 |
| 4 | Separation factor (α) | 1.41 | 1.377 | ------ |
| 5 | Efficiency/length(t.p/m) | 104768 | 115239 | 147312 |
| 6 | HETP (mm) | 0.050 | 0.049 | 0.047 |
| 7 | Resolution (Rs) | ---- | 5.34 | 6.149 |
| 8 | Assymmetry (As) | 0.994 | 0.959 | 0.989 |

*g) Accuracy*

As shown in Table 5.92, the recovery of the investigated components ranged from 98.17 % to 100.69 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed method manifested the reliability and accuracy for the measurement of these components.

**Table 5.92. Recovery Test (n=3):**

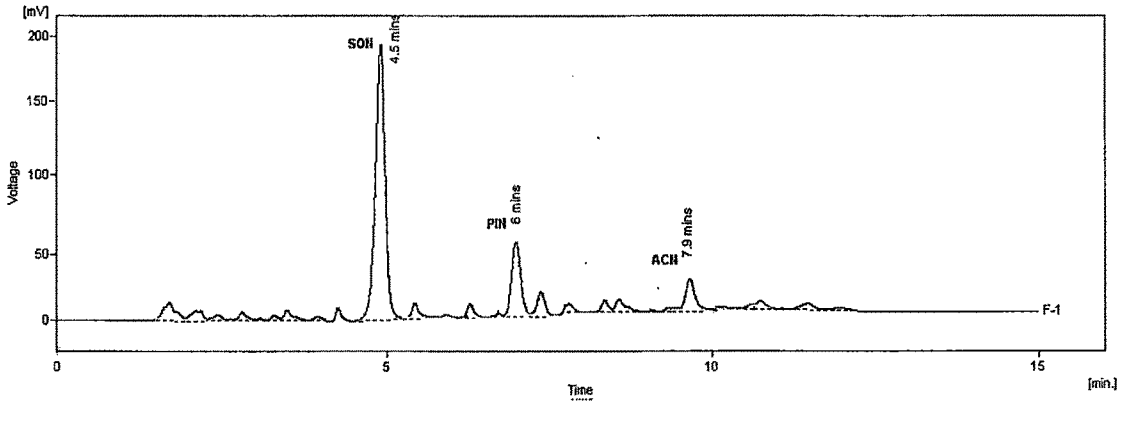
| Components | Quantity added % | Total Quantity Present | Amount Quantity Found | Recovery | % RSD |
|---|---|---|---|---|---|
| Solanine | 0 | 14.23 | 14.17 | 99.64 | 0.75 |
| | 80 | 25.614 | 25.72 | 100.45 | 0.96 |
| | 100 | 28.46 | 28.65 | 100.67 | 1.67 |
| | 120 | 31.306 | 30.82 | 98.45 | 1.39 |
| Piperine | 0 | 47.68 | 47.53 | 99.69 | 0.64 |
| | 80 | 85.825 | 84.34 | 98.28 | 1.46 |
| | 100 | 95.36 | 93.61 | 98.17 | 1.85 |
| | 120 | 104.876 | 103.44 | 98.64 | 1.37 |
| Aconitine | 0 | 1.20 | 1.19 | 99.45 | 0.67 |
| | 80 | 2.16 | 2.14 | 99.21 | 0.86 |
| | 100 | 2.40 | 2.38 | 99.34 | 0.38 |
| | 120 | 2.64 | 2.60 | 98.56 | 0.62 |

***5.6.4.4) Applicability of the developed method in formulations***

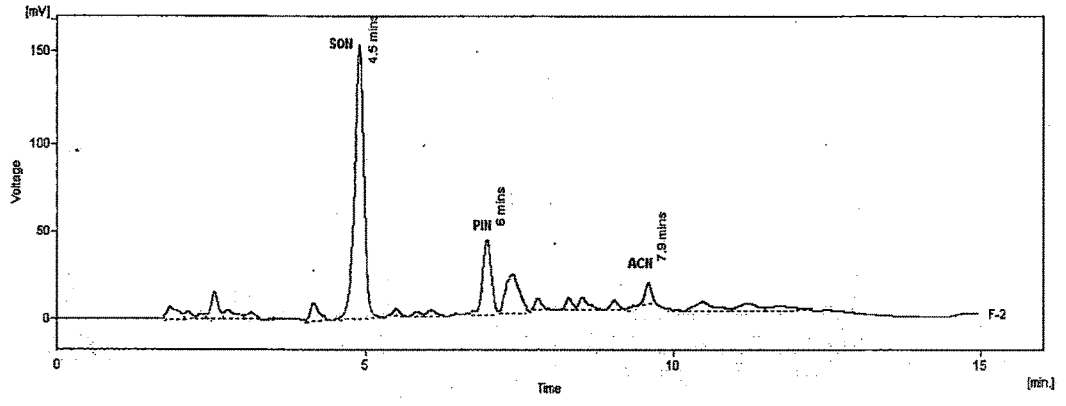
The developed HPLC method was applied to the simultaneous determination of Aconitine, Solanine and Piperine in the Ayurvedic formulations (FORM1, FORM2, FORM3) and the results are presented in Table 5.93. It was observed that the content of Aconitine varied in the three formulations, with the %RSD values of higher than 10 %, which would significantly influence the quality stability because it is the target toxic component for the quality control of *Mahamrutyunjaya rasa* tablets. However, the content of Piperine and Solanine were comparable in all the formulations. Therefore, the simultaneous determination of these three active components contained in *Mahamrutyunjaya rasa* tablets is necessary to improve the safety and quality control level of this Ayurvedic formulation.

**Table 5.93. Contents of the three alkaloids in three proprietary Ayurvedic medicines (n=3)**

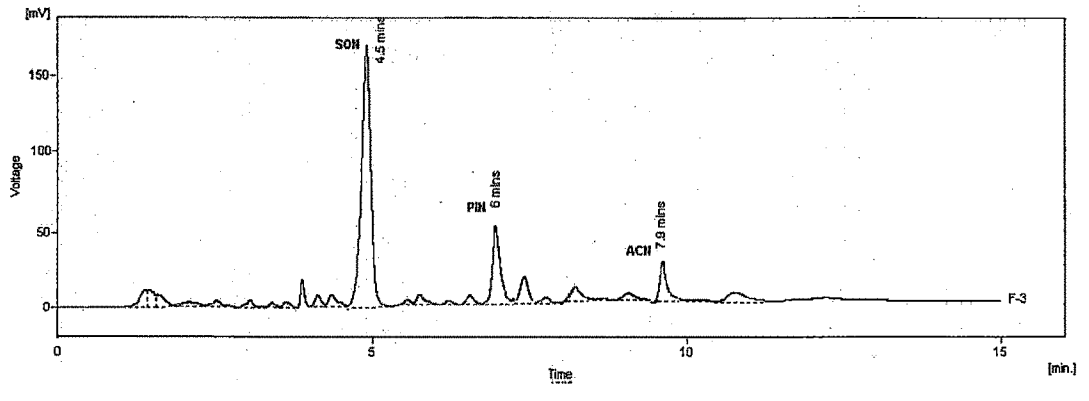
| Formulation | Clinical dosages(mg/day) | Contents, µg/tablet | | |
|---|---|---|---|---|
| | | Solanine | Piperine | Aconitine |
| FORM 1 | 250 | 0.13±0.02 | 0.43±0.04 | 0.11±0.02 |
| FORM2 | 250 | 0.15±0.01 | 0.53±0.02 | 0.14±0.01 |
| FORM3 | 250 | 0.073±0.01 | 0.40±0.03 | 0.49±0.04 |




(a)




(b)




(c)

**Fig. 5.48. Representative chromatogram of (a)FORM-1 , (b)FORM-2 and (c)FORM-3 (F-3).**

### *5.6.5)* Development of analytical methods for simultaneous estimation of aconitine, solanine in *Mahamrutyunjaya rasa.*

#### *5.6.5.1) Chromatographic separation*

*a) Effect of pH value in the mobile phase buffer on the retention time of HPLC.*

The pH dependence of the retention time was investigated in a range of 7-8 pH values by adjusting with 1 % triethylamine. The results of the chromatographic analysis indicated that the pH value has distinct effects, not only for the resolution of the three alkaloids but also for separation of the alkaloids and other unknown compounds in the samples. At the pH value 7.5, all the three alkaloids were baseline separated from each other with resolution values above 2. Therefore, the analysis was carried out at a pH range of 7.5-8.00, where the buffer solution works effectively.

Since alkaloids were to be analyzed in the sample solution, the ion-pair agents such as triethylamine was used to improve the separation of these components.

*b) Effect of mobile phase composition*

The effect of mobile phase composition (i.e. ratio of acetonitrile, $KH_2PO_4$ buffer and methanol was studied at 65:20:15, 70:15:15 and 75:10:15 v/v/v levels) with pH and flow rate of 1.0 ml/min. The resolution was > 3 at 70:15:15, v/v/v level, which is one of the most desirable criteria. Also the retention time was shorter with satisfactory asymmetry values. An adequate theoretical plates is indicative of a good column performance. As can be seen from Table 5.94, the asymmetry was >1.12 at 65:20:15 v/v/v which indicates tailing of the peaks and the resolution was <2 at 75:10:15, v/v/v. Other chromatographic parameters at different composition of mobile phase are also listed in Table 5.94.

*c) Effect of mobile phase flow rate*

The theoretical plates value was highest at flow rate of 1 ml/min. The change in flow rate had no significant effect on resolution and asymmetry while retention time decreased as the flow rate increased. The other system suitability parameters also indicated optimum flow rate of 1 ml/min.

**Table 5.94. Effect of mobile phase pH, composition, and flow rate on various chromatographic parameters.**

| Variable | Value | Retention time | | W@50% | | Assymmetry | | Sep. F | | HETP | | Cap.F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SON | ACN | SON | ACN | SON | ACN | SON | ACN | SON | ACN | SON | ACN |
| pH | 7.0 | 3.8 | 6.5 | 0.170 | 0.24 | 0.98 | 0.92 | 2.10 | ----- | 0.057 | 0.050 | 2.14 | 4.72 |
| | 7.5 | 4.0 | 6.9 | 0.147 | 0.23 | 0.99 | 0.98 | 2.10 | ----- | 0.05 | 0.047 | 2.03 | 4.60 |
| | 8.0 | 4.3 | 8.0 | 0.160 | 0.21 | 0.99 | 1.02 | 1.92 | ----- | 0.052 | 0.048 | 1.99 | 4.54 |
| M.P. | 20:65:15 | 4.5 | 7.5 | 0.163 | 0.24 | 1.03 | 1.02 | 2.20 | ----- | 0.048 | 0.046 | 2.2 | 4.82 |
| Ratio | 15:70:15 | 4.0 | 6.9 | 0.147 | 0.22 | 0.99 | 0.98 | 2.10 | ----- | 0.05 | 0.047 | 2.03 | 4.60 |
| v/v/v | 10:75:15 | 3.8 | 6.3 | 0.172 | 0.27 | 0.91 | 0.91 | 1.93 | ----- | 0.053 | 0.051 | 1.99 | 4.52 |
| Flow | 0.8 | 4.6 | 7.4 | 0.163 | 0.25 | 0.87 | 0.89 | 2.32 | ----- | 0.05 | 0.047 | 2.2 | 4.81 |
| rate | 1.0 | 4.0 | 6.9 | 0.147 | 0.23 | 0.99 | 0.98 | 2.12 | ----- | 0.05 | 0.047 | 2.03 | 4.60 |
| (ml/min) | 1.2 | 3.5 | 6.1 | 0.135 | 0.20 | 1.12 | 1.18 | 1.92 | ----- | 0.54 | 0.053 | 1.93 | 4.32 |

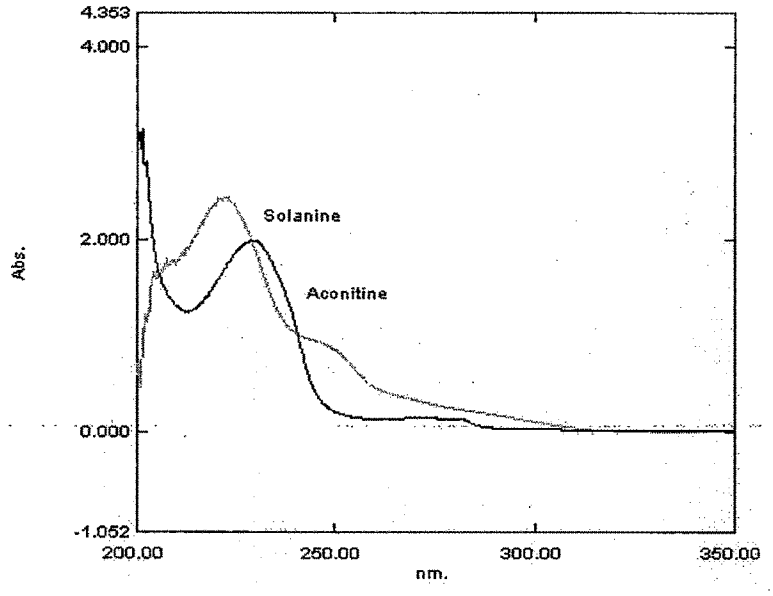



**Fig.5.49. UV spectra of Aconitine (λmax-223nm), Solanine (λmax-218nm).**

***5.6.5.2) Extraction conditions***

The 0.1 N HCl was used to dissolve the alkaloids and ethyl acetate helped to remove the interferences in the aqueous solution. The chemical properties of the alkaloids were considered and extraction in chloroform, ethyl acetate and diethyl ether were employed. The results showed that chloroform was better as more alkaloids were extracted with high recoveries.

***5.6.5.3) Validation***

*a) Calibration curve (linearity)*

The calibration curves (n=3) constructed for the markers were linear over the concentration range of 10-100 µg/ml for each marker. Peak areas of the markers were plotted versus the concentration and linear regression analysis performed on the resultant curve. The coefficients of determination 0.9987, 0.9942 for Solanine and Aconitine respectively with % RSD value was less than 2% across the concentration range studied were obtained following linear regression analysis (Table 5.95).

**Table.5.95. Peak area of Solanine and Aconitine.**

| Concentration(µg/ml) | Peak Area | |
|---|---|---|
| | **Solanine** | **Aconitine** |
| **10** | 327.3 | 212.05 |
| **20** | 573.27 | 297.77 |
| **40** | 949.177 | 435.702 |
| **60** | 1200.857 | 567.676 |
| **80** | 1579.93 | 688.68 |
| **100** | 1924.499 | 821.076 |

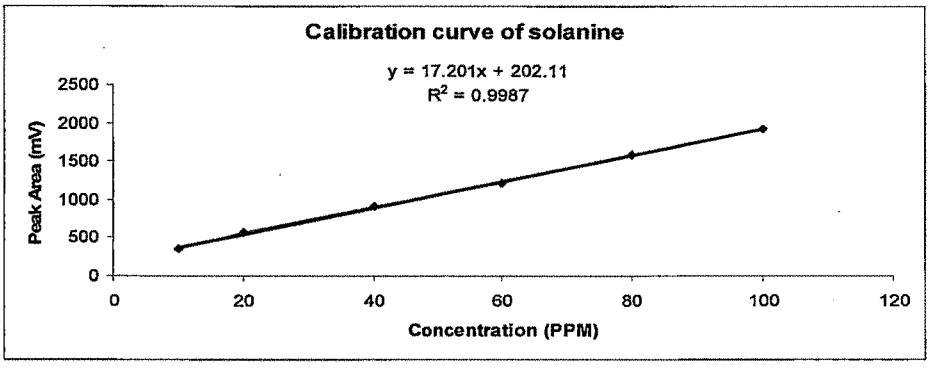



**Fig. 5.50. Calibration curve of Solanine.**

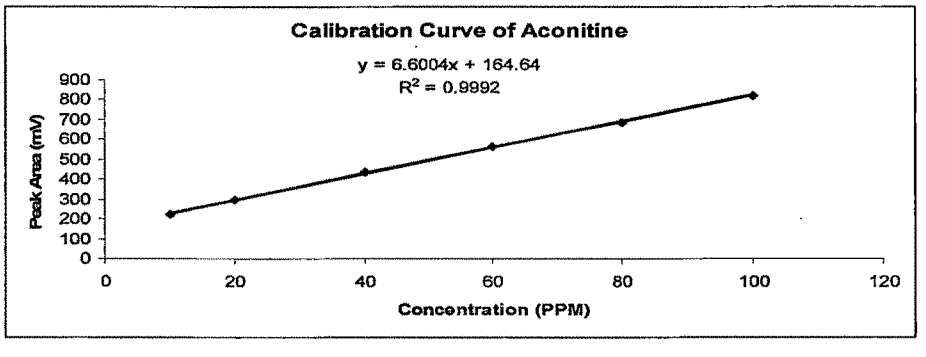



**Fig. 5.51. Calibration curve of Aconitine**

**Fig. 5.52. Representative Chromatograms of Solanine and Aconitine (100µg/ml)**

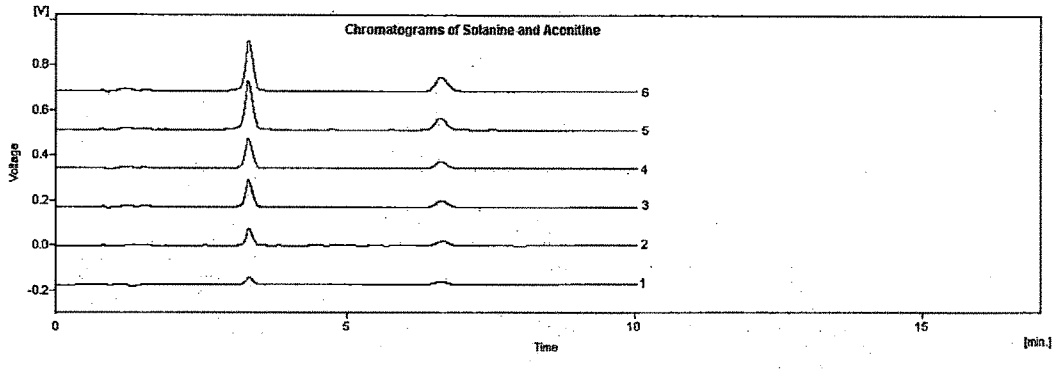



**Table. 5.96. Linear regression data for the calibration curves (n=3)**

| **Parameter** | **Solanine** | **Aconitine** |
|---|---|---|
| Retention Time, min | 4.01 ± 0.1 | 6.91 ± 0.2 |
| Detection wavelength, nm | 223 nm | 223 nm |
| LOD, µg/ml | 0.14 | 0.15 |
| LOQ, µg/ml | 0.462 | 0.495 |
| Linearity Range | 10-100 µg/ml | 10-100 µg/ml |
| Coefficient of Determination (Height) | 0.9959 | 0.9942 |
| Coefficient of Determination (Area) | 0.9984 | 0.9979 |
| Regression equation (Height) | Y=6.670x +159.17 | y=0.5525x +13.903 |
| Regression equation (Area) | Y=17.201x +202.11 | y=6.6004x+64.64 |
| Slope (Height) | 6.670 | 0.5525 |
| Slope (Area) | 17.201 | 6.6004 |
| Intercept (Height) | 201.37 | 28.67 |
| Intercept (Area) | 202.11 | 164.64 |

*b) Precision*

The precision result of the solution at medium concentration is presented in Table 5.97, and it was shown that the %RSD values of retention time were less than 1%, while the %RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision(Intra 4 h six injections, inter 6 days).

