CHAPTER 2

ISOLATION, PURIFICATION AND CHARACTERISATION OF RESERPINE FROM ROOT OF *Rauvolfia serpentina* WITH SPECIAL INTEREST ON THE ESTABLISHMENT OF AN EASY AND RAPID COLORIMETRIC METHOD FOR ITS QUANTITATIVE ESTIMATION

INTRODUCTION

The dried root of *Rauvolfia serpentina* commonly known as serpentina root, in Sanskrit as sarpagandha and in Hindi chandrabhaga is one of the most important drugs used in modern medicine. Once it was a commonly accruing local plants in the plains of Darjeeling District but due to ruthless collection from the natural habitat it has faced endangered condition. This plant is commercial source of reserpine, an indole alkaloid, which is one of the most important therapeutically useful drug.

The importance of Rauvolfia serpentina in modern medicine was recognised in 1952 when Muller succeeded in isolating pharmacodynamic principle reserpine in the roots of the plant which revolutionized the therapeutic use of the drug as antihypertension and sedative. More than 20 different indole alkaloids have been isolated so far from this plant and most of the alkaloids are confined to the roots of the plant. The total alkaloid content in the root range from 1.7 to 3.0 percent which are mostly concentrated in the bark (about 90%). Alkaloids are also present in leaves, stem and seeds but not in significant amount as compared to roots. Siddiqui and Siddiqui (1931, 1935) isolated reserpine and other indole alkaloids at the very beginning when the importance of Rauvolfia

serpentine was known. They were the pioneer workers in this line of investigation, but they followed cumbrous method. They collected different solvent parts following the method of fractional separation. Each fraction contained a mixture of alkaloids and the modern method of chromatography was not used to separate pure alkaloids very easily.

Besides most of the methodologies so far recorded in connection with the quantitative estimation of reserpine have been observed to involve costly instruments, which are generally not available in most of the laboratory. More over all these involved cumbrous process without the consideration of purification of reserpine.

Thus an attempt has been made to work out an easy and rapid colorimetric method for quantitative determination of reserpine involving the newly developed methodology for purification of the natural product.

MATERIALS AND METHODS

MATERIALS

Root bark of *Rauvolfia serpentina* was collected from Sukna, Mirik area.

METHODS

Extraction : 500 gms of dry powdered bark of *Rauvolfia serpentina* was extracted using soxhlet apparatus with chloroform for 5 hours per day and continued for 3 days. The extracted chloroform part was separated and evaporated to dryness.

Purification under column chromatography: A glass column was packed with first absorbent cotton plug then alumina (Al_2O_3) in wet condition in

petroleum ether. The concentrated mass of chloroform extract was absorbed. Various following solvents and their mixtures were eluted one after another as represented below:

Solvents/ solvent mixtures	**Ratio**
Petroleum ether	Pure
Petroleum ether : Benzene	(3:1)
Petroleum ether: Benzene	(1:1)
Petroleum ether: Benzene	(1:3)
Benzene	Pure
Benzene: Chloroform	(3:1)
Benzene: Chloroform	(1:1)
Benzene: Chloroform	(1:3)
Chloroform	Pure
Chloroform: MeOH	(3:1)
Chloroform: MeOH	(1:1)
Chloroform: MeOH	(1:3)
MeOH	Pure

Various fractions after elution were collected in a conical flask and evaporated to dryness on electrically operated hot plate. Various solid materials obtained after elution were subjected to paper chromatography for identification of chemicals.

Identification of chemicals by paper chromatography: A piece of chromatography paper (Whatman no. 1) was taken and concentrated mass of

chemicals dissolved in chloroform was spotted on the paper along with the authentic marker and ran under different solvents or their mixtures in ascending type of chromatography. After running of chromatography for desirable time when solvent front has run for a considerable length. The paper was taken out and dried under hot air woven and it was kept in iodine chamber so the area containing chemicals became dark in colour and then Rf was calculated.

