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Chapter-2

Materials and Methods

Investigation of the stress degradation, degradation kinetics and impurity profiling of ramosetron HCl, ondansetron HCl, granisetron HCl, tropisetron HCl and palonosetron HCl was the focus of this thesis. All the components of the materials and methods which were the integrated part of this research work are described broadly in this section.

2.1 Materials and methods for stress degradation studies

Purposeful degradation was conducted through various stressed conditions such as aqueous, acid, base, oxidation and photolytic treatments to achieve maximum degradation of 20.0%.

2.1.1 Drug substance and reagents

Pure hydrochloride salt of ramosetron, ondansetron, granisetron, tropisetron and palonosetron bulk, manufactured by SMS Pharmaceutical Ltd., India, was obtained from Incepta Pharmaceutical Ltd. (Dhaka, Bangladesh). Methanol (HPLC grade), acetonitrile (HPLC grade), triethylamine (reagent grade), hydrogen peroxide, dipotassium hydrogen phosphate anhydrous (reagent grade) and sodium hydroxide (reagent grade) were purchased from Scharlau (Scharlau S.L., Spain). HPLC grade water was prepared by PALL purification system (PALL, cascada AN, USA). Hydrochloric acid (37% commercial grade) and orthophosphoric acid (reagent grade) were purchased from Labscan (ACI Labscan, Thailand). LC-MS grade methanol was procured from Panreac (Panreac, E.U).

2.1.2 Equipment used for studies of stress degradation

An Agilent Technologies 1260 series HPLC system (Agilent, Infinity 1260, Germany) equipped with integral autosampler (model 1260 HiP ALS) and quaternary gradient pump (model Quat Pump VL) with an on-line degasser was used. The column compartment (model 1260 TCC) having temperature control and a diode array detector (model 1260 DAD VL+) were employed throughout the analysis. Chromatographic data was acquired using Agilent Open LAB software. A hot air oven (Memmert, Mumbai, India) was used to maintain constant temperature. The stress photodegradation was carried out in a photostability chamber (Oswarld OPSH-G-16-GMP series,

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Oswarld scientific, Mumbai, India) equipped with illumination bank made of light source as described in the ICH guideline Q1B. An ultrasonicator from Power Sonic-405 (Hwashin Technology, Seoul, Korea) and pH meter from pH tutor (Eutech Instruments, Singapore) were used.

4000 QTRAP LC-MS-MS mass spectrometer (4000, AB SCIEX, USA) coupled with ESI ion source was used to generate MS data. The data were acquired using analyst software 1.5.2.

2.1.3 Chromatographic conditions used for studies of stress degradation

Chromatographic separation was achieved at a temperature of 40°C on a bonded phase cyano column (250 x 4.6 mm; CN; (250 x 4.6 mm; CN; Kromasil, Waters or Supelcosil) using a mobile phase comprising of a mixture of acetonitrile-methanol-buffer (50 mM dipotassium hydrogen phosphate anhydrous containing 1 ml of triethylamine per liter with pH 7.0 adjusted by dilute orthophophoric acid) in the ratio (3 : 1 : 6). During the change of HPLC column of new brand remaining the same column packing, both the organic phases (acetonitrile and methanol) were changed by 5% to achieve acceptable resolution. The mobile phase so prepared was filtered through 0.45 µm membrane filter and degassed by sonication. Flow rate of 1.0 ml/min was maintained. The injection volume was 20 µL for all the analyses. The detection was carried out at the wavelength of 210 nm.

2.1.4 Procedure for stress degradation study

Stress degradation of the drug substances were conducted in aqueous, acid, base, oxidative and photolytic conditions. Photo degradation of the drug substances were conducted in solid state. The concentration of the solutions kept for degradation under different stressed conditions was 1.0 mg/ml. The final concentration of the stressed solutions was 0.2 mg/ml in mobile phase.

2.1.5 Preparation of standard solution

The first dilution of the standard solution was prepared in HPLC grade methanol to get a concentration of 5.0 mg/ ml. The second dilution was done by mobile phase to get a final concentration of 0.2 mg/ml. This standard solution was prepared on the day of analysis.