**Table 5.97. Intra-day and inter-day precision of HPLC method (n=6)**

| Amount (µg/mL) | Intra-day precision | | | | Inter- day precision | | | |
|---|---|---|---|---|---|---|---|---|
| | Solanine | | Aconitine | | Solanine | | Aconitine | |
| | Mean area | % RSD | Mean area | % RSD | Mean area | % RSD | Mean area | % RSD |
| 10 | 325.32 | 1.78 | 208.78 | 1.67 | 328.12 | 1.43 | 212.78 | 1.65 |
| 60 | 1195.23 | 1.75 | 563.90 | 1.78 | 1203.23 | 1.56 | 569.56 | 1.56 |
| 100 | 1916.45 | 1.98 | 819.56 | 1.34 | 1926.34 | 1.98 | 1920.78 | 1.01 |

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (%RSD values of the retention time and peak area were both less than 3%).

*c) Limit of detection and limit of quantification*

The LOD and LOQ were found to be 0.14 and 0.462 µg/ml, respectively for solanine, 0.15 and 0.495 µg/ml for aconitine.

*d) Specificity*

Satisfactory results were obtained, indicating the high specificity of the proposed method for the determination of the markers in binary mixture and formulations. No interferences were observed as shown in overlaid chromatograms of standard solution containing both the compounds. Good resolution and absence of interferences between the drugs determined are shown in Fig.5.53..

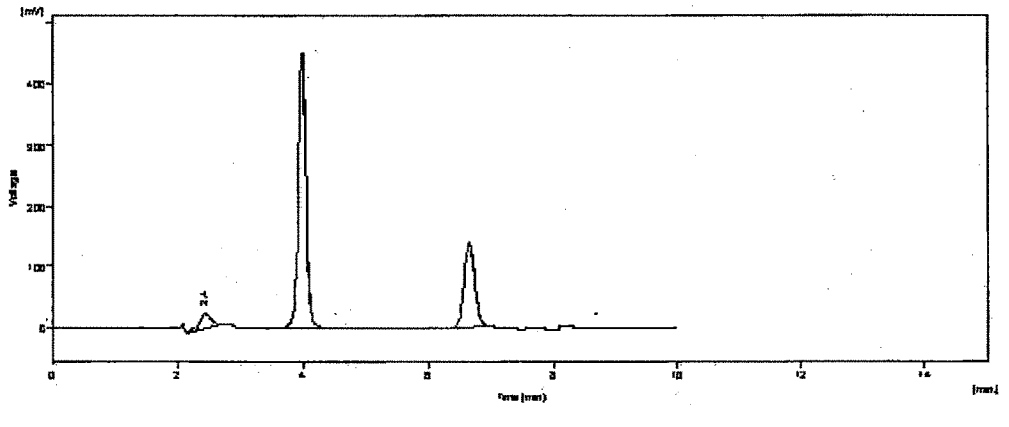


**Fig. 5.53.Representative chromatogram of Solanine and Aconitine.**

*e) Robustness*

Table 5.98 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean were between 98 % and 102 % for solanine and aconitine. Therefore it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

**Table 5.98. Robustness of the method**

| **Chromatographic change** | | **Recovery, %** | |
|---|---|---|---|
| **Factor** | **Level** | **Aconitine** | **Solanine** |
| **Detection Wavelength, nm** | | | |
| 220 | -1 | 99.34 ± 0.65 | 100.45 ± 0.34 |
| 223 | 0 | 98.78 ± 0.78 | 99.89 ± 0.24 |
| 226 | 1 | 101.23 ± 0.87 | 98.67 ± 0.54 |
| **Acetonitrile Brand** | | | |
| Rankem | 1 | 100.97 ± 0.45 | 100.65 ± 0.37 |
| Spectrochem | 2 | 99.89 ± 0.56 | 99.87 ± 0.48 |
| Qualigens | 3 | 98.32 ± 0.57 | 98.99 ± 0.54 |
| **Column Brand** | | | |
| Phenomenex | 1 | 98.47 ± 0.98 | 99.21 ± 0.73 |
| Hypersil | 2 | 99.12 ± 0.66 | 99.67 ± 0.92 |

*f) System suitability*

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table 5.99). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

**Table 5.99. System suitability parameters**

| Sr. No. | Parameter | Solanine | Aconitine |
|---|---|---|---|
| 1 | Retention time (min) | 4.01 | 6.92 |
| 2 | Capacity factor (k') | 2.03 | 4.60 |
| 3 | Separation factor (α) | 2.10 | -------- |
| 4 | Efficiency/length(t.p/m) | 137162 | 208349 |
| 5 | HETP (mm) | 0.05 | 0.045 |
| 6 | Resolution (Rs) | 7.657 | 13.168 |
| 7 | Assymmetry (As) | 0.974 | 1.061 |

*g) Accuracy*

As shown in Table 5.100, the recovery of the investigated component ranged from 98 % to 101 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed methods manifested the reliability and accuracy for the measurement of solanine.

**Table 5.100. Recovery studies (n=3)**

| Excess drug added to the analyte(%) | Theoretical content (µg) | Recovery (%) | % RSD |
|---|---|---|---|
| Aconitine | | | |
| 0 | 9.45 | 99.67 | 1.23 |
| 80 | 17.01 | 99.80 | 1.67 |
| 100 | 18.9 | 98.56 | 1.34 |
| 120 | 20.79 | 98.23 | 1.70 |
| Solanine | | | |
| 0 | 13.62 | 100.34 | 1.89 |
| 80 | 24.51 | 100.12 | 2.01 |
| 100 | 27.24 | 98.67 | 1.98 |
| 120 | 29.964 | 99.11 | 1.78 |

***5.6.5.4) Applicability of the developed method in formulations***

The developed HPLC method was applied to the simultaneous determination of aconitine and solanine in the Ayurvedic formulations (FORM1, FORM2, FORM3) and the results are presented in Table 5.101.

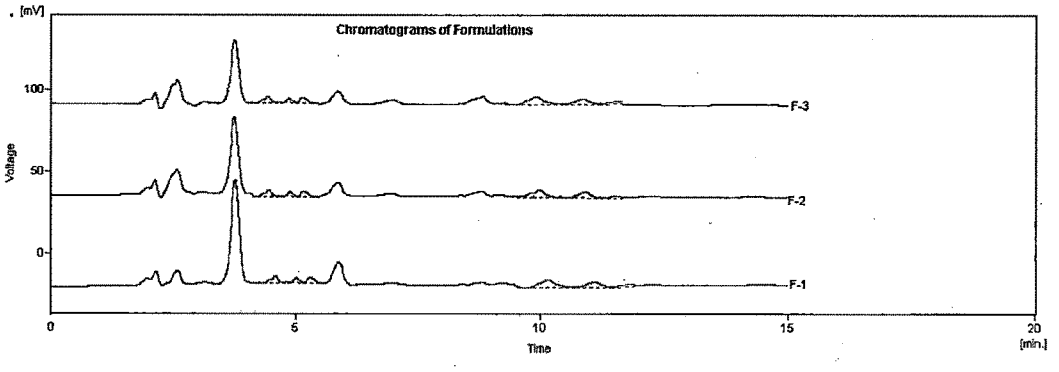



**Fig. 5.54. Representative Chromatograms of Form-1(F-1), Form-2 (F-2), Form (F-3).**

**Table 5.101. Results of the analysis:**

| Sr.No. | Formulation | Content, µg per tablet | |
|---|---|---|---|
| | | **Aconitine** | **Solanine** |
| 1 | Form1 | 0.114±0.02 | 0.123±0.06 |
| 2 | Form2 | 0.186±0.03 | 0.134±0.03 |
| 3 | Form3 | 0.49±0.02 | 0.075±0.04 |

## 5.6.6) Development of HPLC method for the simultaneous estimation of solanine and piperine.

### *5.6.6.1) Optimization of Chromatographic cinditions*

*a) Effect of pH value in the mobile phase buffer on the retention time of HPLC.*

The pH dependence of the retention time was investigated in a range of 7-8 pH values by adjusting with 1 % triethylamine. The results of the chromatographic analysis indicated that the pH value has distinct effects, not only for the resolution of the three alkaloids but also for separation of the alkaloids and other unknown compounds in the samples. At the pH value 7.5, all the three alkaloids were baseline separated from each other with resolution values above 2. Therefore, the analysis was carried out at a pH range of 7.5-8.00, where the buffer solution works effectively. Since alkaloids were to be analyzed in the sample solution, the ion-pair agents such as triethylamine was used to improve the separation of these components.

*b) Effect of mobile phase composition*

The effect of mobile phase composition (i.e. ratio of acetonitrile, $KH_2PO_4$ buffer and methanol was studied at 50:25:15, 55:20:15 and 60:15:15 v/v/v levels) with pH and flow rate of 1.0 ml/min. The resolution was > 3 at 55:20:15, v/v/v level, which is one of the most desirable criteria. Also the retention time was shorter with satisfactory asymmetry values. An adequate theoretical plates is indicative of a good column performance. As can be seen from Table 5.102, the asymmetry was >1.12 at 50:25:15 v/v/v which indicates tailing of the peaks and the resolution was <2 at 60:15:15, v/v/v. Other chromatographic parameters at different composition of mobile phase are also listed in Table 5.102.

*c) Effect of mobile phase flow rate*

The theoretical plates value was highest at flow rate of 1 ml/min. The change in flow rate had no significant effect on resolution and asymmetry while retention time decreased as the flow rate increased. The other system suitability parameters also indicated optimum flow rate of 1 ml/min.

**Table 5.102 Effect of mobile phase pH, composition, and flow rate on various chromatographic parameters.**

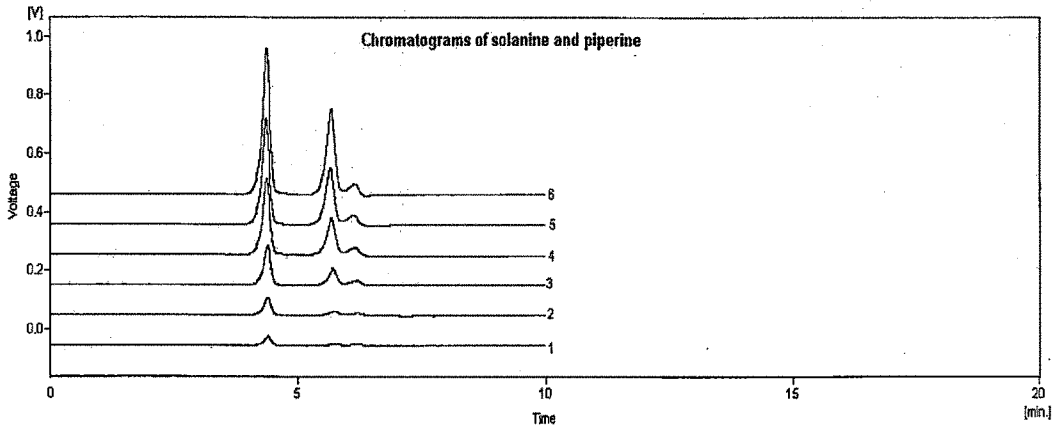
| Variable | Value | Retention time | | W@50% | | Assymmetry | | Sep. F | | HETP | | Cap.F | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | SON | PIN | SON | PIN | SON | PIN | SON | PIN | SON | PIN | SON | PIN |
| **pH** | 7.0 | 4.2 | 5.4 | 0.170 | 0.24 | 0.98 | 0.92 | 1.35 | ---- | 0.057 | 0.050 | 2.86 | 3.82 |
| | 7.5 | 4.5 | 5.9 | 0.147 | 0.23 | 0.99 | 0.98 | 1.346 | ---- | 0.05 | 0.047 | 2.86 | 3.85 |
| | 8.0 | 4.8 | 6.3 | 0.160 | 0.21 | 0.99 | 1.02 | 1.32 | ---- | 0.052 | 0.048 | 2.92 | 3.84 |
| **M.P.** | 50:20:15 | 4.7 | 6.3 | 0.163 | 0.24 | 1.03 | 1.02 | 1.36 | ---- | 0.048 | 0.046 | 2.66 | 3.82 |
| **Ratio** | 55:15:15 | 4.5 | 5.9 | 0.147 | 0.22 | 0.99 | 0.98 | 1.346 | ---- | 0.05 | 0.047 | 2.85 | 3.85 |
| **v/v/v** | 60:10:15 | 4.1 | 5.5 | 0.172 | 0.27 | 0.91 | 0.91 | 1.36 | ---- | 0.053 | 0.051 | 2.93 | 3.81 |
| **Flow** | 0.8 | 4.9 | 6.4 | 0.163 | 0.25 | 0.87 | 0.89 | 1.54 | ---- | 0.05 | 0.047 | 2.82 | 3.82 |
| **rate** | 1.0 | 4.5 | 5.9 | 0.147 | 0.23 | 0.99 | 0.98 | 1.35 | ---- | 0.05 | 0.047 | 2.86 | 3.85 |
| **(ml/min)** | 1.2 | 4.1 | 5.5 | 0.135 | 0.20 | 1.12 | 1.18 | 1.32 | ---- | 0.54 | 0.053 | 2.82 | 3.84 |

***5.6.6.2) Validation***

*a) Calibration curve (linearity)*

The calibration curves constructed for the markers were linear over the concentration range of 10-100 µg/ml for each marker. Peak areas of the markers were plotted versus the concentration and linear regression analysis performed on the resultant curve. The coefficients of determination 0.9969, 0.9981 for Solanine and Piperine respectively with % RSD values ranging from 0.5 to 2% across the concentration range studied were obtained following linear regression analysis (Table 5.55).

**Fig. 5.55 Representative chromatograms of HPLC in linearity range.**



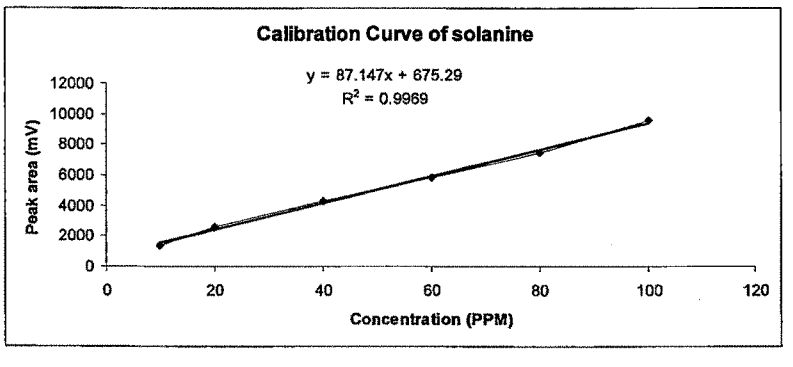




**Fig. 2.54 Calibration curve of Solanine**

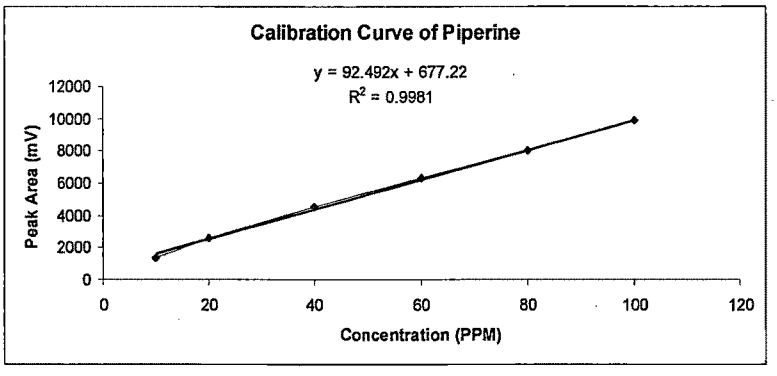



**Fig. 2.56 Calibration curve of Piperine.**

**Table. 5.103 Peak area of Solanine and Piperine.**

| Concentration (µg/ml) | Peak area (mV) | |
|---|---|---|
| | Solanine | Piperine |
| 10 | 1390.96 | 1392.75 |
| 20 | 2580.26 | 2592.9 |
| 40 | 4308.2 | 4578.2 |
| 60 | 5812.83 | 6289.35 |
| 80 | 7441.62 | 8026.23 |
| 100 | 9533.52 | 9856.32 |

**Table. 5.104. Linear regression data for the calibration curves (n=3).**

| Parameter | Solanine | Piperine |
|---|---|---|
| Retention Time, min | 4.54 ± 0.1 | 5.92 ± 0.2 |
| Detection wavelength, nm | 223 nm | 343 nm |
| LOD, µg/ml | 0.18 | 0.067 |
| LOQ, µg/ml | 0.592 | 0.221 |
| Linearity Range | 10-100 µg/ml | 10-100 µg/ml |
| Coefficient of Determination (Height) | 0.9936 | 0.9953 |
| Coefficient of Determination (Area) | 0.9969 | 0.9981 |
| Regression equation (Height) | Y=86.217x+707.18 | y=5.5551x+27.584 |
| Regression equation (Area) | Y=87.147x+675.29 | y= 92.492x+677.22 |
| Slope (Height) | 86.217 | 5.5551 |
| Slope (Area) | 87.147 | 92.492 |
| Intercept (Height) | 707.18 | 27.584 |
| Intercept (Area) | 675.29 | 677.22 |

*b) Repeatability, precision and stability*

The precision result of the solution at medium concentration is presented in Table 5.105, and it was shown that the %RSD values of retention time were less than 1%, while the %RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision(Intra 4 h six injections, inter 6 days).

**Table 5.105. Intra-day and inter-day precision of HPLC method (n=6)**

| Amount | Intra-day precision | | | | Inter- day precision | | | |
|---|---|---|---|---|---|---|---|---|
| (µg/mL) | Solanine | | Piperine | | Solanine | | Piperine | |
| | Mean area | % RSD | Mean area | % RSD | Mean area | % RSD | Mean area | % RSD |
| 10 | 318.32 | 0.86 | 1318.73 | 0.79 | 326.78 | 1.45 | 1341.30 | 1.25 |
| 60 | 1089.23 | 1.09 | 6238.97 | 0.68 | 1112.37 | 1.56 | 6251.43 | 1.43 |
| 100 | 1845.45 | 1.25 | 9845.98 | 0.48 | 1896.75 | 1.21 | 9857.14 | 1.90 |

For the stability test, the same sample was analyzed within 24 h at the room temperature, and the solution was found to be stable (%RSD values of the retention time and peak area were both less than 3%).