Determination of melting point: Fine crystals were taken in a capillary tube blunt on one end it was tapped so that the crystals accumulated at the bottom of the capillary tube and melting point was determined using electrically operated melting point apparatus.

Detection of alkaloid under UV light: The paper containing the Rauwolfia alkaloids were exposed under UV light in a dark room. The spots containing Rauwolfia alkaloid was detected by green fluorescence.

Identification and characterisation of alkaloid by IR spectrum: The isolated chemical was identified and characterised to be reserpine after comparing the absorption peak in IR spectrum of isolated product with those of authentic sample.

Preparation of stock solution of reserpine: Authentic reserpine (Sigma company) of 10 mg was dissolved in 0.5 ml of chloroform and acetic anhydride was taken to make it a volume of 10 ml to make 1000 ppm of stock solution.

Preparation of different grades of stock solution: From 1000 ppm of stock solution different grades of 900, 800, 700, 600, 500, 400, 300, 200, and 100 ppm were prepared by dilution with acetic anhydride.

Preparation of reaction mixture: 1 ml of reserpine solution in acetic anhydride was mixed with 1 ml of concentrated sulphuric acid (H_2SO_4). It was shaken properly and kept for 10 minutes and then brown colour appeared.

Determination of absorption maxima of the reaction: The O. D. value of the reaction mexture was measured at different wave lengths starting from 400–800 nm. The values were taken with the help of spectrocolorimeter (C. Z. instrument) and plotted on graph paper and the absorption maxima was derermined. Maximum absorption was at 620 nm.

Determination of standard curve of reserpine solution: To each of 1 ml of reserpine solution from 1000 ppm to 100 ppm, 1 ml of concentrated sulphuric acid (H_2SO_4) was added and optical density for each of the reaction mixture was determined with the help of spectrocolorimeter of 607 nm.

Isolation and purification of reserpine obtained from *R. serpentina* root by paper chromatography method: 500 mg of the root of *R. serpentina* was crushed into powder and was subjected to chloroform extract under reflux condition. The extraction was continued for 20 minutes and then filtered. The chloroform was evaporated to 0.5 ml of the extract. The crude extract was taken in a pipette and was subjected to paper (Whatman N 1) in a streak. The paper was run first in petroleum ether and then in pure methanol to obtain pure reserpine on chromatographic paper and its scheduled Rf. The paper was dried and then reserpine zone on the paper was cut into pieces. The pieces of paper were extracted with boiling chloroform and was filtered. The chloroform extract was evaporated to 0.5 ml. Acetic anhydride was added to make the total volume of 1 ml. 1 ml concentrated sulphuric acid (H_2SO_4) was added and O. D. value was determined at 807 nm. The percentage of reserpine was determined by dry weight basis.

RESULT AND DISCUSSION

The dried root of *Rauvolfia serpentina* has been used in indegenous system in medicine from ancient times in India. However its importance in modern medicine was recognised in 1952 only after the isolation of a pharmacodynamic

principle, reserpine in the roots of the plant which revolutionized the therapeutic use of the drug as antihypertensive and sedative. Since then a number of alkaloids have been isolated from the roots of the plant. the synthesis of reserpine in 1956 followed as a natural corollary to this necessity. But even after the marketing of the synthetic reserpine the natural product hold its place due to the lower price.

As reserpine is a secondary product its productivity is expected to vary in plants subjected to various treatments and conditions. Thus during investigation the quantitative estimation of reserpine in a large number of samples is very much needed.

Numerous methods have been reported for the quantitative estimation of reserpine after using UV spectrophotometry (Nguyen *et al.*, 1989), HPLC (Cieri, 1983), LC (Cieri *et al.*, 1987) fluorescence analysis (Balon-Almda *et al.*, 1986) and radio immunoassay (Arens *et al.*, 1978). But all these methods are cumbersome and sometimes involve costly instruments not available in all the laboratories. No colorimetric method has so far been established for quantitative estimation of reserpine. Thus an attempt has been made to work out an easy and rapid colorimetric method for the quantitative estimation of the natural product in pure condition.

