Chapter 7.

SUMMARY

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Ayurveda is accepted to be the oldest treatises in medicinal system, which came into existence in about 900 B.C. Generally Ayurvedic formulations are multi-component mixtures, containing plant and animal-derived products, minerals and metals.

Quality control of traditional medicines is a critical and essential issue to be considered in assuring the therapeutic efficacy, safety and to rationalize their use in health care. Quality assurance is an integral part of traditional medicine, which ensures that it delivers the required quantity of quality medicament. In former days these traditional medicinal formulations were prepared by *Vaidyas* and delivered to the patients in the fresh form where quality assurance was not needed in the present way.

To establish the potentiality of Ayurvedic medicine, research needs to be conducted based on the following aspects:

- To standardize materials, methods and measures for preparation, and preservation of Ayurvedic drugs.
- To provide proper scientific validation and significance to the fundamental principles of the system.
- To rationalize the utility of positive and judicious use of modern scientific methods that pertains to the development of Ayurveda.

In order to assess the quality of herbal products, WHO has focused the development of methods towards safety, efficacy, and quality. The safety and efficacy are mainly dependent on presence and assessment of biological marker and toxicity profile, whereas, quality comprises of the assessment of the labeled ingredients in the formulation, for which analytical methods are to be developed.

Ayurvedic treatments have been developed for various diseases e.g. diabetes, neurological disorders, and cardiovascular *(Hrudroga)* conditions. 'Cardiovascular Disease' is now becoming a major health problem even in the developing countries. *Ayurveda* offers a number of formulations that are therapeutically active against the CVS disorders.

Therefore, the present studies were conceived to develop standardization tools to study the safety, quality and efficacy of *Mahamrutyunjaya rasa.* It is a

compound herbo-mineral cardioactive ayurvedic formulation. It comprises of four kinds of herbs: *Aconitum ferox, Solanum indicum, Piper nigrum* and *Piper longum* and three minerals: *Cinnabar, Sulphur* and *Sodium metaborate.* Ayurvedic literature records the formula of *Mahamrutyunjaya rasa* as: 1 part each of powdered processed *Aconitum ferox, Solanum indicum , Piper nigrum* and *Piper longum,* sieved and then mixed with 1 part each of purified sulphur and purified sodium metaborate. To this mixture 2 parts of purified cinnabar is added and mixed uniformly. Aconitine, a diester-diterpene alkaloid is the marker of *Aconitum ferox,* solanine, a glyco-alkaloid is the marker compound of *Solanum indicum* and piperine is the marker compound of *Piper nigrum* and *Piper longum.*

Therefore, the present investigations were undertaken to develop certain methods for the standardization of *Mahamrutyunjaya rasa* in context with these marker and mineral components.

The plant material except the roots of *Solanum indicum were* purchased from a local store and *Solanum indicum* was collected from the local area of Baroda, Gujarat and authenticated in the Botany department, of The M.S. University of Baroda.

The collected plant materials were dried and powdered. The plant materials were studied as per the WHO guidelines. The macroscopical and microscopical examination of the crude intact drugs and powdered drugs was done. The histological characters of the roots of *A. ferox* and *S. indicum* was studied with reference to the standard monographs and recorded. The determination of ash values, extractive values, moisture content, volatile matter, pesticide residues, heavy metals, microbial content was also performed. All the parameters were found to comply with the limits prescribed in the WHO guidelines.

The physically standardized plant materials were extracted in petroleum ether, methanol and water. These extracts were then subjected to preliminary phytochemical analysis using chemical tests and TLC studies which revealed the presence of alkaloids, steroids, carbohydrates, saponins, phenolics and amino acids.

Aconitine and Piperine were isolated from the standardized plant materials using reported methods and characterized by TLC studies and melting point.

The standardization of *Mahamrutyunjaya rasa* was performed on three formulations from different manufacturers. Two proprietary formulations from Baidyanath Pvt. Ltd. (FORM2) and Pune Rasashala (FORM3) were studied. These formulations were compared with the laboratory prepared formulation (FORM1). The formulation in the laboratory was prepared using the standardized plant materials as per the traditional method.