*c) Limit of detection and limit of quantification*

The LOD and LOQ were found to be 0.18 and 0.592 µg/ml, respectively for Solanine, 0.067 and 0.221 µg/ml, respectively for piperine.

*d) Specificity*

Satisfactory results were obtained, indicating the high specificity of the proposed method for the determination of the markers in binary mixture and formulations. No interferences were observed as shown in overlaid chromatograms of standard solution containing both the compounds. Good resolution and absence of interferences between the drugs determined are shown in Fig.5.57.

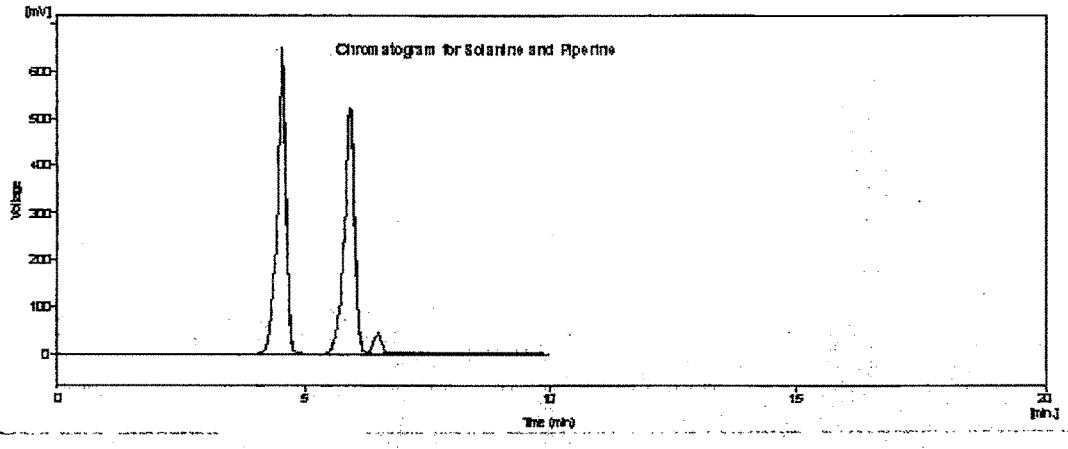



**Fig. 5.57 Representative Chromatograms of Solanine and Piperine Standards**

*e) Robustness*

Table 5.106 shows the mean obtained (n=6) for each factor studied, indicating that the selected factors remained unaffected by small variations of these parameters. The recovery obtained individually and the mean were between 98 % and 102 % for Solanine and Piperine. Therefore it can be concluded that the method is consistent for detection wavelength, selected column and solvent brand.

**Table 5.106. Robustness of the method (n=3)**

| **Chromatographic Change** | | **% Recovery** | |
|---|---|---|---|
| **Factor** | **Level** | **Solanine** | **Piperine** |
| **Acetonitrile Brand** | | | |
| Spectrochem | 1 | 98.45 ± 0.34 | 99.04 ± 0.68 |
| Rankem | 2 | 99.02 ± 0.66 | 99.53 ± 0.85 |
| Qualigens | 3 | 99.54 ± 0.71 | 99.74 ± 0.93 |
| **Detection wavelength, nm** | | | |
| λmax -2 | -1 | 101.24 ± 1.84 | 100.43 ± 1.06 |
| λmax | 0 | 99.42 ± 0.69 | 99.48 ± 1.26 |
| λmax +2 | 1 | 98.27 ± 1.23 | 98.10 ± 0.75 |
| **Column Brand** | | | |
| Phenomenex | 1 | 98.32 ± 0.59 | 99.36 ± 1.16 |
| Hypersil | 2 | 99.24 ± 0.71 | 99.11 ± 0.92 |

**5.6.7) Development of Colorimetric method for the estimation of Sulphur of *Mahamrutyunjaya rasa.***

Sulphur in presence of Hydrazine hydrate gets converted to water soluble hydrazine sulphide forming a yellow coloured solution with a λmax of 368 nm. Hydrazine hydrate contains a water molecule which is replaced by sulphide group to form hydrazine sulphide. The complex formed is stable in presence of 0.1N NaOH for 24 h.

$$NH_2NH_2.H_2^{++} + S^{--} \longrightarrow NH_2NH_2.H_2S$$

The formation of the hydrazine sulphide was rapid and the yellow color was stable upto 24 h without any change in color intensity when kept in dark at room temperature.

***5.6.7.1) Spectral characteristics***

The absorption spectra of hydrazine sulphide, was measured in the range 350–550 nm against the blank solution. The colorless reagent blank has practically no absorption at these wavelengths in all cases. The absorption spectrum of hydrazine sulphide is shown in Figure 5.59. The absorbances of the formed hydrazine sulphide was measured at 368 nm. Investigations were carried out to establish the most favorable conditions to get the most intense colour and to achieve maximum color development for the quantitative determination of sulphur. The influence of each of the following variables on the reaction was tested. The variables like concentration of Sodium hydroxide and Hydrazine hydrate concentration were studied to determine the optimum concentrations and their effect on the position of their λmax and the shape of these absorption spectra. Other variables like pH and temperature etc. did not affect the position of their λmax and the shape of these absorption spectras. These results indicate that only one type of compound is formed. The measurements for the formulations were also made at these wavelengths.

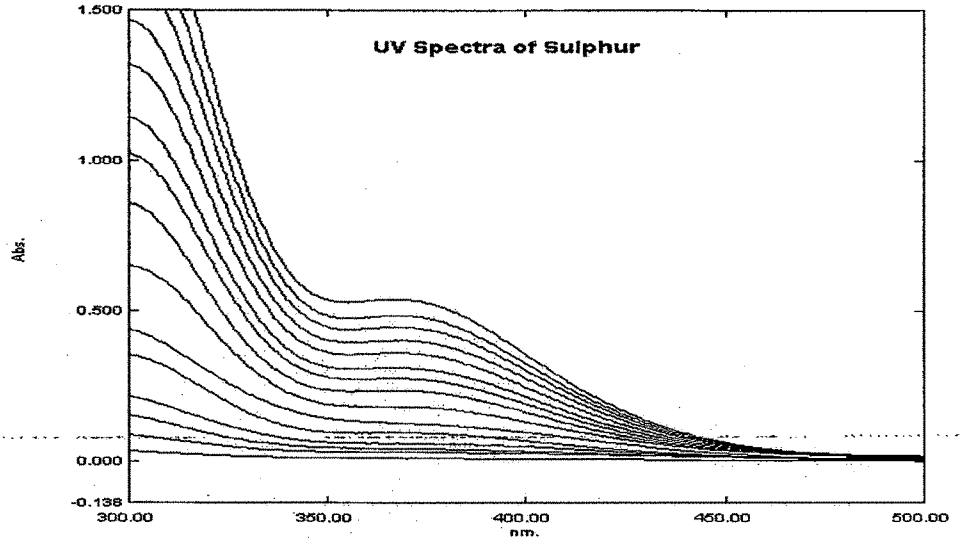



**Fig.5.59 Overlain spectra for Sulphur.**

### *5.6.7.2) Optimization of conditions*

*5.6.7.2.1) Effects of Hydrazine hydrate concentration*

The effect of the concentration of hydrazine hydrate was tested by adding different volumes of hydrazine hydrate in the range 0.5-5.0 mL, to a constant amount of Sulphur. Fig.5.60 shows that 2.5 ml of hydrazine hydrate in 10 ml of 0.1N NaOH gave maximum absorbance. Thus, 25 ml of hydrazine hydrate in 100 ml 0.1N NaOH was used for the preparation of coloured solution. The solution was found to be stable for 24 h.

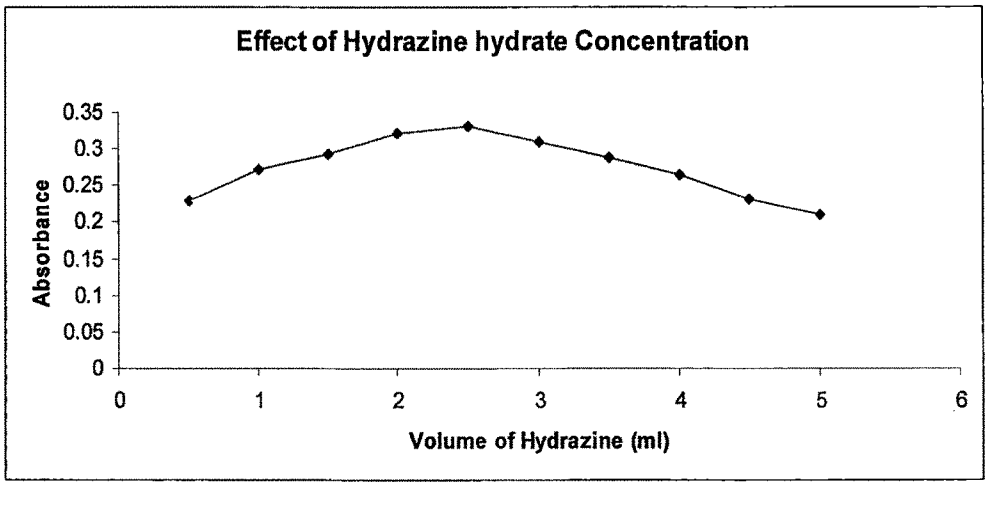



**Fig.5.60. Effect of Hydrazine hydrate concentration.**

*5.6.7.2.2) Effect of NaOH concentration*

The effect of concentration of NaOH on the hydrazine sulphate was studied over the concentration range of 0.01N- 0.5N NaOH. The concentration of 0.1N NaOH was found to be most suitable as it gave maximum absorbance. The absorbance decreased with increase in normality above 0.1N NaOH.

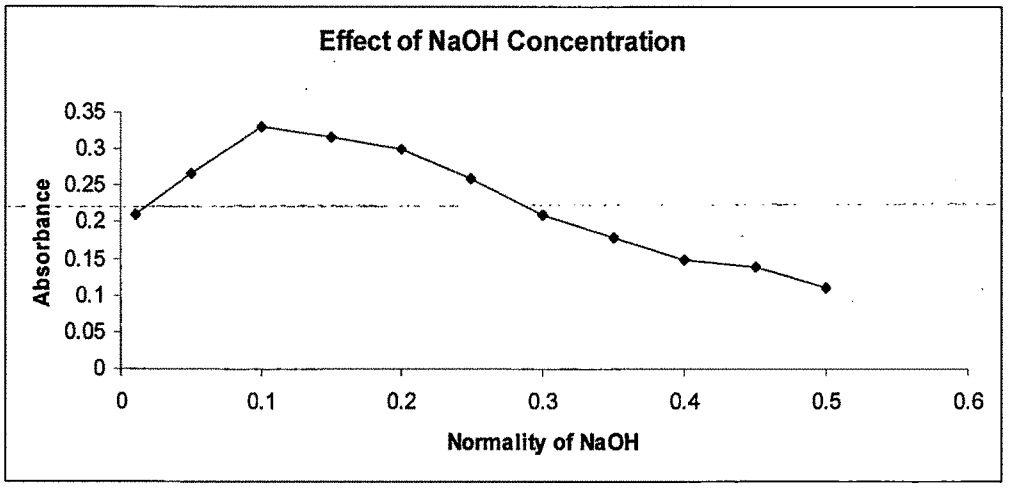



**Fig. 5.61. Effect of Sodium hydroxide Concentration on absorbance of hydrazine sulphide.**

*5.6.7.2.3) Selection of the diluting solvents*

Three solutions, HCl, NaOH and water were used to study the suitable diluting solvents for hydrazine sulphide formed. The yellow colouration was not found to be stable in the presence of water, while in presence of Hydrochloric acid the compound degraded to give an unstable coloured solution. In the presence of Sodium hydroxide the complex was found to be stable in terms of absorbance and shape of the UV spectra. Thus sodium hydroxide was used as the optimized diluting solvent.

*5.6.7.2.4) Composition of ion-pair extraction*

The composition of the compound was established by Job's method (1) of continuous variation (Figure 5.62).

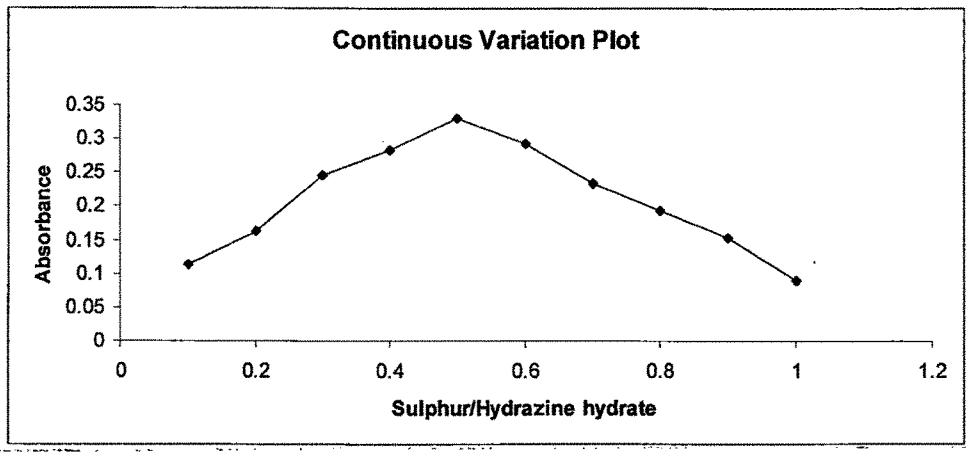



**Fig. 2.62 Continuous variation plot for Hydrazine sulphide.**

***5.6.7.3) Optimization of Extraction Conditions***

Maximum extraction was observed with toluene. The extraction conditions like the volume of toluene and sonication time period were also optimized. It was found that 50 ml toluene gave maximum extractive value. With increase in volume there was no further increase in concentration. The sonication period was optimized to be 20 minutes.

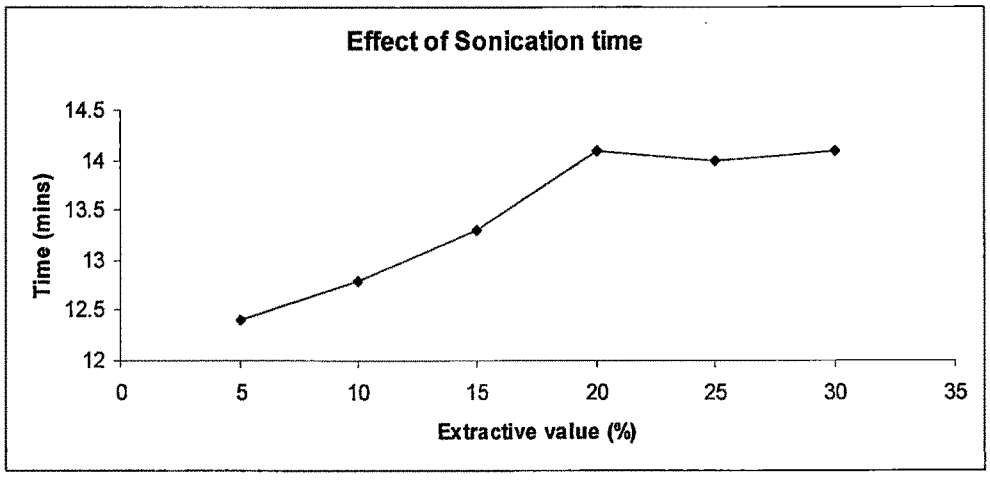



**Fig2.63. Effect of Sonication time on the extractive value of Sulphur.**

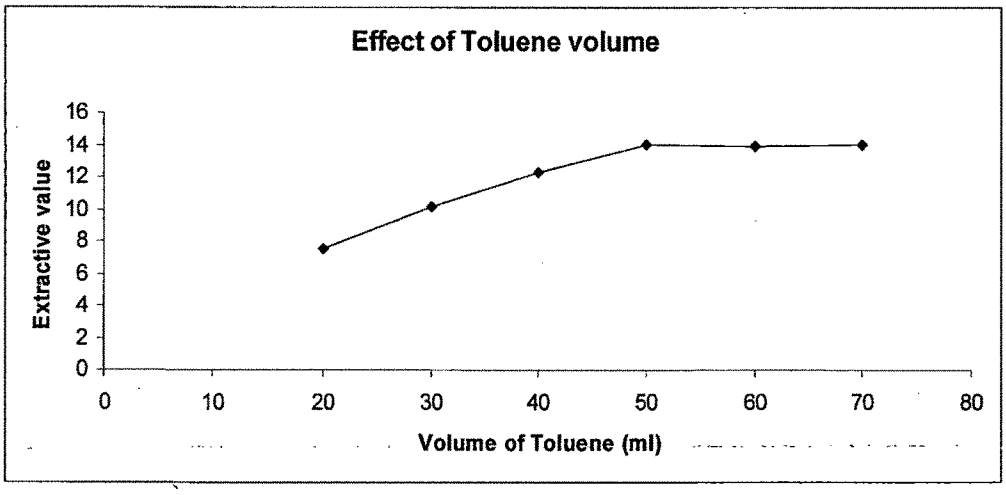



**Fig2.64. Effect of Toluene volume on the extractive value of sulphur.**

***5.6.7.4) Validation of the Methods***

*a) Linearity and range*

The linearity of the method was evaluated by analyzing a series of different concentrations of sulphur. In this study, eleven different concentrations of sulphur were chosen within the linearity range, and each was repeated three times. The calibration curve was found to follow Beer's law in the concentration range of 20-70 µg/ml. The optical characteristic such as Beer's law limits, molar absorptivity and Sandell's sensitivity and regression equation (slope, intercept and correlation coefficient) determined for each method are given in Table 5.110. The high molar absorptivities of the resulting colored complexes indicate the high sensitivity of the proposed methods. The line equation was y = 0.0066x + 0.0038 with a slope (a) = 0.0066, intercept (b) = 0.0038 and coefficient of determination ($r^2$) =0.9993.