Previously Siddiqui and Siddiqui (1931, 1935) isolated reserpine following the method of fractional separation and for obvious reason the fraction contained a mixture of alkaloids. The modern method of chromatography was not used during isolation of reserpine. It has been observed that during quantitative estimation of reserpine most of the authors did not apply chromatography for its purification. Here in this part of the work column chromatography and paper chromatography have been applied for isolation of pure reserpine on large and microscale respectively. During column chromatography a glass column was packed with alumina (Al_2O_3) in wet condition in petroleum ether. The chloroform extract of root of *R. serpentina* was concentrated and adsorbed on alumina. Different solvent

and their mixtures used during column chromatography have been shown in Table 2. Crystals of reserpine were obtained after elution with benzene : chloroform, 3:1 (Table 2). The pure reserpine was isolated very easily after recrystallisation from chloroform-methanol mixture. The isolated product showed m.p. 262°C similar to that of authentic reserpine. Moreover, the isolated product was confirmed to be reserpine after comparing the IR spectra of both the isolated product and reserpine. The absorption peaks (λ max) at 3370 (NH), 1690 (C=O), 1600, 1570 (aromatic C=C), 1440 (CH_2), 1400, 1360, 1320 (C–N) 1260, 1230, 1210, 1100 (C–O), 1050, 1020, 990, 790, 760 (o-substituted benzene + indole aromatic ring) and 730 cm^{-1} of the isolated product were observed to be similar to those of authentic reserpine (Fig. 2).

Table 2 : Purification of crude extract of root bark of *R. serpentina* by column chromatography.

Solvent and their mixtures	**Ratio**	**Fraction number**	**Residue obtained after evaporation**
Petroleum ether	Pure	1 – 5	Oil
Petroleum ether:Benzene	3:1	6 – 10	Oil
Petroleum ether: Benzene	1:1	11 – 15	Oil
Petroleum ether: Benzene	1:3	16 – 20	No residue
Benzene	Pure	21 – 25	No residue
Benzene. : Chloroform	3:1	26 – 30	Crystal
Benzene. : Chloroform	1:1	31 – 35	No crystal(residue)
Benzene. : Chloroform	1:3	36 – 40	No residue
Chloroform	Pure	41 – 45	No residue
Methanol	Pure	46 – 50	No residue