The procedure of the formulation preparation was standardized by studying the purification techniques of the ingredients as per the standard procedures. Aconite roots, cinnabar, sulphur and sodium metaborate were purified before incorporation in the ayurvedic formulations. Samples were collected at different steps of the purification procedure and analyzed using XRD, DSC, HPTLC and IR.

The Aconite roots were processed with cow urine and washed with cow milk and hot water. The alkaloidal fractions of the roots were subjected to HPTLC and IR studies. The physicochemical analysis demonstrated that this processing helps in conversion of the toxic alkaloids to other innocuous alkaloids. The HPTLC studies showed a clear change in the concentration of various alkaloids. The IR studies also depicted the loss of a C=O group and C-O group. This change may be attributed to the loss of an ester group from the diester alkaloids which has been reported in earlier studies. The diester alkaloids are prone to hydrolysis in alkaline conditions. However, due to the presence of a large number of alkaloids in the roots of aconite, the exact physicochemical changes cannot be predicted. It has been reported that the monoester alkaloids have similar pharmacological activity with lower toxicity as compared to the diester alkaloids. The toxic effects of the diester alkaloids is presumably due to the induction of arrhythmias by increase in the ectopic impulse formation, making representative triggered activities due to early as well as delayed after-depolarization. The monoester alkaloids have a slow increase in the ectopic impulse formation. Therefore, it becomes important to monitor the purification procedures. Thus, for the quality

control of aconite roots as the raw materials, HPTLC finger prints and IR spectra can be used as a standard.

Sulphur was processed with cow ghee, melted and washed with cow milk and hot water. Samples were collected at different steps and subjected to XRD and DSC studies in order to study the physicochemical changes. Sulpur was found to be in the orthorhombic crystalline form with small amounts of monoclinc crystalline forms, displayed by two sharp endothermic peaks in the differential thermogram. The physicochemical changes were observed which may be due to the purification of sulphur. Sulphur in physically and chemically pure form may produce adverse effects because they are heterogeneous to the body tissue. 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 XRD and DSC patterns of the final product displayed that the structure reverts back to the S_8 orthorhombic sulphur. Thus, from the above study it can be concluded that the processing of sulphur using the traditional methods brings about purification of sulphur by ghee, reducing the toxic nature of sulphur. It can also be concluded that the unwanted components are reduced which may be observed by the intensity of the peaks in the X-ray diffraction pattern of the final product. Thus, it can be concluded that the processing of sulphur using the traditional methods brings about purification of sulphur, reducing the toxic nature of sulphur. Further, the unwanted components are reduced in the final product. Thus the XRD and DSC data may be used as a standard reference for the quality control of the raw materials.

The *Shodhana* of cinnabar was done using lemon juice. The juice was replaced seven times by washing it each time with hot water. The samples were collected at different intervals and subjected to XRD studies inorder to understand the changes in the crystal structure. The d-spacing values of the final product matched with the reference data showing high purity of cinnabar in the trigonal trapezohedral cystalline form. The purification of cinnabar may be done in order to remove all the extraneous material so that mercury has the desired therapeutic effect without any side effects due to increased bioavailability. 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.
Thus, for the quality control of cinnabar as raw materials, the XRD pattern can be used as a standard.
The crude samples of sodium metaborate were heated until constant weight was achieved. The samples were collected at different intervals and subjected to XRD and DSC studies. The XRD spectra of sodium metaborate depicted that there was a gradual purification of sodium metaborate. A gradual decrease in the number of peaks was observed with decrease in the sharpness of the peaks. The differential thermograms also showed an extra peak at 74.71 °C in the raw material which disappeared in the final product which may be due to the loss of a hydrate group. Further, the endothermic peak at 137 °C became sharper with every sample (even when lesser amount of samples were analyzed) clearly showing the purification of the raw material. Thus, for the quality control of sodium metaborate as raw material, the XRD pattern and DSC thermograms can be used as a standard.