**Table.5.110 Linearity data of Sulphur.**

| Sr. No. | Concentration (µg/ml) | Absorbance |
|---|---|---|
| 1 | 20 | 0.137 |
| 2 | 25 | 0.172 |
| 3 | 30 | 0.203 |
| 4 | 35 | 0.231 |
| 5 | 40 | 0.264 |
| 6 | 45 | 0.298 |

| | | |
|---|---|---|
| 7 | 50 | 0.332 |
| 8 | 55 | 0.361 |
| 9 | 60 | 0.402 |
| 10 | 65 | 0.432 |
| 11 | 70 | 0.468 |

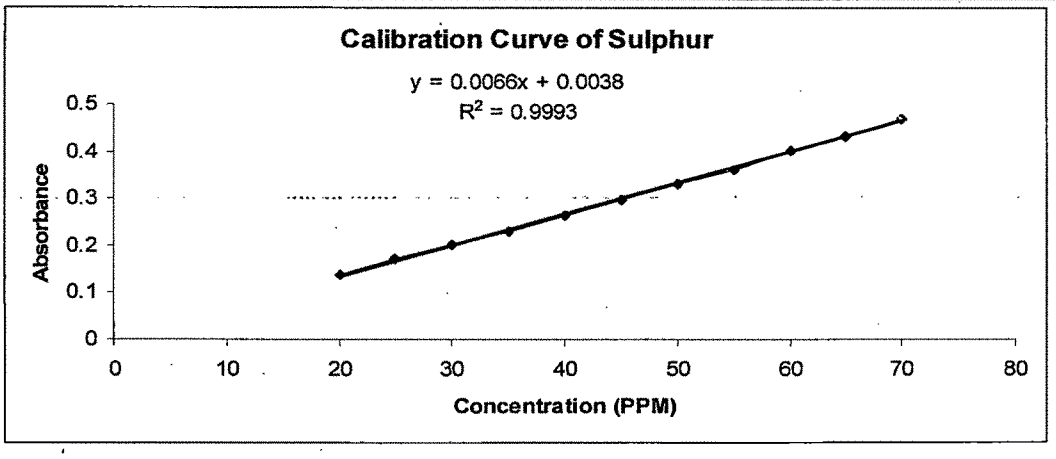



**Fig2.65. Calibration plot of Sulphur.**

**Table 5.111. Optical characteristics and statistical data of the regression equations for determination of sulphur using hydrazine hydrate.**

| Sr.No. | Parameter/Characteristics | Result |
|---|---|---|
| 1 | Colour | Yellow |
| 2 | λ max (nm) | 368.0 |
| 3 | Stability (h) | 24 |
| 4 | Molar absorptivity (1 $mol^{-1}$ $cm^{-1}$) | $3.951 \times 10^3$ |
| 5 | Sandell's sensitivity (µg/$cm^2$) | 0.07656 |
| 6 | Linearity Range | 20-70 µg/ml |
| 7 | Regression equation | Y=0.0066x+0.0038 |
| 8 | Slope | 0.0066 |
| 9 | Intercept | 0.0038 |
| 10 | Limit of Detection (µg/mL) | 0.1456 µg/ml |
| 11 | Limit of Quantification (µg/mL) | 4.394 µg/ml |
| 12 | Coefficient of Determination ($r^2$) | 0.9993 |
| 13 | Regression coefficient. | 0.9996 |
| 14 | Relative standard deviation (%) | 0.621 |

*b) Detection and Quantitation Limits*

The LOD and LOQ values for the method were calculated from the calibration curve as kSD/b where k= 3 for LOD and 10 for LOQ, SD is the standard deviation of the intercept and b is the slope of the calibration curve.

*c) Precision*

For evaluation of the precision, within the day (intra-day) and between-day (inter-day) precision variability was performed at three concentration levels (20, 40 and 70 µg/mL) for sulphur. The experiments were repeated six times a day for intra-day precision and on three different days for inter-day precision. Table represents the results which measured in terms of % RSD for within-day and between-day variability studies. As evident the % RSD values of the data obtained were well below 2% indicating that the method was sufficiently precise. Thus, it was concluded that there was no significant difference in the assay done for within-day and between-day.

**Table. 5.112. Intra-day and inter-day precision studies (n= 6)**

| Sulphur (µg/mL) | Intra-day precision | | Inter-day precision | |
|---|---|---|---|---|
| | Measured ± SD, RSD (%) | Std Error | Measured ± SD, RSD (%) | Std Error |
| 20 | 19.47 ± 0.09, 0.55 | 0.007 | 19.72 ± 0.10, 0.45 | 0.016 |
| 40 | 40.09 ± 0.12, 0.81 | 0.050 | 39.84 ± 0.10, 0.91 | 0.039 |
| 70 | 79.82 ± 0.09, 0.63 | 0.049 | 79.63 ± 0.08, 0.53 | 0.051 |

*d) Accuracy*

To confirm the accuracy of proposed method, recovery study was performed by the standard addition technique. Three different levels of standards were added to pre-analyzed samples, and each level was repeated three times. The average percent recoveries obtained were quantitative (97-100%), indicating good accuracy of the method. Results indicate that the methods is accurate and precise, and also that there is no interference due to the other components present in the formulation.

**Table.5.113. Recovery studies of Sulphur**

| Excess drug added to the analyte(%) | Theoretical content (µg) | Recovery (%) | % RSD |
|---|---|---|---|
| 0 | 50.23 | 98.13 | 1.85 |
| 80 | 90.207 | 99.72 | 1.70 |
| 100 | 100.46 | 97.81 | 0.79 |
| 120 | 110.299 | 98.09 | 1.34 |

***5.6.7.4) Analysis of pharmaceutical preparations***

The proposed methods have been successfully applied to the determination of sulphur in *Mahamrutyunjaya rasa*. The label claim of sulphur is 10.93 mg per tablet. The results obtained are shown in Table 5.114. Five replicates determinations were made. Satisfactory results were obtained for drug. (Table 5.114). The results were reproducible with low %RSD values. The results of analysis of the formulations and the recovery study of drug suggested that there is no interference from the other components, which are present in the formulation.

**Table. 5.114. Analysis of pharmaceutical preparation**

| Sr.No. | Formulation | Content, mg per tablet |
|---|---|---|
| 1 | Form-1 | 10.06 ± 0.72 |
| 2 | Form-2 | 11.09 ± 1.02 |
| 3 | Form-3 | 11.94 ± 0.83 |

* mean of five determinations

### 5.6.8) Estimation of Boron by Spectrofluorimetry in *Mahamrutyunjaya rasa.*

Boron present in sodium metaborate was analyzed using a spectrofluorimetric method. A reported simple and sensitive spectrofluorimetric method was used for the determination of boron with Alizarin Red. The method was further adapted and optimized for the determination of boron in *Mahamrutyunjaya rasa.* The fluorescence intensity of the complex formed in aqueous solution developed immediately. A buffer solution prepared by mixing disodium hydrogen phosphate with potassium dihydrogen phosphate was added to an aqueous solution of sodium metaborate. To this mixture Alizarin red S solution was added. In the presence of Alizarin red S at pH 7.4, a complex was formed which gave flourescence at 570 nm. The compex formed was Borosulfoalizarin which was stable at pH 7.4. The formation of the complex was rapid and the flourescence intensity was stable for 5h without any change in composition when kept in dark at room temperature.

#### *5.6.8.1) Spectral characteristics*

The solutions containing boron-Alizarin red S complex were scanned in the range of 475 to 900 nm. The fluorescence excitation and emission spectra of the boron-Alizarin Red S complex are shown in Fig. 5.69. The blank solution had practically no flouresence at the wavelength scanned in all cases. The fluorescent species were found to have excitation and emission maxima at 470 and 570 nm, respectively.

#### *5.6.8.2) Effect of pH*

The pH of the medium had a large effect on the fluorescence intensity. Experiments indicated that the optimum pH range for complex formation was 6.7-8.0. The fluorescence decreased markedly at both lower and higher pH values. A disodium hydrogen phosphate-potassium dihydrogen phosphate buffer of pH 7.4 was selected.

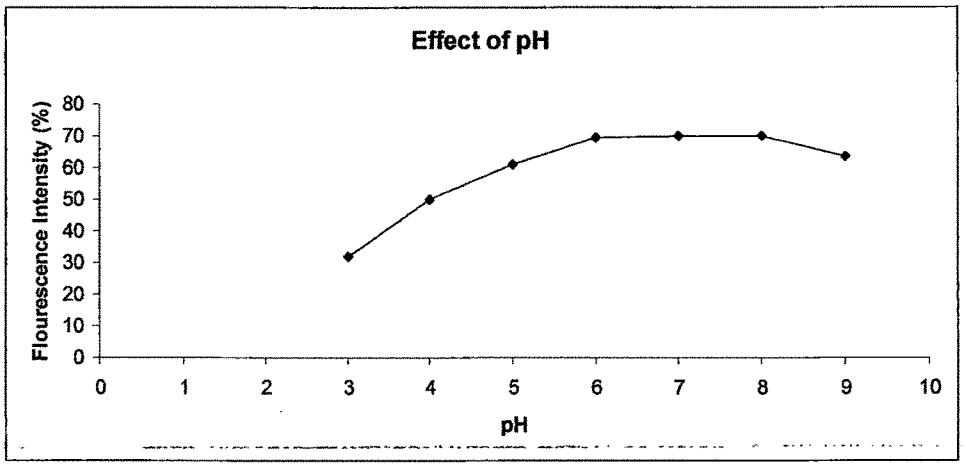



**Fig.5.66. Effect of pH on the flourescence intensity (%).**

***5.6.8.3) Influence of Reagent Concentration***

The effect of reagent concentration on the fluorescence intensity for solutions containing 10 µg/ml of boron was studied. The fluorescence intensity increased with the reagent concentration up to 0.112 mM, then remained constant between 0.112 mM and 0.2 mM, the last being the highest concentration studied. A 0.15 mM reagent concentration was selected to ensure a sufficient excess of the reagent. The stoichiometry of the complex was studied under the established conditions by the classical method of Yoe and Jones[226]. From this study it was concluded that the composition of the complex was 1 : 1.

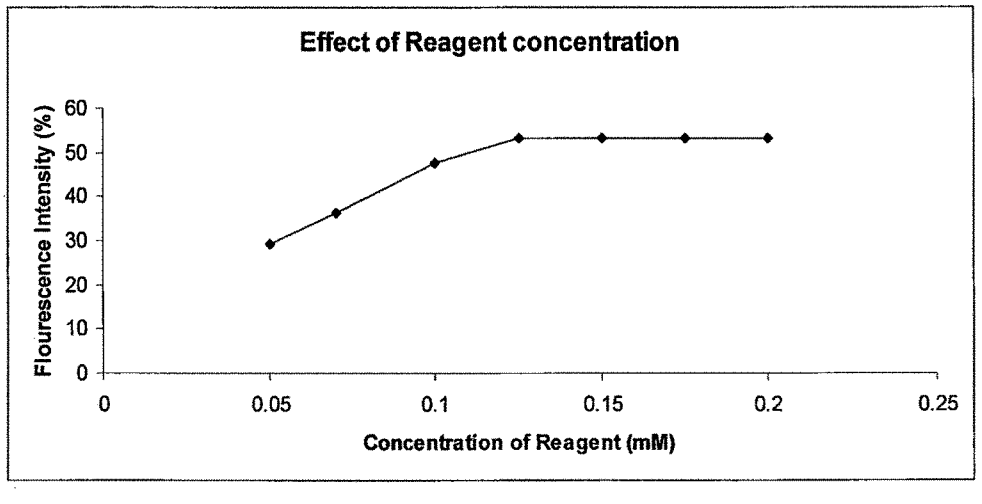



**Fig.5.67. Effect of reagent concentration on the flourescence intensity (%).**

***5.6.8.4) Effect of Temperature***

The effect of temperature on the measurements was studied by heating thermostatically a series of samples between 5 and 50°C. An increase in temperature from 5 to 50°C reduced the fluorescence intensity, it was found necessary to maintain the temperature at 25 °C.

***5.6.8.5) Stability of the samples***

The fluorescence intensity of the boron-Alizarin Red S complex developed instantaneously and remained almost constant for 5h after sample preparation.

***5.6.8.6) Order of Addition of Reagents***

The order of addition of the reagents was investigated and was found to have no effect on the fluorescence intensity.

***5.6.8.7) Influence of Foreign Ions***

Most of the ions tested were found to interfere with the measurement of fluorescence intensity and as the tolerance level for many metal ions was increased by the addition of EDTA solution as a masking agent, the influence of interferents was studied in the presence of an EDTA concentration of 0.01 M.

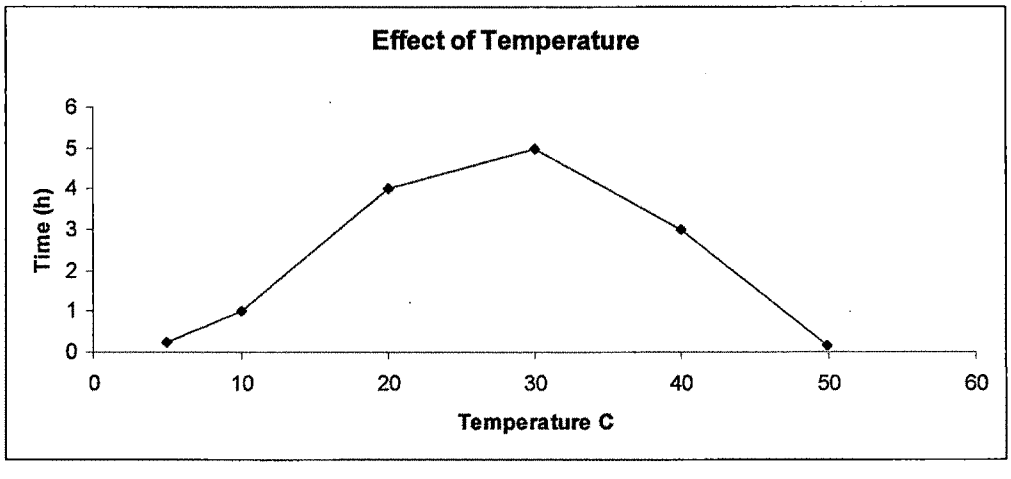



**Fig.5.68. Effect of temperature on the flourescence intensity (%).**

*f) System suitability*

A system suitability test was performed to evaluate the chromatographic parameters (capacity factor, separation factor, column efficiency, number of theoretical plates, HETP asymmetry of the peaks and resolution between two consecutive peaks) before the validation runs (Table 5.107). Three replicate injections of the standard solution and three injections of the solution prepared for the specificity procedure were used.

**Table 5.107. System suitability parameters**

| Sr. No. | Parameter | Solanine | Piperine |
|---|---|---|---|
| 1 | Retention time (min) | 4.52 | 5.92 |
| 2 | Capacity factor (k') | 2.86 | 3.85 |
| 3 | Separation factor (α) | 1.346 | -------- |
| 4 | Efficiency/length(t.p/m) | 73279 | 84780 |
| 5 | HETP (mm) | 0.051 | 0.048 |
| 6 | Resolution (Rs) | ------ | 4.095 |
| 7 | Assymmetry (As) | 0.980 | 0.973 |

*g) Accuracy*

As shown in Table 6, the recovery of the investigated component ranged from 98% to 100 %, and their %RSD values were all less than 2 %. It was known from recovery tests that the developed methods manifested the reliability and accuracy for the measurement of solanine and piperine simultaneously.

**Table 5.108. Recovery studies (n=3)**

| Excess drug added to the analyte(%) | Theoretical content (µg) | Recovery (%) | % RSD |
|---|---|---|---|
| Solanine | | | |
| 0 | 13.94 | 98.37 | 0.84 |
| 80 | 25.092 | 98.67 | 0.62 |
| 100 | 27.88 | 99.08 | 0.77 |
| 120 | 30.668 | 98.35 | 0.54 |
| Piperine | | | |
| 0 | 52.16 | 98.68 | 0.94 |
| 80 | 93.888 | 98.74 | 0.75 |

| | | | |
|---|---|---|---|
| 100 | 104.32 | 99.02 | 1.09 |
| 120 | 114.752 | 98.41 | 1.15 |

***5.6.6.3) Applicability of the developed method in formulations***

The developed HPLC method was applied to the simultaneous determination of solanine and piperine in the Ayurvedic formulations (FORM1, FORM2, FORM3) and the results are presented in Table 5.109.

**Table 5.109. Results of analysis.**

| Sr. No. | Formulation | Content, µg/tablet | |
|---|---|---|---|
| | | Solanine | Piperine |
| 1 | Form1 | 0.128±0.02 | 0.42±0.02 |
| 2 | Form2 | 0.140±0.04 | 0.55±0.05 |
| 3 | Form3 | 0.068±0.02 | 0.36±0.04 |

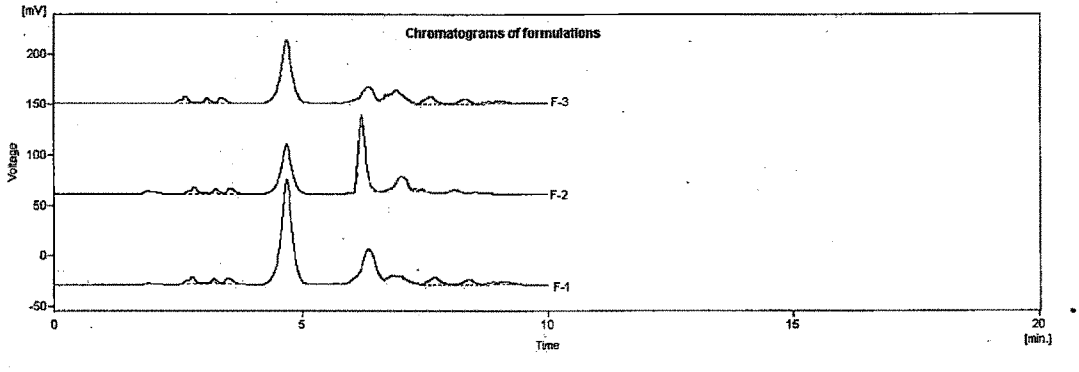



**Fig. 5.58. Representative chromatogram of Form-1(F-1), Form-2 (F-2)and Form-3 (F-3).**

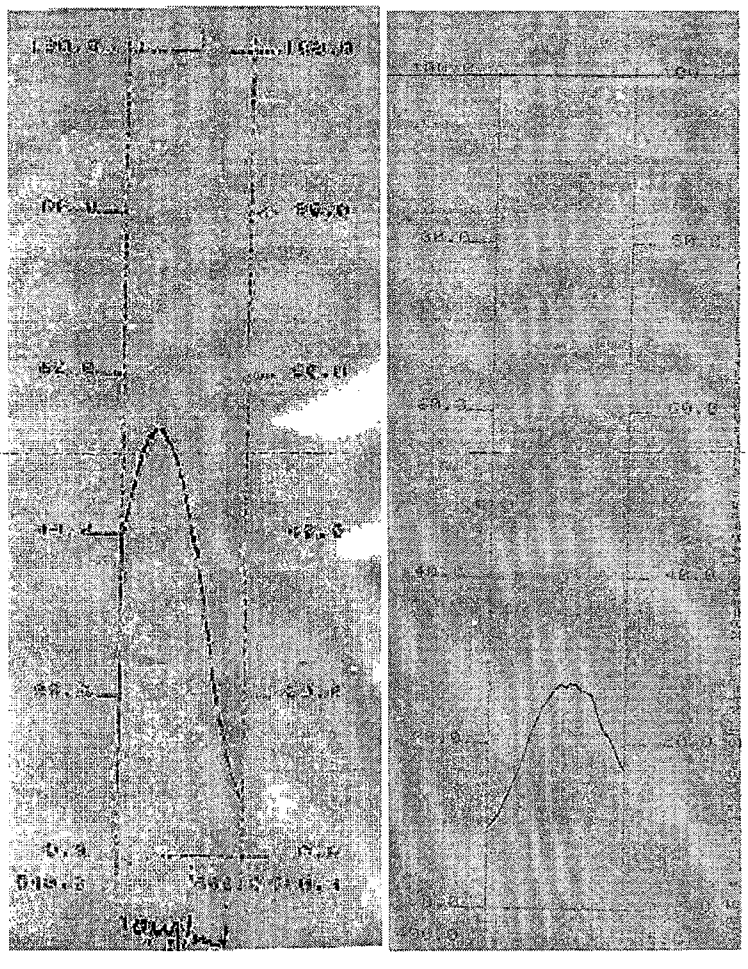


**Fig.5.69. Excitation of Boron at 470 nm and emission at 570 nm.**

***5.6.8.7) Method validation***

*a) Linearity and sensitivity*

The linear calibration graphs were obtained under the optimum experimental conditions. The analytical results obtained from this investigation are summarized in Table 5.116. The calibration data were fitted by least square method and the regression equations. A linear relationship was found between fluorescence intensity and concentration in the ranges given in Table 5.115. LOD was 74 ng/ml and LOQ was found to be 222 ng/ml, respectively.