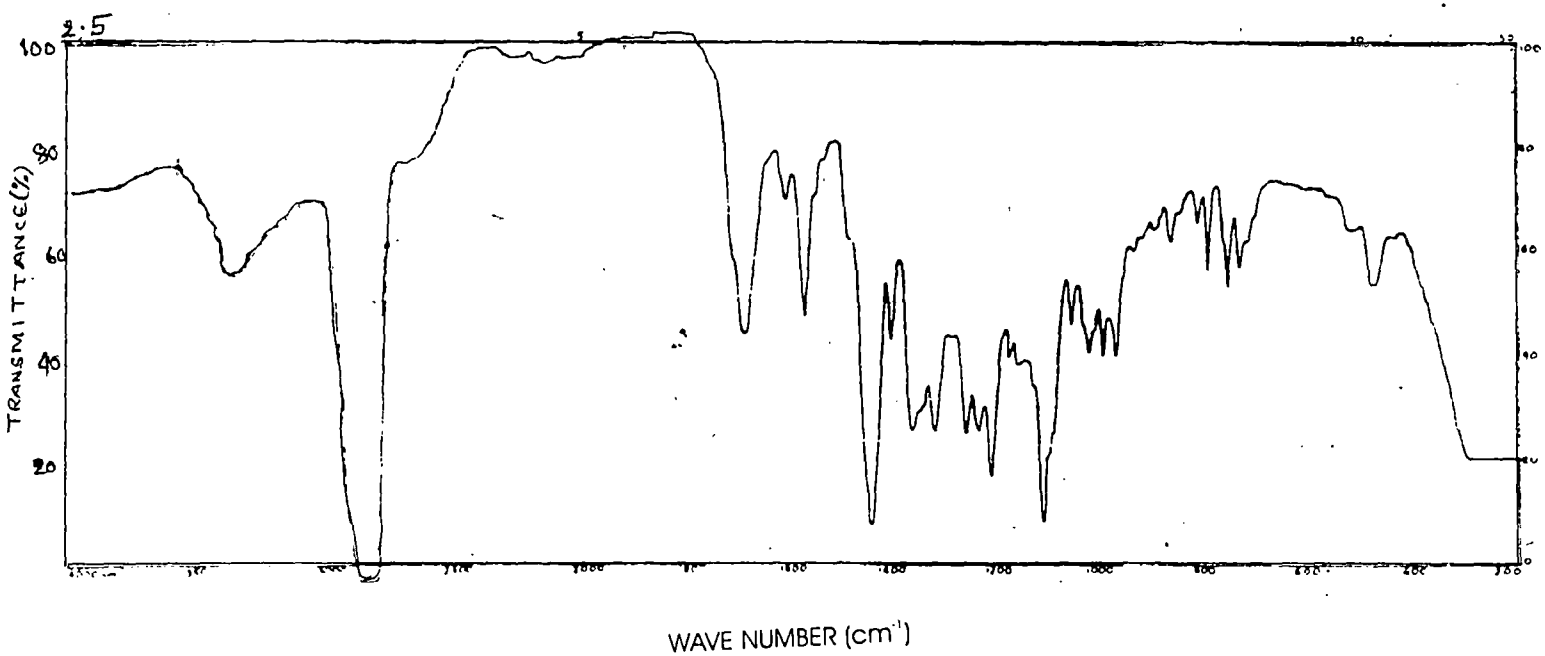

Fig. 2 IR spectrum of reserpine

Table 3 shows the Rf values of the natural product isolated from root bark of *R. serpentina* and which are similar to those of authentic reserpine in the same solvent or solvent mixtures during paper chromatography. These informations will be of much help during purification of extract containing reserpine on microscale by paper chromatography. In order to isolate pure reserpine the paper bearing reserpine was dried and the zone of paper bearing natural product corresponding to Rf of authentic reserpine was taken out and cut into pieces. These were subjected to hot chloroform treatment, filtered and chloroform was evaporated to obtain solid mass of reserpine which was used for its quantitative estimation with the help of new method proposed in this part of work.

The methodology was based on the principle that the reaction mixture containing reserpine, acetic anhydride and conc. H_2SO_4 shows uniform brown colour.

The colour complex was observed to show absorption maxima at 620 nm (Fig. 3) and the colour became stable after standing the mixture, for 15 to 20 minutes and continued to last for one hour. The reserpine content was calculated from the prepared standard curve ranging from 100 ppm to 1000 ppm and which was observed to obey Beer's Law (Fig. 4).

The proposed method is supposed to be advantageous because of the fact that reserpine content can be determined from a low concentration of 100 ppm solution. Moreover with this method only a small amount of dried plant issue was observed to be sufficient for estimation of reserpine content within a very short time.

The proposed method may be treated as first time to report in the field of quantitative estimation involved in pharmacognosy. It is claimed to be an easy method because it is very easy to separate reserpine from the crude extract by paper chromatography and the determination could be done with the help of colorimeter which is easily available in all the laboratories.

Table 3 : Rf value of authentic reserpine and unknown sample isolated from root bark of *R. serpentna* during paper chromatography in different solvents/solvent mixtures.