The physical standardization of the formulations was done and compared by the methods prescribed in the pharmacopoeia. The uniformity of weight, disintegration test, hardness, diameter, thickness, friability, ash values, extractive values, pesticide residues, heavy metal analysis, microbial contamination studies were performed. Differences in the physical properties of the marketed formulations was observed. FORM2 was found to be very hard with long disintegration time, whereas FORM3 had higher friability. Presence of organochlorinated pesticides was observed in the FORM2. Presence of mercury was also found in heavy metal analysis due to the cinnabar in the formulation. All the other results were within limits and complied with the standard limits.
The formulations were extracted in methanol. The extracts were then subjected to preliminary phytochemical analysis using chemical tests and TLC studies

which revealed the presence of alkaloids, steroids, saponins, volatile oils and phenolics in the extracts of all three formulations.

The chemical standardization of the formulations were performed by developing new analytical methods for the determination of the marker compounds and the minerals in the three formulations. Different analytical methods were developed depending on the number of markers and instruments available. Various techniques like HPLC, HPLTC, Spectroflourimetry, Spectrophotometry and Voltammetry. All the methods were validated with respect to parameters including Linearity, Precision, Accuracy, Limit of Detection (LOD), Limit of Quantification (LOQ), System suitability and Robustness.

The chemical marker aconitine was used for the standardization of Aconitum roots present in the formulation. A HPLC method was developed for the estimation of aconitine in the polyherbal formulation. The separation was performed on a Phenomenex C_{18} column using ammonium bicarbonate buffer (pH 8.00) and acetonitrile (40:60, v/v) at room temperature with a flow rate of 1 ml/min at 223nm. About 1gm each of the three formulations were powdered and extracted in hydrochloric acid by sonication and treated to remove the non-alkaloid components. Chloroform was used to extract the alkaloids after basification. The standard solutions of aconitine were prepared in acetonitrile in the range of 10-100 µg/ml. The results showed that the content of aconitine in the three formulations varied considerably. The validation parameters showed that the method was precise, accurate, sensitive and robust. 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 and safety because it is the target toxic component for the quality control of *Mahamrutyunjaya rasa.*

HPTLC method was also developed for the analysis of aconitine in *Mahamrutyunjaya rasa.* A 1 mg/ml stock solution of aconitine was prepared in methanol. It was further used for the preparation of the calibration curve. A 10 mg/ml solution of alkaloid fraction of FORM1, FORM2 and FORM3 was dissolved in 0.5 ml chloroform and diluted further in methanol. Different volumes from 2-10 µl of stock solution were applied. The plate was developed in

a solvent system consisting of Toluene, Ethyl Acetate and Diethyl amine (7:2:1) to a distance of 8.5 cm in a twin trough chamber. The plate was scanned and quantified. The plate was sprayed with Dragendorff's reagent, immediately scanned and quantified using the Camag TLC Scanner-3. The method was found to be precise, accurate, sensitive and robust.

A spectrofluorimetric method for the analysis of aconitine in *Mahamrutyunjaya rasa* was also developed. The instrumental parameters were as follows: Scan Speed: Medium, Sensitivity: High, Abscissa scale: 2, Ordinate scale: 3. Aconitine gave strong fluorescence having excitation and emission wavelength of 270 nm and 505 nm in methanol. The linearity range was 100-600 pg/ml. The method was validated and found to be precise, accurate, sensitive and robust. It was also suitable for the estimation of aconitine in the alkaloid fraction of *Mahamrutyunjaya rasa.*

A stability indicating HPLC method for aconitine was also developed using the same LC system and Hypersil C_{18} column (particle size 5 µm; 250 mm X 4.6 mm ID) column. 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. The forced decomposition studies under a variety of conditions like pH, light, oxidation, dry heat, relative humidity, etc. were carried out as per the ICH guidelines. Aconitine degraded in the alkaline conditions, in oxidative conditions, in photolytic and in humid conditions. It however degraded slowly in higher concentrations of hydrochloric acid and at high temperature. The method was validated and found to be precise, accurate, sensitive and robust. The peak purity analysis of aconitine was also done using a PDA detector to study the specificity of the method. Thus, a validated stability-indicating HPLC analytical method has been developed for the determination of aconitine in the ayurvedic medicine. The method was also applied to the formulations. The results of stress testing undertaken revealed that the method is selective and stability indicating.