**Table 5.115. Data of Calibration Curve for Boron**

| Concentration of Boron (µg/mL) | # Relative Fluorescence Intensity | % RSD |
|---|---|---|
| 7.5 | 45.1 | 1.23 |
| 10 | 53.3 | 0.86 |
| 12.5 | 63.4 | 0.65 |
| 15 | 69.7 | 1.02 |
| 17.5 | 79.9 | 1.42 |
| 20 | 87.7 | 0.93 |
| 22.5 | 93.1 | 0.92 |
| 25 | 102.1 | 1.08 |

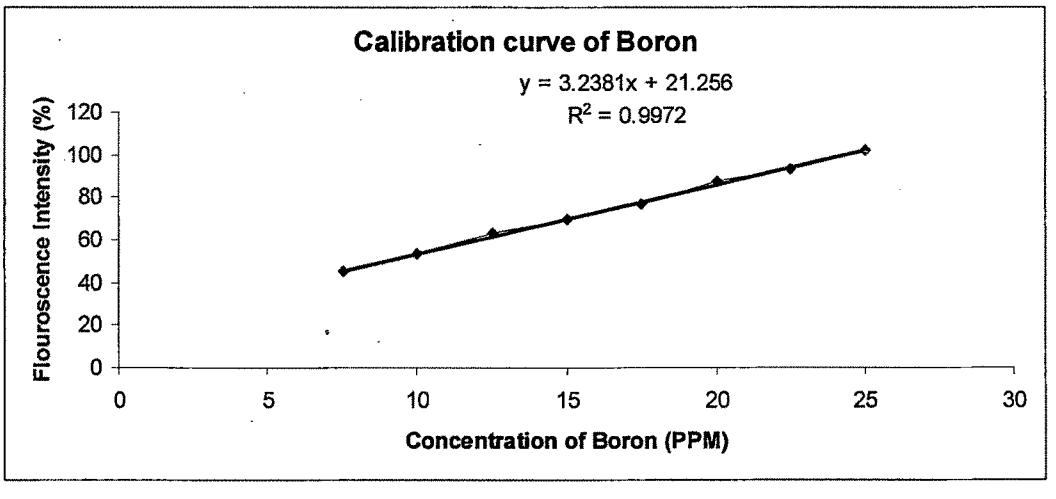



**Fig.5.70.Calibration curve of Boron using spectroflourimetry.**

**Table. 5.116. Optical Characteristics and Analytical Data**

| Parameters | Results |
|---|---|
| Excitation wave length | 470 nm |
| Emission wave length | 570 nm |
| Scan Speed | Medium |
| Sensitivity | High |
| Abscissa scale | 2 |
| Ordinate scale | 6 |
| Linearity range (ng/mL) | 7.5-25 µg/ml |
| Regression equation (Ya) | Y = 3.2381x + 21.256 |
| Slope (b) | 3.2381 |

| | |
|---|---|
| Intercept (a) | 21.256 |
| Coefficient of determination (r2) | 0.9972 |
| Correlation coefficient (r) | 0.9985 |
| Limit of Detection | 74 ng/ml |
| Limit of Quantification | 222 ng/ml |

*b) Precision*

The precision of the method was tested with regard to both the intra-day and inter-day precision of the assay. The intra-day variability of the assay was determined by repeated analysis of three concentrations ($n$ = 6). Similarly, the inter-day variability of the assay was determined through replicate analysis of three concentrations ($n$ = 3), and the results are listed in Table 3. Both the intra-day and inter-day precision of the presented methods were fairly good.

**Table 5.117. Intra-day and inter-day precision of spectrofluorimetric method. (n=6)**

| **Amount (µg/ml)** | **Intra-day precision** | | |
|---|---|---|---|
| | **Fluorescence Intensity** | **S.D.** | **% RSD** |
| 7.5 | 44.3 | 1.2 | 1.4 |
| 15 | 70.02 | 0.85 | 1.13 |
| 25 | 101.8 | 0.57 | 0.92 |
| | **Inter-day precision** | | |
| **Amount (µg/ml)** | **Fluorescence Intensity** | **S.D.** | **% RSD** |
| 7.5 | 45.3 | 1.1 | 1.15 |
| 15 | 69.8 | 0.94 | 1.02 |
| 25 | 102.0 | 0.73 | 0.97 |

*c) Recovery*

The proposed method when used for estimation of boron from the herbal dosage form after spiking with 80, 100 and 120% of additional drug afforded recovery of 98–101% as listed in Table 5.118.

Table. 5.118. Data of sample analysis and recovery studies:

| Excess drug added to the analyte(%) | Theoretical content (µg/ml) | Recovery (%) | % RSD |
|---|---|---|---|
| 0 | 9.8 | 98.21 | 1.16 |
| 80 | 17.64 | 98.69 | 1.62 |
| 100 | 19.6 | 98.10 | 1.42 |
| 120 | 21.56 | 98.91 | 1.08 |

*d) Robustness*

No significant difference could be observed in the results found out. The recovery obtained individually and the mean was between 98 % and 101 % boron. Therefore, it can be concluded that the method is consistent for the brand of the reagents used.

***5.6.8.8) Applicability of the developed method in formulations***

The developed method was applied to the determination of boron in the Ayurvedic formulations (FORM1, FORM2, FORM3) and the results are presented in Table 5.119. The label claim of Boron in the formulation is about 4.3 mg per tablet. It was observed that the content of boron varied in the three formulations to some extent which would significantly influence the quality stability because it is one of the target toxic component for the quality control of *Mahamrutyunjaya rasa* tablets.

Table.5.119. Results of Analysis

| Sr. No. | Formulation | Boron, mg per tablet |
|---|---|---|
| 1 | Form-1 | 4.35 ± 0.17 |
| 2 | Form-2 | 4.2 ± 0.21 |
| 3 | Form-3 | 5.3 ± 0.11 |

### *5.6.9)* Estimation of Boron by Voltammetry.

***5.6.9.1) Voltammetric peak characteristics of the boron-ARS complex.***

The differential pulse voltammograms of Alizarin red S and boron-Alizarin red S complex are shown in Fig.5.71, 5.72. A peak was observed at -590 mV in a solution of Alizarin red S at pH 7.0 when the potential was scanned towards anodic direction. After the addition of Boron to this solution a new peak at -520

mV appears. This new peak proportionally increases with increasing concentration of Boron while the peak at -590 mV significantly decreases. The peak at -590 mV practically disappears when the excess boron was added to the solution higher than 0.5 µg/ml. This indicates that Alizarin red S reacts with boron to form a complex, borosulfoalizarin and a new anodic peak related to this complex appears. No peak was observed corresponding to boron-Alizarin red S complex when the potential scanning was done towards more negative values.

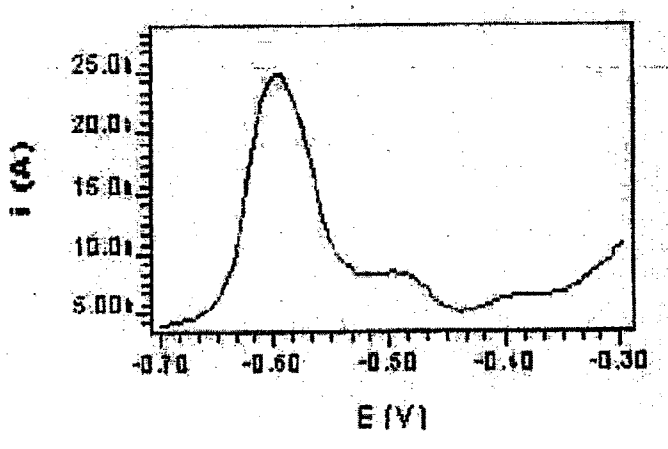



**Fig.5.71. Differential voltammogram of Alizarin red S.**

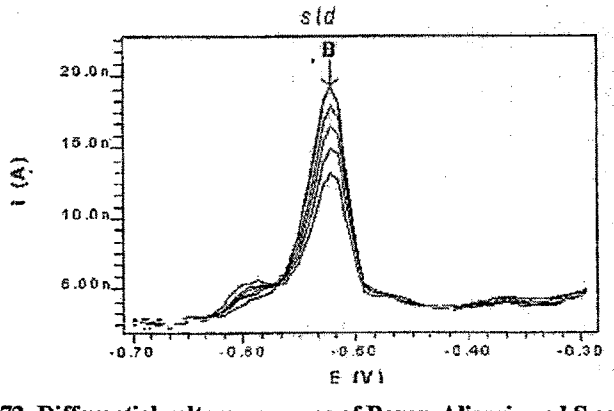



**Fig.5.72. Differential voltammograms of Boron-Alizarin red S complex.**

As the anodic peak height of boron-Alizarin red S complex was plotted versus potential scan rate, the peak current linearity changed with potential scan rate indicating that it is an adsorption process. In this case pre-treatment conditions such as accumulation time and potential were investigated.

The variation of peak current as a function of accumulation time at -0.7 V is shown in Fig.5.73 .As follows from the figure, the current increases with

accumulation time and reaches a maximum at 5s and then, decreases. Although, this behaviour quite disagrees with the conventional adsorptive stripping phenomenon, here the competitive adsorption of the complex and the free ligand should be considered. For accumulation times longer than 5s, massive adsorption of the free ligand on the electrode surface takes place and hinders the anodic peak of the complex. The precision of the method was poor for accumulation time of 5s, therefore, the measurements were performed without applying any accumulation time.

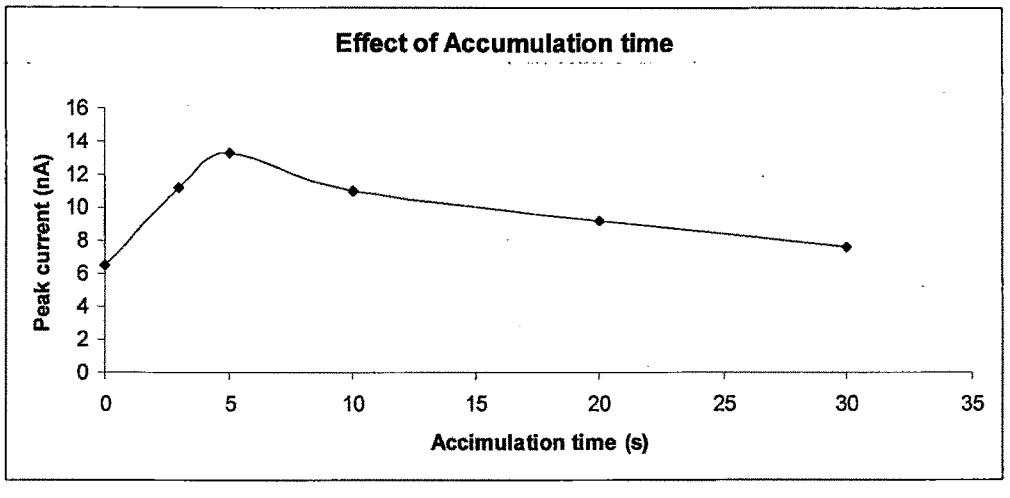



**Fig.5.73. Effect of Accumulation time on the peak current (nA).**

On the other hand, as the scan rate is 5 mV/s and the starting potential is -0.7V, 36 s is virtually passed during the scan to potential -0.521 where the anodic peak is observed. Accumulation at a certain degree is maintained during this period. Infact, the scan rate is quite critical for the peak height as it determines the time passed during the scan and so adsorption /desorption ratio of the complex and free ligand. The competitive adsorption of the free ligand and the complex can be clearly observed in the scan where the scan rate was 5 mV/s. At higher scan rates , the anodic peak of free ligand increases and the complex peak appears as a shoulder on the former peak in particular for low boron concentrations. This results in bad resolution threatening the selectivity of the method.

Besides, the effect of temperature on the peak current was investigated. The reaction mixture including boron, alizarin red S at pH 7.0 was heated for 15 min at 40, 60 and 80 °C and then allowed to stand for 15 min, 4 and 10h. No

significant difference was observed between the results of these experiments and of the ones performed at room temperature.

The effect of accumulation potential on the peak current was investigated for a very short accumulation time (3s) at different potential values but the scan was initiated from -0.7 V in each experiment. Fig.5.74 shows the dependence of the peak current on the accumulation potential. The peak current gives a maximum at -700 mV and thereafter, this potential was chosen.

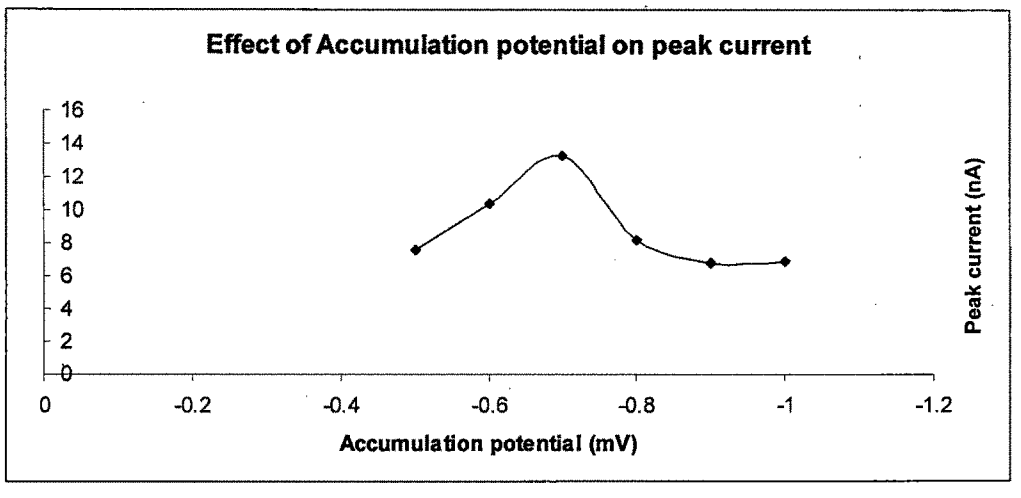



**Fig.5.74. Effect of Accumulation potential on the peak current (nA).**

***5.6.9.2) Effect of pH and supporting electrolyte***

Fig.5.75 shows the effect of pH on the peak current. The peak current slightly increases from pH 5.0 to 7.0 and then, decreases with increasing in pH. A positive shift in the peak potential of the complex was observed with decrease in pH. No signal related to the boron complex was obtained below pH 5.0. The optimum pH was selected as 7.0 to carry out subsequent experiments.

Additionally, different electrolytes at pH 7.0 were tested as a supporting electrolyte (ammonium acetate, phosphate, ammonium acetate-phosphate mixture, sodium acetate, ammonium nitrate, ammonium chloride, potassium perchlorate, sodium bromate). Among these, 0.1M ammonium acetate - 0.1M phosphate mixture (pH 7.0) has given the maximum peak current and the best resolution.

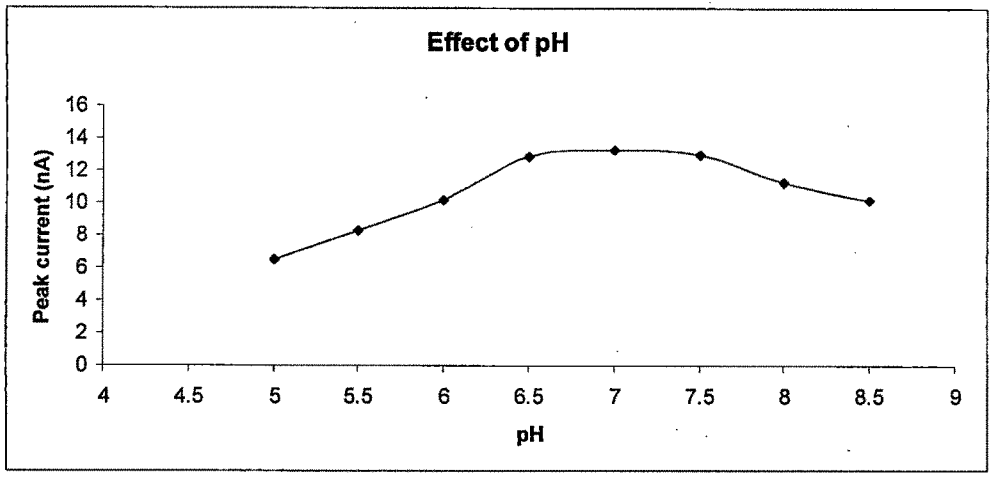



**Fig.5.75. Effect of pH on the peak current (nA).**

***5.6.9.3) Effect of Alizarin red S concentrations.***

The effect of the ligand concentration on peak current of the boron alizarin red S complex was tested. Various concentrations of ligand ranged from 0.1 to 10 μM in the presence of 100 μg/L Boron and supporting electrolyte at pH 7.0 were studied. The results have shown that peak height of boron-Alizarin red S complex increases with the ligand concentration. However, the free ARS peak overlaps with the peak of boron-Alizarin S complex for low boron concentrations. Thus, 1 μM of Alizarin red S was selected for subsequent experiments to obtain good resolution between the alizarin red S peak and boron alizarin red S peak.

**Table. 5.120. Voltammetric parameters for determination of boron**

| **Parameter** | **Description** |
|---|---|
| Accumulation potential (mV) | -700 |
| Accumulation time (s) | 0 |
| Supporting Electrolyte, pH | Ammonium acetate-phosphate buffer, 7.0 |
| Concentration of ARS (mM) | 0.001 |
| Scan rate (mV/s) | 5 |
| Pulse amplitude (mV) | 50 |
| Pulse duration (ms) | 20 |

***5.6.9.4) Method Validation***

*a) Linearity and Range*

Under the recommended conditions in the Table.5.121, a calibration curve for boron was constructed. Fig.5.76 shows the voltammograms obtained for the calibration curve. The curve is linear in the concentration range of 1-10 µg/ml in boron and the regression equation of the curve is y=0.8015x + 8.3787, where y is the peak height (in nA) and x is the concentration of Boron (in µg/ml). The correlation coefficient was 0.9975 for n=6.