2.1.6 Preparation of stock sample for degradation study

The stock solution of the API was prepared in HPLC grade methanol to get a concentration of 5.0 mg/ml.

2.1.7 Preparation of analytical sample for stress degradation under aqueous degradation

An aliquot of stock sample prepared for degradation study was diluted to 5 ml with purified water to get a concentration of 1.0 mg/ml. This solution was kept in a dry oven at 60°C for 7- and 21-days. These stressed treated samples were further diluted with mobile phase to get a final concentration of 0.2 mg/ml.

2.1.8 Preparation of analytical sample for stress degradation under acid degradation

An aliquot of stock sample prepared for degradation study was diluted to 5 ml with 0.1N HCl, 0.5N HCl, 1.0N HCl and 2.0N HCl solutions separately to get a concentration of 1.0 mg/ml. These solutions were kept in a dry oven at 60°C for 7- and 21-days. These stressed samples were neutralized with equimolar strength and volume of sodium hydroxide before further dilution with mobile phase to get a final concentration of 0.2 mg/ml.

2.1.9 Preparation of analytical sample for stress degradation under basic degradation

An aliquot of stock sample prepared for degradation study was diluted to 5 ml with 0.1N NaOH, 0.5N NaOH, 1.0N NaOH and 2.0N NaOH solutions separately to get 1.0 mg/ml. These solutions were kept in a dry oven at 60°C for 7 days. These stressed treated samples were neutralized with equimolar strength and volume of hydrochloric acid, respectively before further dilution with mobile phase to get a final concentration of 0.2 mg/ml.

2.1.10 Preparation of analytical sample for stress degradation under oxidation

An aliquot of stock sample prepared for degradation study was diluted to 5 ml with 3.0% H_2O_2, 5.0% H_2O_2 and 10.0% H_2O_2 solutions separately to get a concentration of 1.0 mg/ml. These solutions were kept in a dark place for 1 hour, 2 hours and 3 hours. These stressed treated samples were further diluted with mobile phase to get a final concentration of 0.2 mg/ml.

2.1.11 Preparation of analytical sample for stress degradation under photolysis

Bulk powder of the API was evenly spread on aluminum foil and kept in the photostability chamber for direct exposure of fluorescence light (1.2 and 3.6 million lux) and UV light (200 and 600 watts hour/m^2) along with control sample wrapped with similar aluminium foil. A portion of the exposed and control samples were dissolved in 1.0 ml of HPLC grade methanol and diluted with mobile phase to get a final concentration of 0.2 mg/ml.

2.2 Materials and methods for studies of degradation kinetics

Degradation kinetics studies were conducted on ramosetron HCl, ondansetron HCl, granisetron HCl and tropisetron HCl at 60°C, 70°C and 80°C for different time interval of 1 hour, 2 hours, 3 hours, 4 hours and 5 hours. Different solvents were used for different APIs considering the magnitude of degradant products. Ondansetron HCl, granisetron HCl and tropisetron HCl were dissolved in 2.0N sodium hydroxide. On the other hand, 0.1N sodium hydroxide was used to dissolve ramosetron HCl.

2.2.1 Drug substance and reagents

Ramosetron HCl, ondansetron HCl, granisetron HCl and tropisetron HCl APIs, manufactured by SMS Pharmaceutical Ltd., India, were obtained from Incepta Pharmaceutical Ltd. (Dhaka, Bangladesh). HPLC grade methanol, HPLC grade acetonitrile, reagent grade triethylamine, hydrogen peroxide, dipotassium hydrogen phosphate anhydrous and sodium hydroxide were purchased from Scharlau (Scharlau S.L., Spain). HPLC grade water was prepared by PALL purification system (PALL, cascada AN, USA). Hydrochloric acid (37% commercial grade) and orthophosphoric acid (reagent grade) were purchased from Labscan (ACI Labscan, Thailand).