In all the methods developed for the analysis of aconitine the assay results showed less deviation in the formulation samples, though the concentration in

different formulations varied greatly, thus showing the applicabilty of all the methods for the analysis of aconitine.

Solanine was used for the standardization of Solanum roots in *Mahamrutyunjaya rasa.* An HPLC method was developed in which separation was performed using a Phenomenx C_{18} column. The mobile phase comprised of Tris buffer (10 mM; pH 6.0 adjusted with 1% Triethylamine) - acetonitrile (60: 40 v/v) at 1 ml/min with detection at 218 nm. The standard solutions of solanine were prepared in methanol in the range of 10-100 µg/ml. The solanine content in all the formulations also showed slight variation and the method can be applicable to different formulations containing solanine.

A HPTLC method was also developed for the estimation of solanine in *Mahamrutyunjaya rasa.* A 1 mg/ml stock solution of solanine was prepared in methanol. It was further diluted to 100 µg/ml in methanol The reference standard marker solanine from potato sprouts purchased from Sigma Aldrich Pvt. Ltd. was taken as standard. FORM1, FORM2 and FORM3 were analyzed by this HPTLC method. A 10 mg/ml solution of methanol extract of the formulation was prepared. The plate was developed in a solvent system consisting of Chloroform, Methanol and Ammonia (7:3:0.5) to a distance of 8.5 cm in a twin trough chamber. The plate was sprayed with Dragendorff's reagent immediately and scanned and quantified. The plate was sprayed with Dragendorff's reagent, immediately scanned and quantified using the Camag TLC Scanner-3. The method was found to be precise, accurate, sensitive and robust.

The HPLC and HPTLC methods developed showed similar results of the assay performed in the formulations. Thus both the methods can be applied for assay of solanine in *Mahamrutyunjaya rasa.*

A HPTLC method was also developed for the estimation of piperine in *Mahamrutyunjaya rasa.* A 1 mg/ml stock solution of piperine was prepared in methanol. It was further diluted to 100 µg/ml in methanol The reference standard marker piperine (95% purity) was purchased from Sigma Aldrich Pvt. Ltd. was taken as standard. FORM1, FORM2 and FORM3 were analyzed by this HPTLC method. A 10 mg/ml solution of methanol extract of the formulation was prepared. The plate was developed in a solvent system consisting of

Toluene and Ethyl acetate(7:3) to a distance of 8.5 cm in a twin trough chamber. The plate was scanned. The plate was sprayed with Dragendorff's reagent, immediately scanned and quantified using the Camag TLC Scanner-3. The method was found to be precise, accurate, sensitive and robust.

A stability indicating HPLC method was developed for piperine using the same LC system and column. The best resolution was achieved on a Hypersil C_{18} column using a mobile phase consisting of acetonitrile - KH_2PO_4 buffer (10 mM, pH 7± 0.1) (35:65 v/v). The assay was performed at 343 nm at 0.7ml/min flow rate. The forced decomposition studies under a variety of conditions like pH, light, oxidation, dry heat, relative humidity etc were carried out as per the ICH guidelines. . All solutions prepared for use in forced degradation studies were prepared to yield starting concentrations of piperine of 100µg/ml. Piperine degraded in the alkaline and photolytic conditions and in the presene of water . It however degraded slowly in higher concentrations of hydrochloric acid and hydrogen peroxide. The method was validated and found to be precise, accurate, sensitive and robust. The peak purity analysis of piperine was also done using a PDA detector to study the specificity of the method. Thus, a validated stability-indicating HPLC analytical method has been developed for the determination of piperine in the ayurvedic medicine. The method was also applied to the formulations. The results of stress testing undertaken revealed that the method is selective and stability indicating.

The HPTLC and stability indicating HPLC methods developed showed similar results of the assay performed in the formulations. Thus both the methods can be applied for assay of piperine *Mahamrutyunjaya rasa.*

Mahamrutyunjaya rasa is a multicomponent preparation with a number of active chemical constituents. In such formulations, the development of methods for the simultaneous estimation of various components is of immense value.