*b) Detection and Quantitation Limits*

The limits of detection (LOD) and quantitation (LOQ) calculated at a signal-to-noise (S/N) ratio of 3 and 10 were 0.2 and 0.66 µg/L, respectively.

**Table. 5.121 Linearity data of boron alizarin red S complex.**

| Sample | Potential (V) | Peak Current (nA) | i. mean | i.delta |
|---|---|---|---|---|
| 1-1 | -0.521 | 8.96 | 9.02 | 1.02 |
| 1-2 | -0.521 | 9.08 | | |
| 2-1 | -0.521 | 10.11 | 10.07 | 1.71 |
| 2-2 | -0.521 | 10.03 | | |
| 3-1 | -0.521 | 11.59 | 11.57 | 1.73 |
| 3-2 | -0.521 | 11.55 | | |
| 4-1 | -0.521 | 13.72 | 13.34 | 1.56 |
| 4-2 | -0.521 | 12.96 | | |
| 5-1 | -0.521 | 14.5 | 14.9 | 1.12 |
| 5-2 | -0.521 | 15.3 | | |
| 6-1 | -0.521 | 16.58 | 16.22 | 1.32 |
| 6-2 | -0.521 | 16.22 | | |

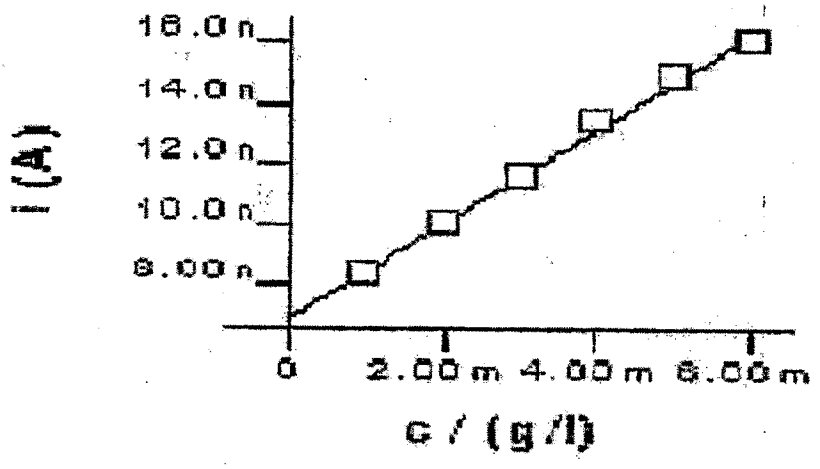



**Fig.5.76. Calibration curve of Boron-alizarin red S using Voltammetry.**

*c) Precision*

The precision of the method was tested with regard to both the intra-day and inter-day precision of the assay. The intra-day variability of the assay was determined by repeated analysis of three concentrations ($n$ = 6). Similarly, the inter-day variability of the assay was determined through replicate analysis of three concentrations ($n$ = 3), and the results are listed in Table 5.122. Both the intra-day and inter-day precision of the presented methods were fairly good.

**Table.5.122 Intra-day and inter-day precision of spectrofluorimetric method. (n=6)**

| **Amount (µg/ml)** | **Intra-day precision** | | |
|---|---|---|---|
| | **Peak Current (nA)** | **S.D.** | **% RSD** |
| 1 | 9.01 | 0.56 | 1.2 |
| 5 | 12.63 | 0.34 | 1.32 |
| 10 | 16.18 | 0.72 | 0.96 |
| **Amount (µg /ml)** | **Inter-day precision** | | |
| | **Peak Current (nA)** | **S.D.** | **% RSD** |
| 1 | 9.05 | 1.1 | 0.93 |
| 5 | 12.52 | 0.42 | 0.72 |
| 10 | 16.31 | 0.31 | 0.91 |

*d) Recovery*

The proposed method when used for estimation of boron from the herbal dosage form after spiking with 80, 100 and 120% of additional drug afforded recovery of 98–101% as listed in Table 5.123.

**Table.5.123. Data of sample analysis and recovery studies:**

| Excess drug added to the analyte(%) | Theoretical content (µg/ml) | Recovery (%) | % RSD |
|---|---|---|---|
| 0 | 3.8 | 98.53 | 1.29 |
| 80 | 6.84 | 99.21 | 1.54 |
| 100 | 7.6 | 98.10 | 1.71 |
| 120 | 8.36 | 98.23 | 1.83 |

*e) Robustness*

No significant difference could be observed in the results found out. The recovery obtained individually and the mean was between 98 % and 101 % boron. Therefore, it can be concluded that the method is consistent for the reagent brand.

***5.6.9.5) Applicability of the developed method in formulations***

The proposed method was applied to the determination of boron in the three formulations. The label claim of Boron in the formulation is about 4.3 mg per tablet. The boron concentration in the samples and the recoveries obtained from the spiked samples are shown in Table 5.124. The required dilution was made for all of the samples. The recoveries were in the range 96-104%. The proposed method provides an accurate and sensitive voltammetric determination of boron. The method is comparable with atomic spectrometric methods in terms of simplicity and costs. The method has been successfully applied for boron determination in the formulations. The interference from metal ions can be eliminated by addition of EDTA.

**Table.5.124. Results of Analysis**

| Sr. No. | Formulation | Boron, mg per tablet |
|---|---|---|
| 1 | Form-1 | 4.25 ± 0.19 |
| 2 | Form-2 | 4.15 ± 0.15 |
| 3 | Form-3 | 5.1 ± 0.24 |

The flourimetric method was used for controlling the accuracy of proposed method. Comparative results using Student's t-test (for 95% confidence limit) show that there is a good agreement between the mean concentration obtained by the two methods.

A novel voltammetric method for the determination of boron using Alizarin red S as complexing agent was described. This method is somewhat different from that of the conventional adsorptive stripping analysis as the anodic peaks are evaluated. This difference arises from the nature of the complex between boron and the alizarin red S at pH 7.0. By interpreting the competitive adsorptive virtually applying no accumulation time as the free ligand covers the active sites of the surface. Therefore, the complex is adsorbed during the potential scan from initial potential till the peak potentials.

### 5.6.10) Estimation of Boron in *Mahamrutyunjaya rasa* by ICP-AES:

In this study analytical methods have been applied for determination of boron in *Mahamrutyunjaya rasa.* The results obtained by ICP-AES at 249.43 nm. A calibration plot was prepared using standard Boron solution in the range (1-6 µg/ml). The powdered formulations were incinerated and the ash was used for the analysis. The solutions for analysis were prepared in 0.1N HCl (prepared in double distilled water). The samples were analyzed at 249.43nm and the boron content in all the formulations was found to be similar. The content found was comparable with the content estimated by the spectrofluorimetry and voltammetry method.

**Table.5.125. Operating conditions of ICP-AES.**

| **Parameter** | **Description** |
|---|---|
| Plasma view | 15-mm radial |
| Nebulizer type | Concentric glass |
| Sample uptake rate | 1 mL/min |
| Washing time | 30 s |
| Plasma power | 1300 W |
| Argon flow rate | |
| Plasma | 15 L/min |
| Auxiliary | 0.5 L/min |

| | |
|---|---|
| Nebulizer | 0.8 L/min |
| Replicates | 5 |
| Read time/replicate | 1s |
| Emission wavelength | 249.43 nm |
| Rf power | 1.20 kW |
| Argon gas (high purity) | 99.99 % |

***5.6.10.1) Validation of the method***

*a) Linearity*

The linearity of the present ICP-AES method for boron determination was good (r .0.99) for a boron concentration range of 2.5–100 µg/ml. The results revealed a good linearity.

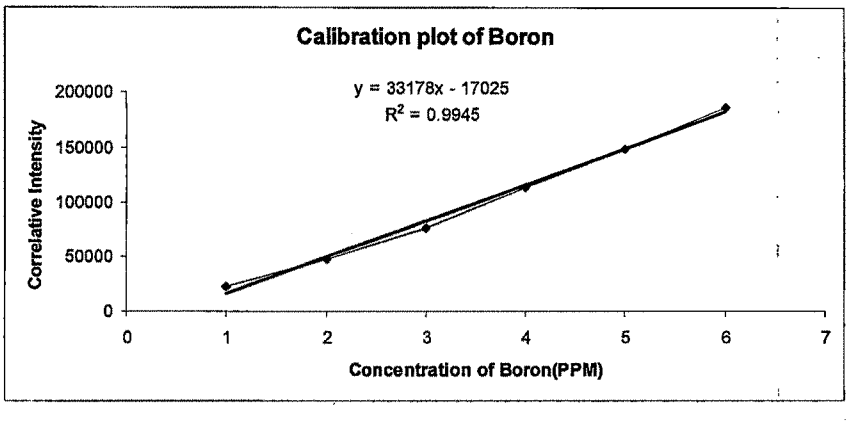



**Fig. 5.77. Calibration curve of Boron.**

**Table 5.126. Data for calibration curve of Boron.**

| Boron Concentration (µg/ml) | G,Correlative Intensity | % RSD |
|---|---|---|
| 1 | 22326 | 0.54 |
| 2 | 47563 | 0.32 |
| 3 | 76190 | 0.18 |
| 4 | 113234 | 0.43 |
| 5 | 148932 | 0.34 |
| 6 | 186342 | 0.56 |

*b) LOD and LOQ*

The limits of detection and quantification were estimated from the SD of the boron signal obtained by repeated measurements (n=6) of a zero calibrator containing the internal standard. The limit of detection was 0.1 PPM , and the limit of quantification was 0.33 PPM (corresponding to the boron signals equivalent to 3 and 10 times the SD of the boron signal in the zero calibrator, respectively).

*c) Recovery Study*

Analytical recovery in the ICP-AES procedure was further studied by supplementing the test samples with standard boron. The observed recoveries were in the range of 96-103%.

***5.6.10.2) Result of Analysis.***

The concentration of boron in the three formulations are shown in Table5.127. The label claim of Boron in the formulation is about 4.3 mg per tablet. The results are in accordance with the results of Voltammetry and Spectroflourimetry. Thus, ICP can used an analytical techqnique for estimation of Boron in the ash of Ayurvedic formulation, *Mahamrutyunjaya rasa.*

**Table.5.127. Results of Analysis**

| Sr. No. | Formulation | Boron, mg per tablet |
|---|---|---|
| 1 | Form1 | 4.25 |
| 2 | Form2 | 4.14 |
| 3 | Form3 | 5.2 |

**5.6.11) Estimation of Mercury in Mahamrutyunjaya rasa by ICP-AES:**

Mercury was estimated in the formulations using inductively coupled plasma-AES (Perkin Elmer, Optical Emission Spectrometer, Optima 2100 DV attached to Winlab 32 software). A calibration plot was prepared using standard mercury solution in the range (1-6 µg/ml). The powdered formulations were treated by acid digestion. The cinnabar is extracted in using aqua regia and diluted in double distilled water. The samples were analyzed at 253.56 nm.

**Table 5.128. Operating conditions of ICP-AES.**

| Parameter | Description |
|---|---|
| Plasma view | 15-mm radial |
| Nebulizer type | Concentric glass |
| Sample uptake rate | 1 mL/min |
| Washing time | 30 s |
| Plasma power | 1300 W |
| Argon flow rate | |
| Plasma | 15 L/min |
| Auxiliary | 0.5 L/min |
| Nebulizer | 0.8 L/min |
| Replicates | 5 |
| Read time/replicate | 1s |
| Emission wavelength | 253.56 nm |
| Rf power | 1.20 kW |
| Argon gas (high purity) | 99.99 % |

**Table 5.129. Data for Calibration curve of Mercury.**

| Mercury Concentration (PPM) | G,Correlative Intensity | % RSD |
|---|---|---|
| 1 | 77,714.8 | 0.39 |
| 2 | 168,549.4 | 0.22 |
| 3 | 246,634.2 | 0.30 |
| 4 | 325,975.3 | 0.16 |
| 5 | 443,692.7 | 0.30 |

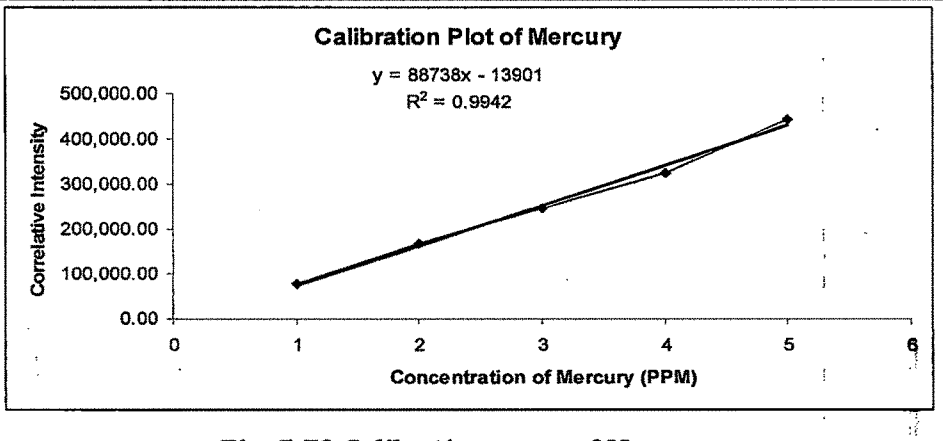



**Fig. 5.78 Calibration curve of Mercury.**

*a) LOD and LOQ*

The limits of detection and quantification were estimated from the SD of the mercury signal obtained by repeated measurements (n=6) of a zero calibrator containing the internal standard. The limit of detection was 0.05 PPM , and the limit of quantification was 0.16 PPM (corresponding to the boron signals equivalent to 3 and 10 times the SD of the boron signal in the zero calibrator, respectively).

*b) Recovery Study*

Analytical recovery in the ICP-AES procedure was further studied by supplementing the test samples with standard mercury. The observed recoveries were in the range of 96-103%.

***5.6.9.2) Result of Analysis.***

The concentration of mercury in the three formulations are shown in Table 4.130. The label claim of Mercury in the formulation is about 4.68 mg per tablet. The results are in accordance with the results of Voltammetry and Spectroflourimetry. Thus, ICP can used an analytical techqnique for estimation of mercury in the ash of Ayurvedic formulation, *Mahamrutyunjaya rasa.*

**Table.4.130. Results of Analysis**

| Sr. No. | Formulation | Mercury, mg per tablet |
|---|---|---|
| 1 | Form1 | 4.57 |
| 2 | Form2 | 5.941 |
| 3 | Form3 | 5.941 |

## 5.7) Biological Standardization of the three formulations.

### *5.7.1) Acute toxicity study*

Mortality was observed in FORM1, FORM2 and FORM3 treated mice at a single oral dose of 2000 and 1550 mg/kg/b.w. While normal behaviour was observed at a single oral administration of FORM1, and FORM2 at 550 mg/kg/b.w. Histopathological examination of the liver, heart and kidney of the mice treated with 550 mg/kg. b.w. of FORM1 and FORM2 appeared normal suggesting lack of any hepatotoxicity, cardiotoxicity or nephrotoxicity. With respect to sections of liver, control liver tissues showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces, and central vein (Fig.5.79(G, H)). Heart and Kidney were also free of pathological findings in FORM1 and FORM2 (Fig. 5.79(A,B,D,E)) treated groups of animals. While mice treated with 550 mg/kg. b.w. of FORM3 showed liver injury on the liver surface to some extent (Fig. 5.79(I)). The hepatic cells appeared as cloudy swelling with slight necrosis. Kidney tubules also exhibited destruction (Fig. 5.79(F)). Tissue glomeruli were affected in addition to the renal corpuscles. Most of renal tubules showed dilation and the epithelial cells tended to be vacuolated with a foamy appearance. Heart sections (Fig. 5.79(C)) also showed infarcted zone with oedema and inflammatory cells and the separated muscle fibres in the myocardium of the FORM3 treated mice. Muscle bands were observed to be in patches and the blood vessels were found to be slightly ruptured in the heart. However, no mortality was observed. Thus, FORM3 was found to be toxic and further the *in vitro* cell viability studies were performed.

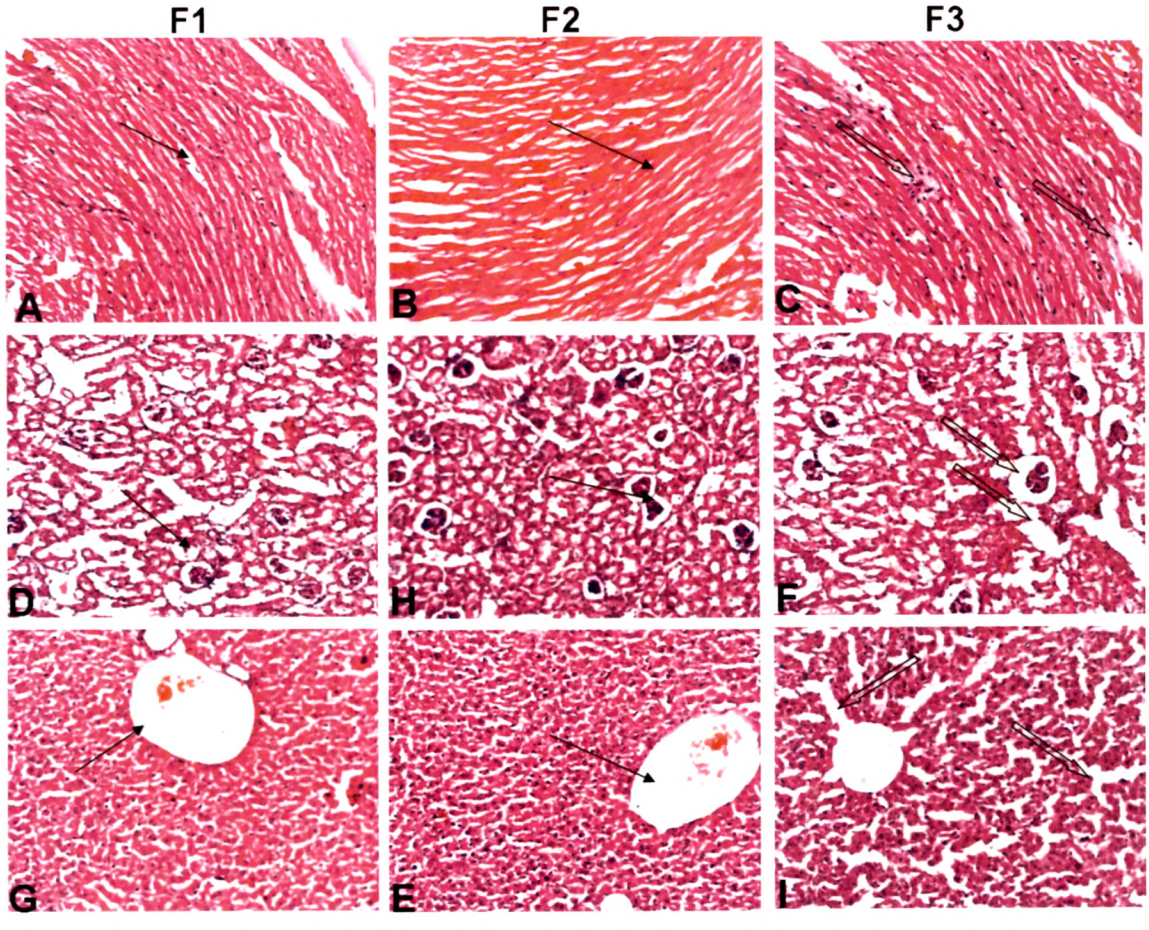



**Fig.5.79. Microscopic images of mice heart, kidney and liver illustrating effect of single dose administration of FORM1, FORM2 and FORM3** (Staining: Hematoxylin and Eosin).