2.2.2 Equipment

Agilent, Infinity 1260 HPLC system, equipped with integral autosampler (model 1260 HiP ALS) and quaternary gradient pump (model Quat Pump VL) with an on-line degasser was used. The column compartment (model 1260 TCC) having temperature control and a diode array detector (model 1260 DAD VL+) were employed throughout the analysis. Agilent Open LAB software was used to integrate chromatograms. A hot air oven (Memmert, Mumbai, India) was used to maintain constant temperature. The stress photodegradation was carried out in a photostability

chamber (Oswarld OPSH-G-16-GMP series, Oswarld scientific, Mumbai, India) equipped with illumination bank made of light source as described in the ICH guideline Q1B. An ultrasonicator from Power Sonic-405 (Hwashin Technology, Seoul, Korea) and pH meter from pH tutor (Eutech Instruments, Singapore) were used.

2.2.3 Chromatographic conditions

A bonded phase cyano column (250 x 4.6 mm; CN; Kromasil , Waters or Supelcosil) was used throughout the analysis maintaining the column oven temperature at 40°C . The mobile phase was a mixture of acetonitrile-methanol-buffer (50 mM dipotassium hydrogen phosphate anhydrous containing 1 ml of triethylamine per liter with pH 7.0 adjusted by dilute orthophophoric acid) in the ratio (3 : 1 : 6). During the change of HPLC column of new brand remaining the same column packing, both the organic phases (acetonitrile and methanol) were changed by 5% to achieve acceptable resolution. The mobile phase so prepared was filtered through 0.45 µm membrane filter and degassed by sonication. Flow rate of 1.0 ml/min was maintained. The injection volume was 20 µL for all the analyses. The detection was carried out at the wavelength of 210 nm.

2.2.4 Procedure for studies of degradation kinetics

Degradation kinetics of the drug substances were conducted in 0.1N NaOH for ramosetron HCl and 2.0N NaOH for ondansetron HCl, granisetron HCl and tropisetron HCl at 60°C, 70°C and 80°C for 1, 2, 3, 4 and 5 hours. The concentration of the solution kept for degradation under different time intervals was 1.0 mg/ml. The final concentration of the degraded samples was 0.2 mg/ml in mobile phase.

To explain kinetics of degradation and to find out the best fitness of the regression coefficient, the relationship between the concentration vs time was drawn for zero, first and second order reaction following Eq. 1, 2, and 3, respectively. Eq. 4 was used to calculate activation energy where, C_t and C_o are the concentrations at time t and zero, respectively. The reaction rate constant for zero, first and second order reactions are k_o, k_1 and k_2, respectively. E_a is the reaction activation energy, A is the frequency factor, R is the molar gas constant and T is the temperature in Kelvin.

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$$C_t = C_0 - tk_0 \quad \text{Eq. 1}$$

$$\log C_t = \log C_0 - tk_1/2.303 \quad \text{Eq. 2}$$

$$1/C_t = 1/C_0 + tk_2 \quad \text{Eq. 3}$$

$$\log k = \log A - E_a/2.303R \,.1/T \quad \text{Eq. 4}$$

2.2.5 Preparation of standard solution for studies of degradation kinetics

The first dilution of the standard solution was prepared in HPLC grade methanol to get a concentration of 5.0 mg/ml. The second dilution was done by mobile phase to get a final concentration of 0.2 mg/ml. The standard solution was prepared freshly.

2.2.6 Preparation of stock sample for studies of degradation kinetics

The stock solution of the API was prepared in HPLC grade methanol to get a concentration of 5.0 mg/ml.

2.2.7 Preparation of analytical sample for studies of degradation kinetics

An aliquot of stock sample prepared for degradation kinetics study was diluted to 5 ml with 0.1N NaOH for ramosetron HCl and 2.0N NaOH for for ondansetron HCl, granisetron HCl and tropisetron HCl in four different volumetric flasks to get a concentration of 1.0 mg/ml. These solutions were kept in a dry oven at 60°C, 70°C and 80°C for 1, 2, 3, 4, and 5 hours. The degraded samples were neutralized with equimolar strength and volume of hydrochloric acid, respectively before further dilution with mobile phase to get a final concentration of 0.2 mg/ml.

2.3 Materials and methods for impurity profiling

Samples produced potential impurities during stress degradation and kinetic studies were considered under impurities investigations.

