2.1. INSTRUMENTATION

PEAK chromatographic system supplied along with LC-7000 isocratic pump was used for the chromatographic separation. Rheodyne injector with 20µl fixed volume loop was utilized to inject the samples. Techcom UV-2301 double beam UV-visible spectrophotometer was employed to perform chromatographic separation. Peak chromatographic software version 1.06 was applied to monitor the output signal. The data was documented by making use of Hitachi software. Sonication of the mobile phase and samples was done on Ultrasonicator (1.5L). Materials were weighed on Denver electronic analytical balance of the model SI-234. Systronics digital pH meter was employed to record the pH of the mobile phase.

2.2. METHOD DEVELOPMENT

Method development was endeavored through a systematic study of the effect of several factors by varying one parameter at a time and keeping remaining parameters constant. Method development in general depends on selection of appropriate wavelength and preference of stationary and mobile phases and it can be acquired by following studies.

2.2.1. Detection of wavelength

The spectrum of diluted solutions of the two active ingredients in methanol was recorded separately. The absorption spectrum of two drugs was scanned on spectrophotometer in UV region i.e. 200-400nm. The wavelength at which the two active ingredients showed maximum overlapping was selected as an optimum wavelength.

2.2.2. Choice of stationary phase

Initial trials were conducted by the use of octadecyl columns with different types, configurations and from different manufactures. The peak area response in each and every case was compared after injecting the standard solution into each and every column.

2.2.3. Selection of mobile phase

Different mobile phases under isocratic conditions were tested in terms of sharp peak and base line separation. System suitability conditions in each case were studied carefully. Various trials were conducted for the selection of mobile phase by changing solvents, composition and flow rates. In each trial, the system suitability conditions were examined in order to obtain good base line separation and sharp peaks without the interference of excipients.

2.2.4. Flow rate

Flow rates of mobile phase were tested from 0.5-1.5ml/min to accomplish optimum separation. Utmost saving of the solvents was achieved when minimum flow rate and minimum run time were maintained. Successful elution of the analytes was reached when the flow rate was maintained at 1ml/min.

2.2.5. Optimized chromatographic conditions

Numerous trials were conducted for the selection of optimum chromatographic conditions.

2.3. METHOD VALIDATION

As per ICH guidelines the method was validated. Linearity, accuracy, precision, robustness, LOD, LOQ etc were the various parameters studied for the method validation.

2.3.1. Specificity

Chromatograms of blank, standard and sample were compared to measure the specificity of the proposed method.

2.3.2. System suitability

System suitability of the proposed method was performed by analyzing repeatability, peak symmetry, theoretical plates of the column, peak area and retention time. Freshly prepared standard solution of the drugs was used to evaluate system suitability conditions.

2.3.3. Linearity

A series of six different concentration levels were prepared to determine the linearity of the method. Calibration plots of concentration against peak area were constructed separately to evaluate linearity. Regression of the plots was calculated by the use of least square method.

2.3.4. Accuracy

Standard addition method was employed to measure the accuracy of the suggested method. Three concentration levels (50%, 100% and 150%) were spiked with the reference solution and the measurements were made in triplicate at each level. Percentage RSD was used to evaluate the recovery of the drugs.

2.3.5. Precision

Precision describes the repeatability of the proposed method. Intra-day precision and inter-day precision were the major steps involved in the measurement of precision.

2.3.5a. Intra-day precision

Six replicates of standard solution were injected into HPLC system to determine the intra-day precision of the method. The percentage RSD was calculated.

2.3.5b. Inter-day precision

Six replicates of standard solution were injected on three successive days to determine the inter-day precision of the method. The percentage RSD of the assay was determined.

2.3.6. Ruggedness

Ruggedness is the measure of reproducibility of the proposed method. Ruggedness was estimated by different analyst using different columns on different days. Six dilutions were tested and the percentage RSD was calculated.

2.3.7. Robustness

Small deliberate changes were introduced with respect to wavelength, pH and mobile phase to measure the robustness of the method. The effect of these changes on chromatographic parameters was observed. In particular retention time, tailing factor and number of theoretical plates were compared with the standard solution. Percentage change in the results was calculated.

2.3.8. Limit of detection and limit of quantification

Limit of detection enables to measure detectable response at the lowest possible concentration. On the other hand, limit of quantification requires the measurement of quantified response with enough accuracy and precision at a minimum concentration level. Solutions of different concentrations were prepared and all the solutions were investigated repeatedly to evaluate LOD and LOQ values.

2.3.9. Solution stability

To measure the stability of drugs, standard solutions were prepared and were kept aside for about two days. At regular intervals of time, the solutions were injected and the chromatographic parameters were compared with the freshly prepared standard solutions.

2.3.10. Formulation

Into the HPLC system 20µl of freshly prepared sample was injected and the corresponding peak response was measured to determine the percentage assay of the commercial formulation.

2.4. FORCED DEGRADATION STUDIES

The stability of formulation under different stress conditions were measured by subjecting the sample solutions to different degradation conditions like aqueous, acidic, basic, peroxide, thermal, light and UV light.

2.4.1. Aqueous degradation

Aqueous sample solution was prepared by dissolving 300mg of sample in double distilled water. The solution was allowed to stand for 48hr and then 5ml of the solution was transferred into 25ml volumetric flask. The solution in the volumetric flask was diluted up to the volume. 20µl of the solution was injected and the degradants were evaluated from the chromatogram and compared with the standard values.

2.4.2. Acid degradation

Initially, 300mg of sample was dissolved in 20ml of hydrochloric acid (0.1N) during the preparation of acid hydrolyzed sample solution. Then, the solution was allowed to rest for 48hr and then 5ml of acid hydrolyzed sample solution was transferred into 25ml volumetric flask. Later, the solution was neutralized with 5ml (0.1N) of sodium hydroxide solution and filled up to the mark with diluents. The solution was injected to HPLC system under chromatographic conditions. The degradants were evaluated from the chromatogram and were compared with the original values.

2.4.3. Base degradation

Base hydrolyzed sample solution was prepared by dissolving 300mg of the sample in 20ml of sodium hydroxide solution (0.1N). After two days, 5ml of base hydrolyzed sample solution was taken into 25ml volumetric flask and neutralized with

5ml of hydrochloric acid solution (0.1N). Later the solution was made up to the mark with diluents. Degradants were evaluated by injecting sample solution into HPLC and were compared with standard values.

2.4.4. Peroxide degradation

Oxidized sample solution was prepared by dissolving 300mg of sample in 20ml of hydrogen peroxide (3%) and the solution was allowed to stand for 48hr. Into 25ml volumetric flask 5ml of the solution was transferred and made up to the mark with the diluents. After injecting the solution, the chromatogram was evaluated to measure the number of degradants.

2.4.5. Thermal degradation

To measure the thermal degradation the sample solution was poured into a petri dish and was maintained at 40^0C to 80^0C in an oven for two days. The solution after thermal exposure was injected to record the chromatogram and the degradents were evaluated on comparing with the original chromatogram.

2.4.6. Light and UV light degradation

In order to measure the degradation by light, the sample was taken into an open petri dish and was placed under ordinary light for 48hr. After the preparation of sample solution, it was injected. Degradants were evaluated on comparing the chromatogram with the original values. Similar procedure was adopted by placing the solution under UV light and the corresponding chromatogram was evaluated to measure the number of degradants. Degradants were evaluated by injecting sample solution into HPLC system and were compared with standard values.