Microscopic images of mice heart from (A) FORM1, (B) FORM2, (C) FORM3 groups; images of mice kidney from (D) FORM1, (E) FORM2, (F) FORM3, images of mice liver from (G) FORM1, (H) FORM2, (I) FORM3 groups. Solid arrow indicates the normal and unfilled arrow indicates the affected/infracted/injured area of kidney/heart//liver.

### *5.7.2) Effect of Formulations on % Cell viability of H9c2 cells*

Effect of FORM1, FORM2 and FORM3 treatments on viability of H9c2 cells were studied in comparison to the untreated (control) cells. Cells pretreated with FORM1 for 12 h showed increased cell viability in the concentration range of 2-200 μg/ml. After 24h of treatment cell viability was decreased by 7.15, 13.89, 15.35, 16.94, 26.59, 33.6, and 47.49%, while after 48 h of treatment viability

was reduced by 3.58, 4.19, 5.19, 13.57, 24.26, 38.04 and 56.34% at the concentration 2, 5, 10, 20, 50, 100 and 200 µg/ml respectively (Fig.5.80).

Cells pretreated with FORM2 for 12 h showed reduction in cell viability by 14.85% at 200 µg/ml concentrations. After 24h of treatment viability was decreased by 9.79, 8.87, 4.9, 15.08, 21.17, 36.78 and 65.08%, while after 48 h of treatment viability was reduced by 4.19, 5.3, 6.29, 7.72, 16.65, 29.33 and 72.66% at the concentration 2, 5, 10, 20, 50, 100 and 200 µg/ml respectively (Fig.5.81).

Cells pretreated with F3 for 12 h showed reduction in cell viability by 34.71, 36.08, 40.42, 31.97, 25.35, 70.55, and 85.55%, similarly after 24 h of treatment viability was decreased by 7.68, 6.49, 7.94, 13.63, 22.09, 53.84, and 94.98% at the concentration 2, 5, 10, 20, 50, 100 and 200 µg/ml respectively. After 48 h of treatment viability was decreased by 7.23, 9.23, 13.01, 24.48, 58.51, and 96.15% at the concentration of 5, 10, 20, 50, 100 and 200 µg/ml respectively (Fig.5.82).

**Table 5.131. Effect of FORM1 on percentage viability of H9c2 cells at different time interval**

| A(µg/ml) | 12 h | 24 h | 48 h |
|---|---|---|---|
| V.Control | 100±8.69 | 100±4.493 | 100±1.87 |
| 2 | 131.96±3.19 | 92.85±1.71 | 96.58±6.83 |
| 5 | 128.31±3.65 | 86.11±4.891 | 95.81±2.09 |
| 10 | 128.53±10.73 | 84.65±3.57 | 94.81±3.41 |
| 20 | 122.6±7.76 | 83.06±2.11 | 86.43±3.85 |
| 50 | 121.46±6.59 | 73.41±5.82 | 75.74±2.31 |
| 100 | 106.84±2.73 | 66.4±3.43 | 61.96±2.53 |
| 200 | 112.1±9.13 | 52.51±3.83 | 43.66±3.30 |

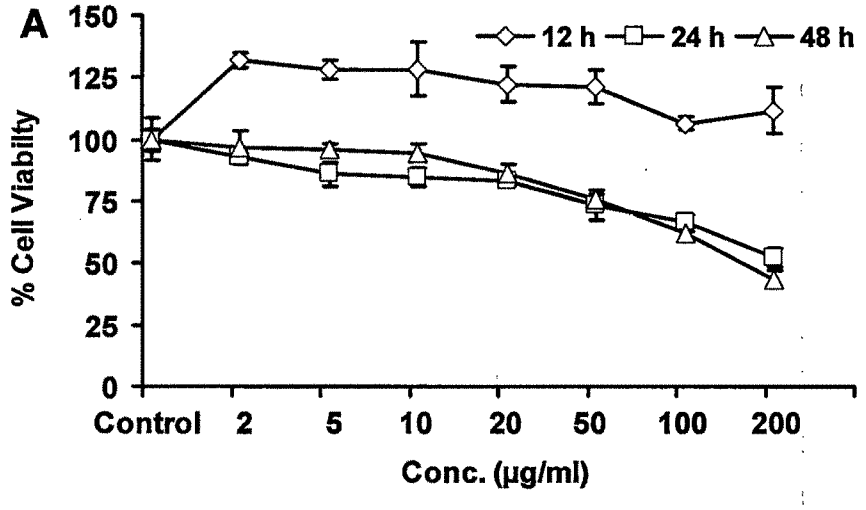



**Fig.5.80. Effect of FORM1 on percentage viability of H9c2 cells at different time interval**

Values are expressed as mean ± SD of % cell viability and are average of three determinations.

**Table.5.132. Effect of FORM2 on percentage viability of H9c2 cells at different time interval**

| B(µg/ml) | 12 h | 24 h | 48 h |
|---|---|---|---|
| V.Control | 100±8.69 | 100±4.49 | 100±1.87 |
| 2 | 99.77±14.84 | 90.21±5.68 | 95.81±2.86 |
| 5 | 94.97±12.4 | 91.13±2.11 | 94.7±2.42 |
| 10 | 97.03±10.50 | 95.1±4.23 | 93.71±2.86 |
| 20 | 107.3±10.95 | 84.92±3.57 | 92.28±1.87 |
| 50 | 103.88±8.67 | 78.83±9.39 | 83.35±2.53 |
| 100 | 92.23±7.76 | 63.22±0.13 | 70.67±2.75 |
| 200 | 85.15±9.13 | 34.92±5.29 | 27.34±6.50 |

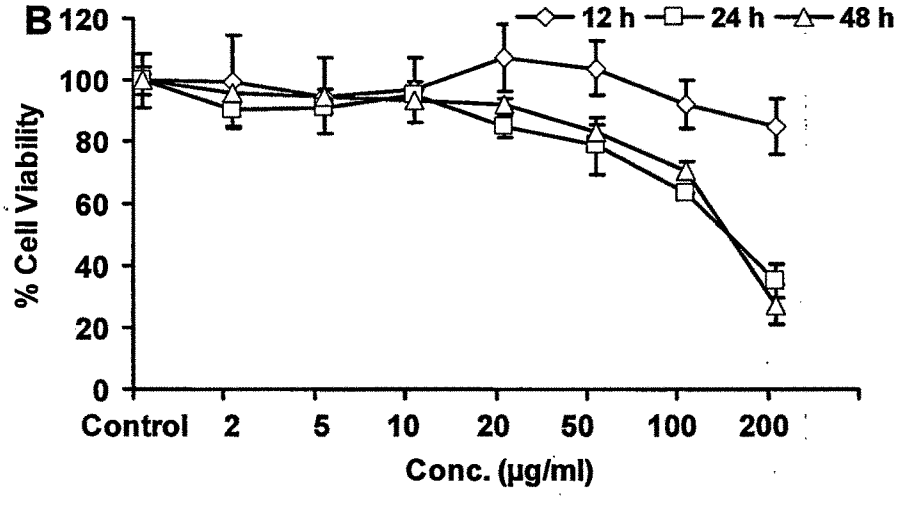



**Fig.5.81. Effect of FORM2 on percentage viability of H9c2 cells at different time interval**

Values are expressed as mean ± SD of % cell viability and are average of three determinations.

**Fig.5.133. Effect of FORM3 on percentage viability of H9c2 cells at different time interval**

| C(µg/ml) | 12 h | 24 h | 48 h |
|---|---|---|---|
| V.Control | 100±8.69 | 100±4.49 | 100±1.87 |
| 2 | 65.29±9.13 | 92.32±7.14 | 100.77±2.42 |
| 5 | 63.92±10.50 | 93.51±5.95 | 92.94±1.89 |
| 10 | 59.58±4.10 | 92.06±5.15 | 90.73±0.99 |
| 20 | 68.03±11.64 | 86.37±1.45 | 86.99±0.77 |
| 50 | 74.65±7.53 | 77.91±5.55 | 75.52±2.86 |
| 100 | 29.45±7.99 | 46.16±3.04 | 41.89±1.76 |
| 200 | 14.15±3.88 | 5.02±0.52 | 3.85±2.09 |

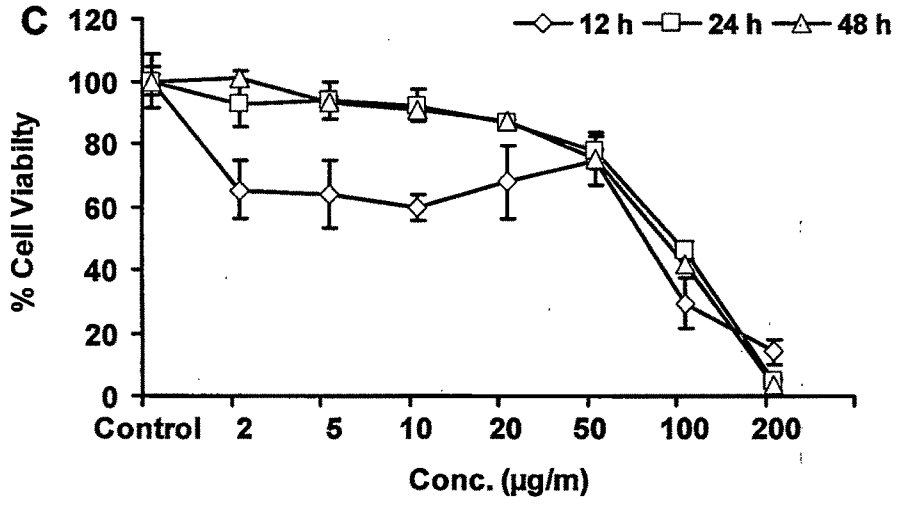



**Fig.5.82. Effect of FORM3 on percentage viability of H9c2 cells at different time interval**

Values are expressed as mean ± SD of % cell viability and are average of three determinations.

### *5.7.3) Effect of FORM1, FORM2 and FORM3 on Isoproterenol induced Myocardial infarction*

FORM1, FORM2 and FORM3 were studied for their effect against ISO induced myocardial infarction in rats at two dose levels (25 mg/kg and 50mg/kg). Group-II injected with ISO (25mg/kg/b.w/s.c) showed increased serum levels of cardiac marker enzymes like CK-MB ($p<0.001$), LDH ($p<0.001$), GOT ($p<0.001$), ALKP ($p<0.001$) and uric acid ($p<0.001$) when compared with Group-I treated with normal saline 2ml/kg/b.w/s.c (Table 5.134).

Group-IV and -V pretreated with FORM1 (25 and 50 mg/kg b.w., respectively) showed significant decrease in the serum levels of CK-MB ($p<0.001$), LDH ($p<0.001$), GOT ($p<0.001$), ALKP ($p<0.01$) and uric acid ($p<0.001$) in a dose dependent manner when compared with Group-II (Table 2).Group-III with FORM1 (50 mg/kg b.w.) alone did not show any significant change in the levels of these marker enzymes when compared with Group-I. (Table 5.134).

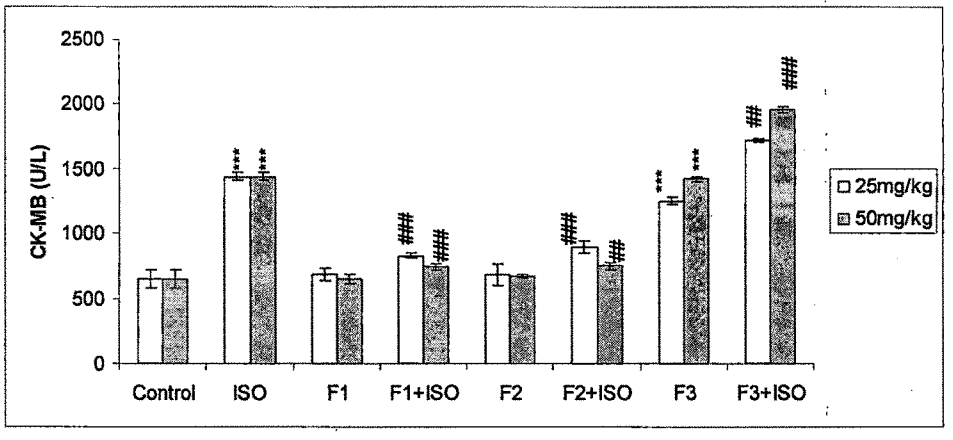
Group-VII and -VIII pretreated with FORM2 (25 and 50 mg/kg b.w., respectively) restored the ISO affected levels of CKMB ($p<0.001$), LDH ($p<0.001$), GOT ($p<0.01$), ALKP ($p<0.001$) and uric acid ($p<0.001$) in a dose dependent manner when compared with Group-II, while Group-VI with FORM2 (50 mg/kg b.w.) alone did not showed any significant change in CK-MB ($p>0.05$), LDH ($p>0.05$), GOT ($p>0.05$), ALKP ($p>0.05$), and uric acid ($p>0.05$) when compared with Group-I (Table 5.134).

Group-X and –XI pretreated with FORM3 (25 and 50 mg/kg b.w., respectively) showed further significant increase in ISO affected levels of CK-MB ($p<0.01$), LDH ($p<0.001$), GOT ($p>0.05$), ALKP ($p<0.001$), and uric acid ($p>0.05$) when compared with Group-II; even Group–IX pretreated with FORM3 (50 mg/kg b.w.) alone showed increased levels of CK-MB ($p<0.001$), LDH ($p<0.001$), GOT ($p<0.001$), ALKP ($p>0.05$), and uric acid ($p<0.01$) when compared with Group-I (Table 5.134).

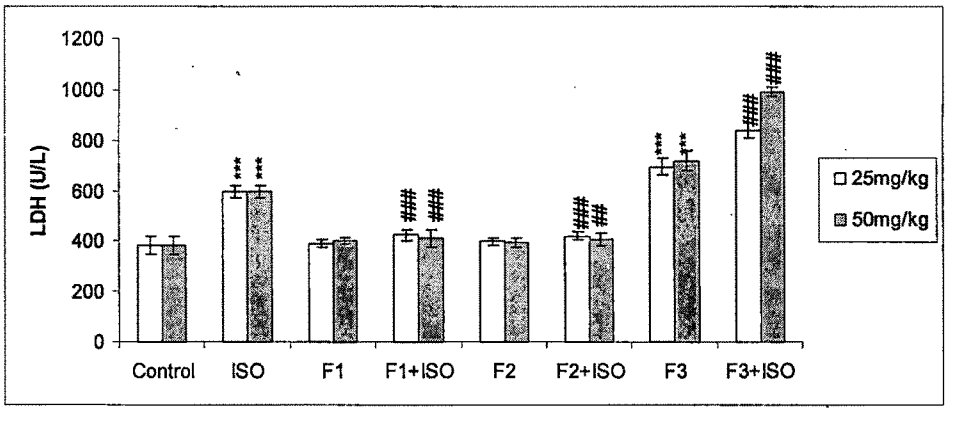
**Table.5.134 . Effect of Formulations on serum cardiac marker enzymes**

| Groups | CKMB† | LDH† | SGOT† | ALKP†† | Uric acid‡ |
|---|---|---|---|---|---|
| **Group-I** | 648.63±68.56 | 383.33±38.98 | 79±5.42 | 276.33±12.86*** | 0.83±0.044 |
| **Group-II** | 1441±27.52*** | 598±22.59*** | 131±2.92*** | 393.67±5.22 | 2±0.049*** |
| **Group-III** | 678.67±46.57 | 392.67±15.67 | 93.18±2.87 | 282.27±17.06 | 0.89±0.073 |
| **Group-IV** | 828.67±16.48### | 426.25±21.34### | 100.35±6.27### | 274.6±18.97### | 1.05±0.018### |
| **Group-V** | 742.12±21.47### | 412.83±32.63### | 92.86±17.89### | 278.86±24.31### | 0.93±0.041### |
| **Group-VI** | 683.13±78.71 | 399±13.13 | 93.24±3.52 | 280.6±11.31 | 1.06±0.031 |
| **Group-VII** | 896.93±48.5### | 423±17.69### | 107.25±2.84## | 280±14.59### | 1.26±0.19### |
| **Group-VIII** | 751.56±27.62## | 408.23±22.81## | 94.29±27.58 | 280.12±13.24## | 0.92±0.012## |
| **Group-IX** | 1255.3±25.16*** | 696.33±31.6*** | 111.18±2.91*** | 287±12.03 | 1.5±0.097** |
| **Group-X** | 1720±12.47## | 840.33±29.94### | 125.87±2.92 | 304±12.77### | 1.84±0.16 |
| **Group-XI** | 1954±24.12### | 992.1±18.92### | 163.23±18.97### | 317.28±36.21 | 2.01±0.015 |

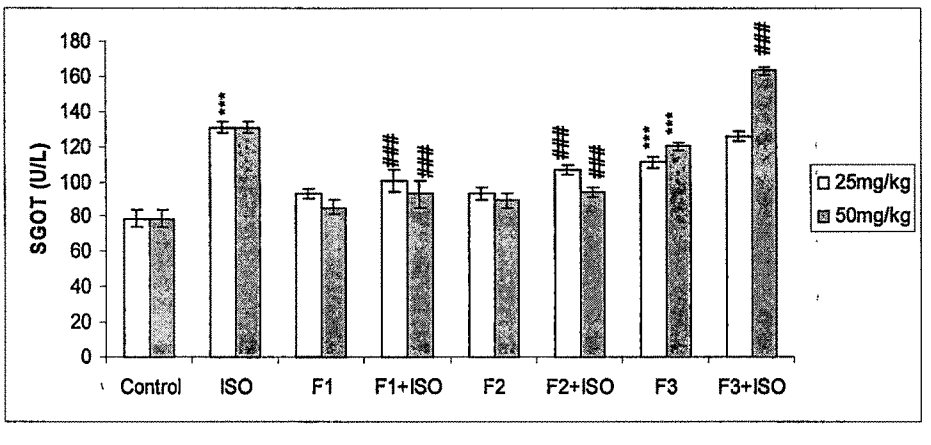
Values are expressed as mean ± SEM; Statistical analysis was done by using One-way Analysis of Variance (ANOVA); Post test applied: Tukey-Kramer multiple comparison test; #Compared with ISO treated group, *Compared with normal group; *p<0.05; **p<0.01; ***p<0.001; p>0.05 = non significant; (n=6); FORM1+ISO vs FORM2+ISO - ns P>0.05; † -U/L; †† -IU/L; ‡ -mg/dL.