2.3.1 Drug substance and reagents

Fully characterized hydrochloride salt of ramosetron, ondansetron, granisetron, tropisetron and palonosetron bulk, manufactured by SMS Pharmaceutical Ltd., India, made available from

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Incepta Pharmaceutical Ltd. (Dhaka, Bangladesh).Methanol (MS grade), acetonitrile (MS grade), triethylamine (extra pure), hydrogen peroxide (extra pure) ,dipotassium hydrogen phosphate anhydrous (extra pure) and sodium hydroxide (extra pure) were purchased from Sigma-Aldrich. HPLC grade water was prepared by PALL purification system (PALL, cascada AN, USA). Hydrochloric acid (37% commercial grade) and orthophosphoric acid (reagent grade) were purchased from Labscan (ACI Labscan, Thailand).

2.3.2 Equipment used for studies of impurity profiling

Agilent HPLC system, equipped with integral autosampler (model 1260 HiP ALS) and quaternary gradient pump (model Quat Pump VL) with an on-line degasser was used. The column compartment (model 1260 TCC) having temperature control and a diode array detector (model 1260 DAD VL+) were employed throughout the analysis. Chromatographic data was integrated using Agilent Open LAB software. A hot air oven (Memmert, Mumbai, India) was used to maintain constant temperature. The stress photodegradation was carried out in a photostability chamber (Oswarld OPSH-G-16-GMP series, Oswarld scientific, Mumbai, India) equipped with illumination bank made of light source as described in the ICH guideline Q1B. An ultrasonicator from Power Sonic-405 (Hwashin Technology, Seoul, Korea) and pH meter from pH tutor (Eutech Instruments, Singapore) were used.

4000 QTRAP LC-MS-MS mass spectrometer (4000, AB SCIEX, USA) coupled with ESI ion source was used to generate MS data. The data were acquired using analyst software 1.5.2.

2.3.3 Chromatographic conditions used for studies of impurity profiling

Chromatographic separation was achieved fixing the column oven temperature at 40°C on a bonded phase cyano column (250 x 4.6 mm; CN; Kromasil, Waters or Supelcosil) using a mobile phase comprising of a mixture of acetonitrile-methanol-buffer (50 mM dipotassium hydrogen phosphate anhydrous containing 1 ml of triethylamine per liter with pH 7.0 adjusted by dilute orthophophoric acid) in the ratio (3 : 1 : 6). During the change of HPLC column of new brand remaining the same column packing, both the organic phases (acetonitrile and methanol) were changed by 5% to achieve acceptable resolution. The mobile phase so prepared was filtered through 0.45 µm membrane filter and degassed by sonication. Flow rate of 1.0 ml/min was

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maintained. The injection volume was 20 μL for all the analyses. The detection was carried out at the wavelength of 210 nm.

2.3.4 Procedure for impurity profiling

For investigation of degradants or impurities, stressed samples of aqueous, acid, base, oxidation and photolytic conditions were analyzed in HPLC and MS. Photo degradation of the drug substance was conducted in solid state. The concentration of the solutions kept for degradation under different stressed conditions was 1.0 mg/ml. The final concentration of the stressed solution was 0.2 mg/ml in mobile phase.

To generate MS scan data, each dilution of stressed sample was prepared by MS grade methanol. The final concentration of the solution used for MS scanning was 40.0 ng/ml. On the other hand, the concentration of photo degradation sample for MS scanning was 10.0 ng/ml.

For investigation of degradants and growing impurities, all chromatograms were evaluated thoroughly. The relative retention time of any degradant or growing impurity observed from HPLC chromatogram of the stressed samples was compared with known relative retention time (RRT) to that of the degradant or growing impurity. Similarly, molecular weight of any degradant or impurity observed from MS spectrum of the stressed samples was compared with known molecular weight to that of the degradant or growing impurity. Molecular weight, intensity (CPS), peak area and RRT are considered together to identify known or unknown growing impurities or degradants. Established impurities are summarized in the tables 5.1-5.4 for ondansetron HCl, granisetron HCl, tropisetron HCl and palonosetron HCl.

2.3.5 Preparation of standard solution for studies of impurity profiling

Required amount of ramosetron HCl, ondansetron HCl, granisetron HCl, tropisetron HCl and palonosetron HCl were weighed in separate volumetric flask. MS grade methanol was added to dissolve the compound then volume was adjusted to the mark to get a concentration of 5.0 mg/ ml. The second dilution was done by mobile phase to get a final concentration of 0.2 mg/ml. This standard solution was prepared on the day of analysis.