An HPLC method for the simultaneous estimation of Aconitine, Solanine and Piperine in *Mahamrutyunjaya rasa* was also developed and validated. The separation of these alkaloids was achieved on an RP C-18 column (250 mm × 4.6 mm ID, 5 µm particle size) in an isocratic system at 1.0 ml/min flow rate with acetonitrile - KH_2PO_4 buffer solution (10 mM, adjusted to pH 7.5 ± 0.1 with 1% triethylamine)-methanol (60:25:15, v/v/v) as mobile phase. The flow rate

was 1 ml/min with UV detection at 223 nm for aconitine and solanine while 343 nm for piperine. The calibration curves were linear with correlation coefficient of 0.999, 0.9942 and0.9989 for solanine, piperine and aconitine respectively. The % RSD values were less than 2% in the concentration range of 10–100µg/ml for all the three alkaloids. The alkaline buffer solution (10 mM Phosphate buffer, pH 7.5) used as mobile phase with acetonitrile and methanol gave satisfactory chromatographic separation of the three alkaloids from their adjacent peaks. The developed LC method was applied to the simultaneous determination of aconitine, solanine and piperine in the Ayurvedic formulations (FORM1, FORM2, FORM3). 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 the formulation. The content of piperine and solanine also varied in all the formulations to some extent. Method validation data indicated that the present method is reliable, reproducible and accurate LC method for the simultaneous determination of the three alkaloids in the proprietary Ayurvedic medicines with optimized extraction and separation conditions.

A HPLC method for simultaneous estimation of Aconitine and Solanine in *Mahamrutyunjaya rasa* was also developed. This method was changed in such a way so as to perform a faster analysis as compared to the method developed for simultaneous estimation of the three components. Analysis was isocratic at 1.0 ml/min flow rate with Phosphate buffer (10mM, pH 7.5): acetonitrile: methanol (15:70:15) as mobile phase on a Phenomenex C18 column. The measurement of aconitine and solanine was carried at room temperature with a flow rate 1 ml/min at 223 nm. The standard and sample solutions were prepared using the same procedure as mentioned in above section. The method was found to be simple, sensitive, precise, accurate and reproducible. The developed method can be applied for the simultaneous estimation of aconitine and solanine in *Mahamrutyunjaya rasa* and the results showed that there was a lot of variation in the concentration of aconitine. Solanine also varied to some extent.

A HPLC method for the simultaneous estimation of Solanine and Piperine in *Mahamrutyunjaya rasa* was developed and validated. This method was changed

in such a way so as to perform a faster analysis as compared to the method developed for simultaneous estimation of the three components. Analysis was performed on Phenomenex C_{18} column in an isocratic system at 1.0 ml/min flow rate with acetonitrile - KH_2PO_4 buffer solution (10 mM, adjusted to pH 7.5 $\pm$ 0.1 with 1% triethylamine) - methanol (55:20:15, v/v/v) as mobile phase. The flow rate was 1 ml/min with UV detection at 218 nm for solanine while 343 nm for piperine. The % RSD values were less than 2% in the concentration range of 10–100 µg/ml for the two alkaloids. The method was applied for the estimation of piperine and solanine in the formulation and the results obtained were similar to the ones obtained by other methods. The analytical performances of the proposed HPLC methods were established and the methods were validated in terms of precision, accuracy, robustness, detection and quantification limits. The method was found to be precise, accurate, reliable, sensitive and robust.

Various methods for the estimation of the inorganic components in the formulations were also developed.

A method for the estimation of sulphur by colorimetry was developed. Sulphur in presence of Hydrazine hydrate gets converted to water soluble hydrazine sulphide forming a yellow coloured solution with a λmax of 368 nm. The solution was found to be stable in 0.1 N NaOH which gave absorbance at 368 nm in the linearity range of 20-70 µg/ml. The method was applied for the estimation of sulphur in formulations. Sulphur was extracted from the formulations using toluene and dried at room temperature. 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. The results indicated that only one type of compound is formed. The results were reproducible with low %RSD values. The method was validated and found to be precise, acurate, reliable, robust and sensitive. Further the step of ashing in sample preparation was not required making the method faster and simple. The results of analysis of the formuations and the recovery study of drug suggested that there is no interference from the other components, which are present in

the formulation. The results were close to the probable concentration present in the formulation, thus proving the utility of the method.