Reserpine/unknown compound	**Solvent used**	**Ratio v/v**	**Rf**
Reserpine	Petroleum ether	Pure	0.00
Reserpine	Petroleum ether:EtOH	9 : 1	0.19
Reserpine	Petroleum ether:EtOH	1 : 1	0.92
Reserpine	Benzene	Pure	0.40
Reserpine	Chloroform.:MeOH	1 : 9	0.92
Reserpine	Methanol	Pure	0.58
Reserpine	EtOH	Pure	0.675
Unknown sample	Petroleum ether	Pure	0.00
Unknown sample	Petroleum ether:EtOH	9 : 1	0.19
Unknown sample	Petroleum ether:EtOH	1 : 1	0.92
Unknown sample	Benzene	Pure	0.45
Unknown sample	Chloroform:MeOH	1 : 9	0.90
Unknown sample	Methanol	Pure	0.58
Unknown sample	EtOH	Pure	0.68

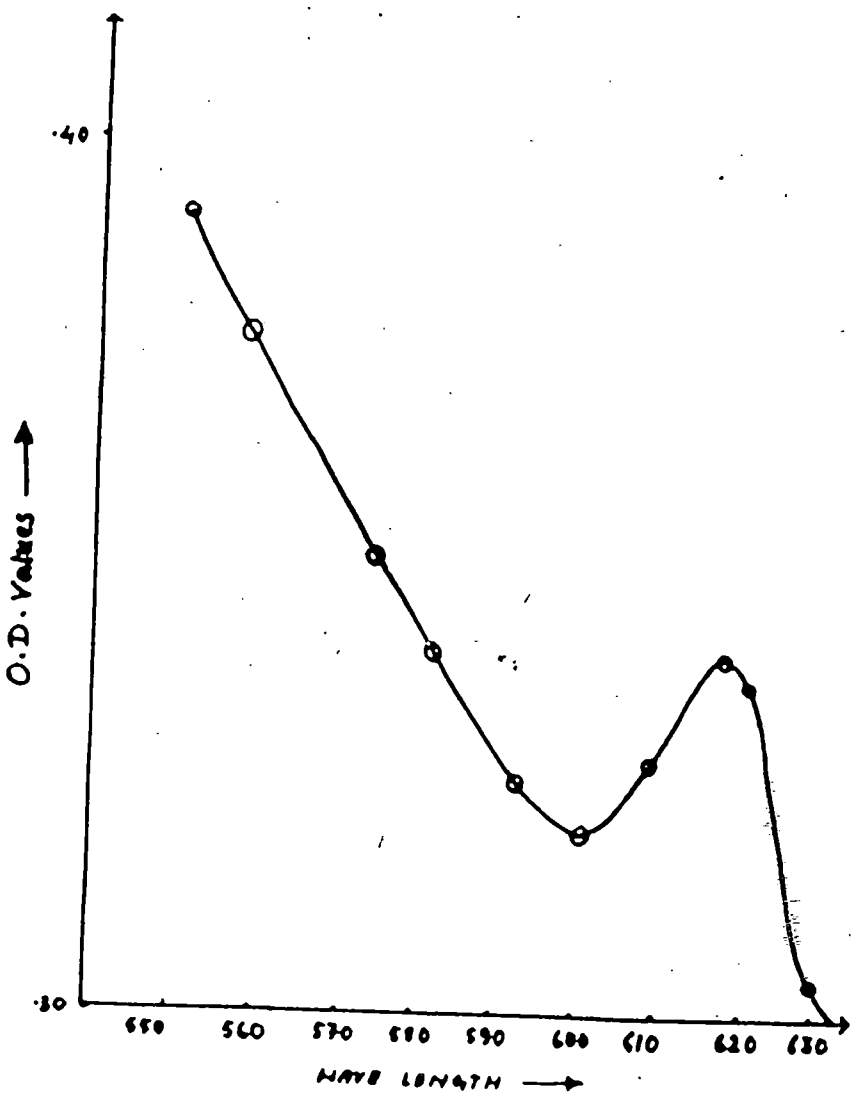

Fig. 3 Absorption maxima of reserpine

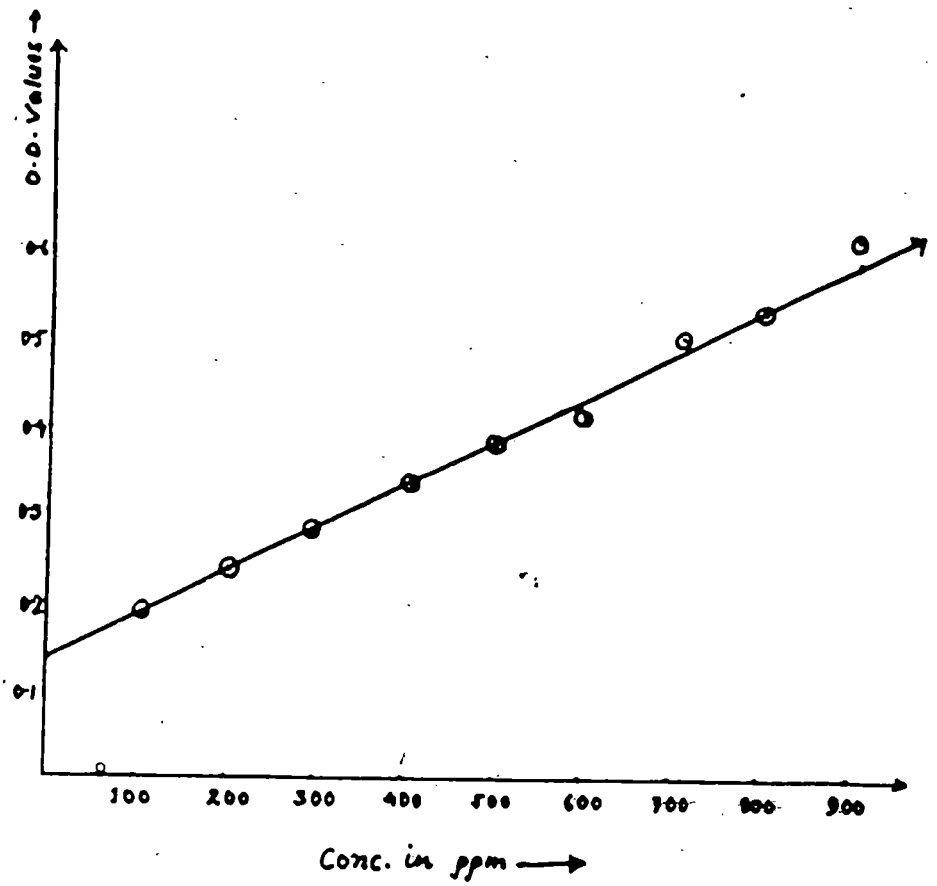

Fig. 4 Standard curve of reserpine

SUMMARY

Root barks of *Rauvolfia serpentina* were collected from different places of Darjeeling District. They were cut into pieces, sun dried and made powder. 500 g of powder was subjected to chloroform extraction with the help of soxhlet apparatus.

The chloroform extract after filtration was concentrated to small volume and subjected to column chromatography taking alumina as adsorbent. The column was eluted with different solvents such as petroleum ether, benzene, chloroform, methanol and their mixtures. Appeciable amount of crystals was obtained after eluting with benzene : chloroform (3:1) and recrystallised from chloroform-methanol mixture. The isolated product was identified to be reserpine having m.p. 262°C and after comparing absorption peaks in IR of isolated product with those of authentic reserpine.

Rfs of isolated product and reserpine in different solvent mixtures during paper chromatography were determined. For quantitative determination of reserpine, a reaction mixture was produced after mixing 1 ml of reserpine solution and 1 ml of concentrated sulphuric acid. It was shaken to produce uniform brown colour.

Absorption maxima of the reaction mixture was determined to be 620 nm.

Standard curve of reserpine from 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm was observed to obey Beer's law.

The brown colour of the reaction mixture attained stability after 15 to 20 minutes and continued to become stable for one hour.