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2.3.6 Preparation of stock sample for studies of impurity profiling

Appropriate aliquot of ramosetron HCl, ondansetron HCl, granisetron HCl, tropisetron HCl and palonosetron HCl were weighed in separate volumetric flask. MS grade methanol was added to dissolve the compound then volume was adjusted to the mark to get a concentration of 5.0 mg/ ml.

2.3.7 Preparation of analytical sample for studies of impurity profiling

An aliquot of stock sample of ondansetron HCl, granisetron HCl and tropisetron HCl prepared for degradation study was diluted to 5 ml with purified water to get a concentration of 1.0 mg/ml. This solution was kept in a dry oven at 60°C for 21-days. These stressed samples were further diluted with mobile phase to get a final concentration of 0.2 mg/ml and analyzed by HPLC.

2.3.8 Preparation of analytical stressed sample for studies of impurity profiling under acid degradation

An aliquot of stock sample of ramosetron HCl, granisetron HCl, tropisetron HCl and palonosetron HCl prepared for degradation study was diluted to 5 ml with 0.1N HCl, 0.5N HCl, 1.0N HCl and 2.0N HCl solutions separately to get a concentration of 1.0 mg/ml. These solutions were kept in a dry oven at 60°C for 7 days. These stressed samples were neutralized with equimolar strength and volume of sodium hydroxide, respectively before further dilution with mobile phase to get a final concentration of 0.2 mg/ml and analyzed by HPLC.

2.3.9 Preparation of analytical stressed sample for studies of impurity profiling under oxidation

An aliquot of stock sample ramosetron HCl, ondansetron HCl and palonosetron HCl prepared for degradation study was diluted to 5 ml with 3.0% H_2O_2 solutions separately to get a concentration of 1.0 mg/ml. These solutions were kept in a dark place for 1 hour, 2 hours and 3 hours. These stressed samples were further diluted with mobile phase to get a final concentration of 0.2 mg/ml and analyzed by both HPLC and MS.

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2.3.10 Preparation of analytical stressed sample for studies of impurity profiling under photolysis

Bulk powder of ramosetron HCl and ondansetron HCl were evenly spread on separate aluminum foil and kept in the photostability chamber for direct exposure of fluorescence light (1.2 and 3.6 million lux) and UV light (200 and 600 watts hour/m^2) along with control sample wrapped with similar aluminium foil. A portion of the exposed and control samples were dissolved in 1.0 ml of HPLC grade methanol and diluted with mobile phase to get a final concentration of 0.2 mg/ml. These stressed samples were analyzed by HPLC and MS.

2.3.11 Preparation of standard solution to analyze samples obtained from the studies of degradation kinetics

The first dilution of the standard solution of ramosetron HCl, ondansetron HCl, granisetron HCl, tropisetron HCl and palonosetron HCl was prepared in MS grade methanol to get a concentration of 5.0 mg/ml. The second dilution was done by mobile phase to get a final concentration of 0.2 mg/ml. The standard solution was prepared freshly.

2.3.12 Preparation of stock sample for studies of degradation kinetics for impurity profiling purpose

The stock solution of each API was prepared in MS grade methanol to get a concentration of 5.0 mg/ml.

2.3.13 Preparation of analytical sample for studies of kinetics for impurity profiling purpose

An aliquot of stock sample of ramosetron HCl, ondansetron HCl, granisetron HCl and tropisetron HCl prepared for degradation kinetics study was diluted to 5 ml with 0.1N NaOH for ramosetron HCl and 2.0N NaOH for ondansetron HCl, granisetron HCl and tropisetron HCl in four different volumetric flasks to get a concentration of 1.0 mg/ml. These solutions were kept in a dry oven at 60°C, 70°C and 80°C for 1, 2, 3, 4, and 5 hours. The degraded samples were neutralized with equimolar strength and volume of hydrochloric acid, respectively before further

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dilution with mobile phase to get a final concentration of 0.2 mg/ml and analyzed by both HPLC and MS.