Three methods were used for the estimation of boron in *Mahamrutyunjaya rasa.* A reported simple and sensitive spectrofluorimetric method was used for the determination of boron with Alizarin Red S. 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. In the presence of Alizarin red S at pH 7.4, a complex was formed which gave flourescence at 570 nm with excitation at 470 nm. The compex formed was Borosulfoalizarin which was stable at pH 7.4. The variables like pH, reagent concentration, order of addition of reagents and temperature were studied to determine the optimum conditions. The method was further validated and found to be precise, accurate, reliable, robust and sensitive. The method was applied satisfactorily for the determination of boron in the ash of the formulations. The label claim was calculated and the results were close to the expected concentration, thus the method was applied successfully for the estimation of boron in herbo-mineral formulation.

A novel voltammetric method for boron determination was developed. Boron was found to complex with Alizarin Red S and the complex, as well as the free ligand, both were adsorbed on a hanging mercury drop electrode. The method was based on the monitoring the anodic peak of the complex at −0.52 V in ammonium acetate–phosphate buffer (pH 7). The maximum peak current was obtained by scanning the potential from −700mV to more positive potentials without accumulation in the presence of 0.15 mM concentration of Alizarin red S. The instrumental parameters were mainly differential pulse mode with a pulse duration of 0.02 s and a scan rate of 5mV/s. The method was applied to the formulation samples for the determination of boron. The label claim of Boron in the formulation is about 4.3 mg per tablet. The results of the analysis showed that the method was successful in determining the correct concentration of boron. The method was also validated and found to be precise, accurate, reliable, robust and sensitive. The boron concentration in the samples and the recoveries showed that there was no interference on addition of EDTA.

The third method for the estimation of Boron in *Mahamrutyunjaya rasa* was by ICP-AES. 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 0.1 N HCl (prepared in double distilled water). The samples were analyzed at 249.43 nm 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 method and voltammetric method.

The estimation of mercury in *Mahamrutyunjaya rasa* was done using ICP-AES. 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 aqua regia and diluted in double distilled water. The samples were analyzed at 253.56 nm. The results showed a lot of variation in the concentration of mercury in the three formulations. The marketed formulations (FORM2 and FORM3) had very high concentrations of mercury as compared to the formulation prepared in the laboratory (FORM1).

Thus, a number of analytical methods have been developed for the estimation of various markers alone as well as in combiantion and for the estimation of the important minerals. The concentration of the three markers in the formulations showed that a lot of variation is there in the concentration of these markers. The concentration of aconitine has to be monitored with care as it has a very narrow therapeutic index. It was observed that FORM1 and FORM2 had comparable concentration of aconitine but FORM3 was found to contain very high concentration of aconitine. The variation in the concentration of aconitine may be due to the improper processing of the raw material. The standardization of the raw material is therefore important when such poisonous components are present in the formulations. Solanine and Piperine concentrations also varied but the % RSD was lesser as compared to aconitine.

The analytical results showed the variations in the concentration in the marker components and thus the biological standardization was performed so as to compare the effects of the three formulations in the biological system.

The biological evaluation of *Mahamrutyunjaya rasa* was performed by acute toxicity studies, *invitro* cell viability studies and the therapeutic potential as

cardio active formulation was studied using isoproterenol induced myocardial infarction in rats.

Toxicity study was carried out on formulations as per the OECD guidelines in female albino mice. Histopathological examination of the visceral organs was performed to observe the toxic effects of formulations. Mortality was observed in FORM 1, FORM 2 and FORM 3 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. Heart and Kidney were also free of pathological findings in FORM1 and FORM2 treated groups of animals. While mice treated with 550 mg/kg. b.w. of FORM3 showed hepatic injury on the liver surface to some extent. Heart sections also showed infarcted zone with oedema and inflammatory cells and the separated muscle fibres in the myocardium of the FORM 3 treated mice. However, no mortality was observed.