CK-MB (U/L)
2500
2000
1500
1000
500
0
Control
ISO
F1
F1+ISO
F2
F2+ISO
F3
F3+ISO
25mg/kg
50mg/kg

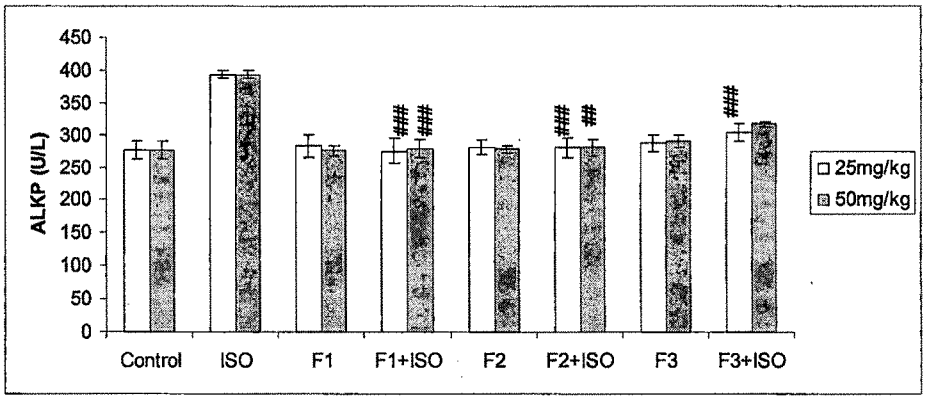
(a)


LDH (U/L)
1200
1000
800
600
400
200
0
Control
ISO
F1
F1+ISO
F2
F2+ISO
F3
F3+ISO
25mg/kg
50mg/kg

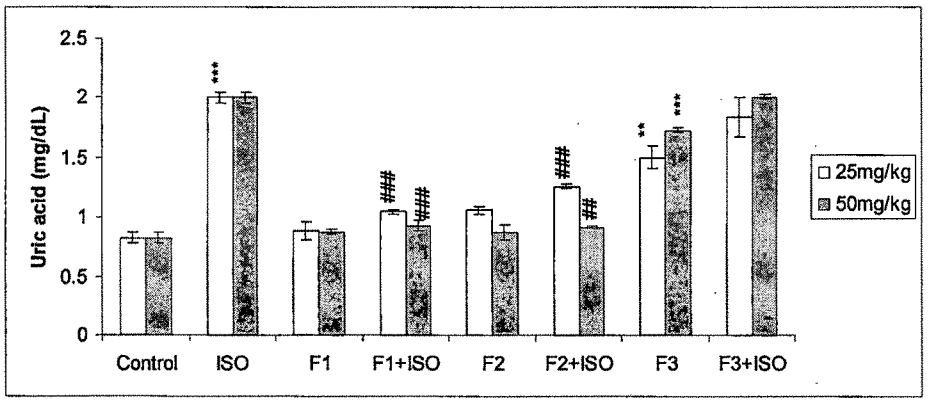
(b)


SGOT (U/L)
180
160
140
120
100
80
60
40
20
0
Control
ISO
F1
F1+ISO
F2
F2+ISO
F3
F3+ISO
25mg/kg
50mg/kg

(c)




(d)




(e)

**Fig. 5.83 Effect of Formulations on (a) CK-MB (b) LDH (c) SGOT (d) ALKP (e) Uric acid.**

Statistical analysis was done by using One-way Analysis of Variance (ANOVA); Post test applied: Tukey-Kramer multiple comparison test; #Compared with ISO treated group, *Compared with normal group; *p<0.05; **p<0.01; ***p<0.001; p>0.05 = non significant; (n=6).

### *5.7.3.1) Effect on Heart weight/ Body weight ratio*

ISO treated rats (Group-II) showed increase in Heart weight /body weight ratio (p<0.01) compared to control rats (Group-I). FORM1 and FORM2 pretreated groups (Group-IV, V, VII, VIII) injected with ISO showed significant reduction in the ratio compared to Group-II in a dose dependent manner, while FORM3 pretreated group showed a significant increase in the ratio of heart weight / body weight compared to Group-I and Group-II (Table 4.135, Fig.5.84).

**Table.5.135. Heart weight to body weight ratio**

| **Groups** | **HW/BW ± SD** | |
|---|---|---|
| **Doses** | **25mg/kg bodyweight** | **50mg/kg bodyweight** |
| Control | 0.00334±0.00016 | 0.00334±0.00016 |
| ISO | 0.0044±0.00012 | 0.0044±0.00012 |
| F1 | 0.00338±0.00010 | 0.00332±0.00009 |
| F1+ISO | 0.003505±0.00018 | 0.00348± 0.00007 |
| F2 | 0.003308±0.00017 | 0.00325±0.00019 |
| F2+ISO | 0.003698±0.00012 | 0.003522±0.00022 |
| F3 | 0.004633±0.0005** | 0.004912 ±0.0005** |
| F3+ISO | 0.005019±0.0003 | 0.005319± 0.0007 |

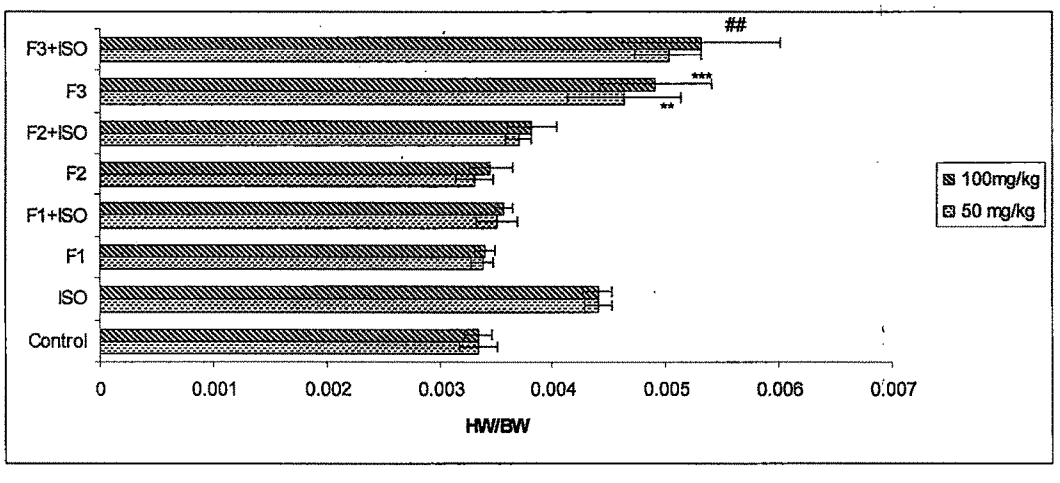
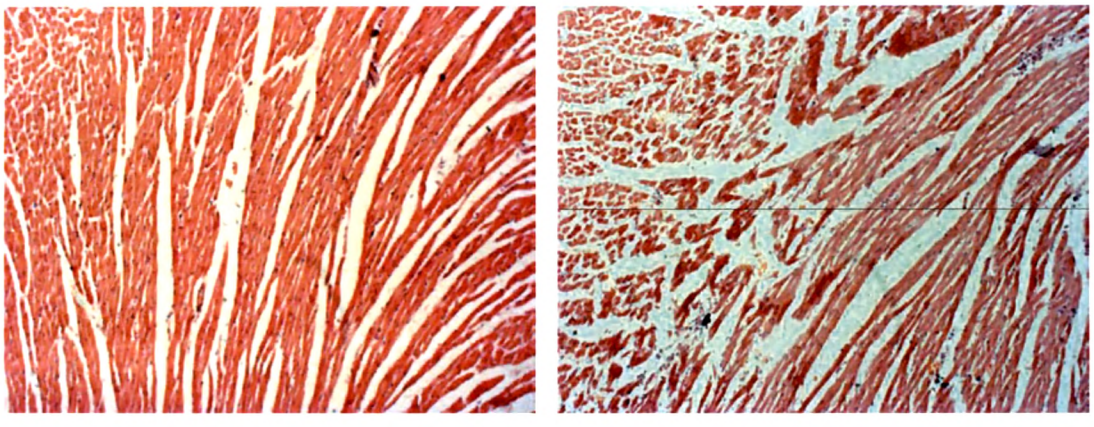



**Fig. 5.84 Effect of Formulations on the Heart weight to body weight ratio.**

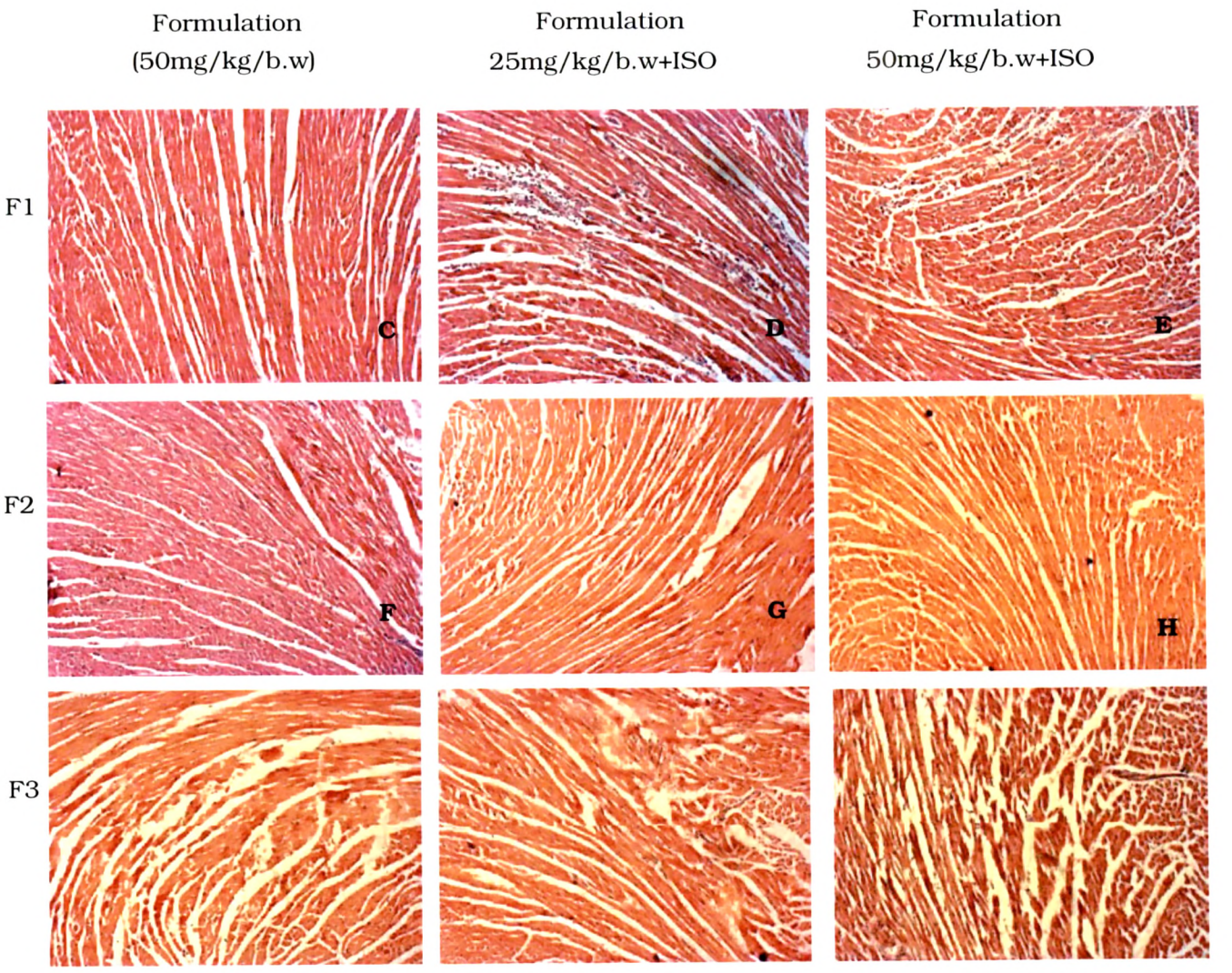
Values are expressed as mean ± SEM; Statistical analysis was done by using One-way Analysis of Variance (ANOVA); Post test applied: Tukey-Kramer multiple comparison test; #Compared with ISO treated group, *Compared with control group; *p<0.05; **p<0.01; ***p<0.001; p>0.05 = non significant; (n=6); FORM2+ISO vs FORM3+ISO -** P<0.01; FORM1-+ISO vs FORM3+ISO- ** P<0.01

### *5.7.3.2) Histopathological studies*



**(A)** **(B)**

**Fig.5.85. Microscopic images of rat heart illustrating effect of pretreatment if FORM1, FORM2 and FORM3 in ISO induced and MI rats** (Staining: Haematoxylin and Eosin). Microscopic images of rat heart from Group-I (A), Group-II (B), Group-III (C), Group-IV (D), Group-V (E), Group-VI (F), Group-VII (G), Group-VIII (H), Group- IX (I), Group-X (J), Group-XI (K).


Formulation
(50mg/kg/b.w)
Formulation
25mg/kg/b.w+ISO
Formulation
50mg/kg/b.w+ISO
F1
F2
F3
C
D
E
F
G
H

Cardiac histopathology of the control group (Fig.2.85A) revealed normal architecture of the myocardium, with intact muscle fibres.

While, the ISO administered heart tissue (Fig.2.85B) showed damage in the cardiac muscles with hyalinization of muscle fibres and focal cellular infiltration or necrosis of muscle fibre. Collection of inflammatory exudate is seen in the focal cells along with atrophy of the remaining normal muscle and also elongation, undulation of the fibers and formation of contractile band lesions characteristic of the pre-infarction stage. It also revealed that there were focal loss of cardiac myofibrils, and cytoplasmic vacuolization in ISO treated rats.

The heart tissue of FORM1 and FORM2 pretreated animals (Fig.2.85C, F) showed no changes in cardiac structure and were similar to that of the control group. There was minimal damage, with mild swelling of muscle cells and focal cardiac muscle fibres. This indicated that both FORM1 and FORM2 were free of toxicity. Further, co-administration of isoproterenol and FORM1 and FORM2 (Fig.2.85D,E,G,H) reduced the damage of cardiac muscle induced by isoproterenol. Pretreatment of rats with FORM1 and FORM2 for 15 days almost completely protected the isoproterenol-induced cardiac muscle damage with little necrotic areas. The FORM1+ISO and FORM2+ISO treated rats showed minimal myocardial degeneration and were completely devoid of any cardiotoxicity. Slight histopathological differences (oedema, vacuoles, myofibrillar disarray, and hemorrhage) were observed between the FORM1+ISO treated FORM2+ISO groups in the hearts on staining. The rats pretreated with FORM1 and FORM2 reversed these changes and showed significant protection against ISO induced myocardial infarction in a dose dependent manner.

Conspicuous damage of the myocardium was present in all FORM3 treated animals. Strips of intensely eosinophilic cells and rather large groups of necrotic cells accompanied by mild mononuclear infiltrate were observed. Myocardial necrosis was located with large areas of necrotic myofibers. Fibrosis and infiltration of mononuclear inflammatory cells was also observed. The FORM3+ISO treated rats also showed acute myocardial necrosis and consisted of variably sized areas of contracted and/or fragmented myofibers with inflammatory infiltrate (Fig. 2.85J, K).

Thus FORM1 and FORM2 pretreatment retains near normal architecture of the myocardium when compared with isoproterenol administered group. While FORM3 is toxic itself and plays no role as a cardio-active.

### *5.7.4) Discussion*

*Mahamrutyunjaya rasa* is a herbo-mineral formulation with a large number of components which have to be purified by *Shodhana* and then incorporated in the formulation. The ingredients may prove to be toxic if not prepared according to the standard text. Further, due to lack of investigations on the toxicity profile and no evidences supporting the pharmacological activity, various *in vitro* and *in vivo* studies were performed to provide an important standardization tool for this formulation.

The data indicates that FORM1, the formulation prepared in the laboratory as per the standard text showed its effect in concentration and time dependent manner on the viability of rat embryonic cardiac cells (H9c2). As both the concentration and time of incubation increased, cell viability was found to be decreased. The results obtained for FORM1 provides a lead for dose fixation for further studies carried out. The formulation was found to be safe at 550mg/kg without any signs of toxic reaction as seen in the histopathological study.

The data for FORM2 formulation indicates that the formulation had similar effect as that of FORM1. Thus, FORM2 showed a concentration dependent and time dependent effect on the cell viability.

FORM3 formulation showed significant toxicity in *in vitro* and *in vivo* studies. The cell viability assay results indicate that FORM3 is highly toxic even at low doses when incubated for 12 h. With the increase in concentration and time of incubation, significant decrease in cell viability was observed. The cell viability studies thus revealed that FORM3 is highly toxic in minute quantities. Further, the acute toxicity studies showed mortality at 2000mg/kg and at the dose of 550 mg/kg changes in the architecture of cellular arrangement of heart, liver and kidney was observed, while no difference in the histopathology of the FORM1 and FORM2 groups was observed in comparison to the control group.

*In vivo* studies are carried out on the selected formulations using ISO induced myocardial infarction in rats. Myocardial necrosis induced by ISO is probably

due to a primary action on the sarcolemmal membrane, followed by stimulation of adenylate cyclase, activation of $Ca^{2+}$ and $Na^{+}$ channels, exaggerated $Ca^{2+}$ inflow, excess of excitation-contraction coupling mechanism, energy consumption and cellular death. Increased serum levels of CKMB, LDH, ALKP and GOT are the diagnostic indicators of myocardial infarction. An increase in the activity of these enzymes in serum is due to their leakage from heart as a result of necrosis induced by ISO. Increase in serum uric acid could be due to excessive degradation of purine nucleotides and proteolysis. Thus, the protective effects of formulations were assessed by analyzing the levels of serum marker enzymes like GOT, ALKP, CK-MB, LDH, and uric acid.

In the above study, isoproterenol treatment resulted in a marked elevation of CK-MB level. Pretreatment of MI rats with FORM1 and FORM2 prevented the maximum increase of the enzyme during the peak infarction in the tissues. Moreover, the activities of other cardio specific enzyme markers like LDH, GOT and ALKP in the serum were also found to be reduced in the FORM1 and FORM2 pretreated MI rats.