The *invitro* cell viability after treatment with test material was assayed by the reduction of 3-(4, 5-dimethylthiazole-2-yl)-2, 5- diphenyl-tetrazoliumbromide (MTT) to formazan. Formulations were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was not more than 0.2% during the experiments. In each experiment, different concentrations of formulations were tested in three separate wells and the cytotoxicity curve was constructed from three different experiments.

The data of the survival curves were expressed as the percentage of untreated controls. Effect of FORM1, FORM2 and FORM3 treatments on viability of rat embryonic cardiac cells (H9c2) were studied in comparison to the untreated (control) cells. Cells pretreated with FORM1 and FORM2 for 12 h showed increased cell viability in the concentration range of 2-200 µg/ml which decreased after 24 h and further after 48 h. While cells pretreated with FORM3 for 12 h showed reduction in cell viability at 200 µg/ml concentrations and decreased greatly in 24 h and 48 h readings.

The data obtained from the cell viability studies on the formulations indicated 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 H9c2 cells. 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 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 the *in vitro* studies. The cell viability assay results indicated 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 also in lower concentration.

In vivo studies were carried out on the selected formulations (25 mg/kg b.w. and 50 mg/kg b.w. administered orally for 15 days) to study the cardio protective action using isoproterenol (ISO) (25mg/kg, s.c.) induced myocardial infarction in rats. Serum levels of creatine kinase isoenzyme (CKMB), lactate dehydrogenase (LDH) and glutamate oxaloacetate transaminase (GOT) were 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. Protective effect of formulations are to be seen by analyzing the levels of serum marker enzymes like GOT, CK-MB, LDH, and uric acid. The study was be supported by histopathological examination of heart.

In the study, isoproterenol treatment resulted in a marked elevation of CK-MB level. Pretreatment of 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.

A significant increase in the serum marker enzymes levels in FORM3 treated rats was observed when compared to the ISO treated group which depicts the highly toxicity nature of the formulation. The reason for high toxicity of FORM3 formulation may be the increased intracellular aggravation of Ca^{2+} which may be due to the high concentration of aconitine. As already mentioned, the

preparation of this formulation involves purification of aconite roots by treatment with cow urine in order to convert the poisonous alkaloids like aconitine to other alkaloids which possesses the same pharmacological activity but are less poisonous in nature. The formulations FORM1 and FORM2 have thus been prepared according to traditional procedures.

The heart tissue of FORM1 and FORM2 pretreated animals showed no changes in cardiac structure and were similar to that of the control group. This indicated that both FORM1 and FORM2 were free of toxicity. Further, administration of FORM1 and FORM2 to the MI rats 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 and FORM2 rats showed minimal myocardial degeneration and were completely devoid of any cardiotoxicity.

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. The FORM 3+ISO treated rats also showed acute myocardial necrosis and consisted of variably sized areas of contracted and/or fragmented myofibers with inflammatory infiltrate.

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 cardioprotective.

It is evident from the results of the study that *Mahamrutyunjaya rasa* has significant cardioactive property when compared with the control groups. But, the differences in the results of toxicological and pharmacological studies prove the need of stringent regulatory control over the manufacture and quality control of this ayurvedic formulation.

Thus, the present work may be concluded as follows: *Mahamrutyunjaya rasa* contains a number of plant parts and mineral components as the principle ingredients and thus the standardization of this cardioactive formulation is difficult. In the present work, an attempt has been made to evaluate the rasa in terms of formulation preparation procedure, physical evaluation, chemical evaluation and biological evaluation. A laboratory formulation has been prepared according to standard text and compared to the proprietary formulations. The physical and chemical changes taking place in purification steps of formulation preparation have been studied, which can form a basis for inprocess evaluation of the formulation. Further the physical evaluation has been performed and standards have been determined as per the WHO guidelines, which can become the basis of preliminary standardization.

The *Rasa* contains various organic and inorganic active components which are toxic in nature and thus require reliable methods for quantification of such components. A number of methods have been developed for the major active marker components like aconitine, solanine and piperine using HPLC, HPTLC and spectrofluorimetry. Method validation data indicate that the methods are reliable, reproducible and accurate methods for determination of the active components in the rasa with optimized extraction and separation conditions. The concentrations of the markers in laboratory formulation were compared with that of the marketed formulations. For the estimation of inorganic components in the formulation, methods have been developed using UV Spectroscopy, Spectrofluorimetry, ICP-AES.

The biological evaluation of the formulations was also performed in terms of the toxicological studies and the pharmacological activity was studied in the isoproterenol induced myocardial infarction model and the cell viability study was also performed on rat embryonic cardiac cells (H9c2). The results of the biological evaluation are in accordance with the concentration of active components obtained on analysis using the developed methods.

Thus, in the present study a number of methods for the standardization of *Mahamrutyunjaya rasa* have been developed in terms of physical, chemical and biological standardization. The results of all the tests were compared and significant variations were observed. The FORM1 prepared by the traditional

method was found to comply with the tests performed for physical standardization. The assay results of all the markers and minerals showed that the concentration of the active components were within acceptable limits. The stability indicating methods when applied to the formulation did not show any deviation from the standard substances. However, aconitine in the samples in humid conditions (75 % RH at 30°C) was found to degrade at a faster rate. The safety and efficacy of FORM1 were further confirmed by the toxicity studies, cell viability studies and pharmacological activity. FORM1 qualified all the tests and was found to be safe as well as effective for use as a cardioactive formulation. The reason being the standardization of the crude plant materials used as raw materials and proper purification of toxic components using standardized methods as per the traditional text.

The FORM2 from Baidyanath showed few deviations from the standard quality. In terms of hardness and disintegration, the values of FORM2 were above the acceptable limits. The presence of organochlorinated pesticides was also observed. The assay results of the markers did not show large deviation in the concentration of aconitine, solanine and piperine when compared to FORM1. The results of the stability studies were also similar to FORM1. However, the concentration of mercury was found to be high as compared to FORM1. The results of biological activity showed similar results as FORM1 and thus was found to be effective cardioactive formulation with less toxicity.

The FORM3 from Pune Rasashala showed significant variations in the results of all the methods of standardization. The formulation was found to have high friability in the friability test and was thus prone to breakage. The assay results showed that aconitine was present in very high concentration as compared to other two formulations. The variation in the concentration of aconitine may be due to the improper processing of the raw material. The standardization of the raw material is therefore important when such poisonous components are present in the formulations. Solanine and Piperine concentrations also varied but the % RSD was lesser as compared to aconitine. The stability indicating assay methods showed similar degradation pattern as FORM1. The concentration of sulphur and boron were also found to be acceptable with less variation. However, the concentration of cinnabar in the FORM3 was high as

compared to the label claim. The results of the biological standardization showed that FORM3 is toxic. The formulation when adminstered for 15 days produced infarcts in the cells themselves and were not at all effective as cardioprotective. The results of toxicity was further confirmed from the cell viability studies. Thus FORM3 plays no role as a cardioprotective.

The results of complete standardization studies showed significant variation in the three medicines. Therefore, from the above study one can conclude that for the preparation of *Mahamrutyunjaya rasa,* standardized traditional methods and raw materials should be used. Further, the concentration of the markers should be assayed which have been observed to have direct effect on the safety and efficacy.

So, it is highly recommended that the standardization of this Ayurvedic medicine must be done as a routine measurement, so as to provide a safe application to patients in clinics, and good manufacture practices. Thus a need arises for the development of reliable standardization tools for effective utility of these traditional medicines.

To establish the potentiality of Ayurvedic medicine, research needs to be conducted on different disciplines of Ayurveda to meet the requirement of the society. This can be done by standardization of materials, methods and measures for preparation, preservation, presentation and administration of Ayurveda drugs. Thus, the rationale and judicious use of modern scientific methods pertain to the development of Ayurveda.