Tlc study of azathioprine and lignocaine hydrochloride biology essay

Science, Biology

4. 3. 1 Physical appearance TestAzathioprine and lignocaine hydrochloride were observed for color, odor and appearance. 4. 3. 2 Melting point DeterminationAzathioprineThe melting point of azathioprine was determined by the capillary method. In this method drug was filled into capillary tube sealed at one end at the height of 3mm from the closed end. The capillary tube was introduced into the digital melting point apparatus. Melting point was noted at temperature at which the drug starts melting till the entire sample gets melted was noted. Thus a range of melting was noted. The average of three determinations was considered for determination of melting point of azathioprine. Lignocaine hydrochlorideSimilar procedure as that for azathioprine was carried out for determination of melting point of lignocaine hydrochloride. 4. 3. 3 TLC Study of Azathioprine and Lignocaine hydrochlorideAzathioprineStationary phase- Silica gel GMobile phase- Chloroform : acetic acid :: 95 : 5ProcedureTLC plate was prepared using pouring method and activated by heating at 110oC for 30 minutes in hot air oven. A stock solution of azathioprine was prepared by dissolving minute quantity of drug in DMSO. Drug spot was applied on TLC plate 2 cm above the bottom edge with the help of thin capillary tubes. Spot was allowed to be completely dried before placing TLC plates in pre-saturated development chamber. After development i. e. when solvent front reaches up to 3/4th of height of TLC plate, plate was removed from chamber and analyzed for drug spot. Rf values of drug was calculated using following formula-RfLignocaine hydrochlorideSimilar procedure was carried out for TLC study of lignocaine hydrochloride using same stationary and mobile phase. 4. 3. 4 Infrared spectral analysis of Azathioprine and Lignocaine hydrochlorideThe IR

spectrum of azathioprine and lignocaine hydrochloride obtained as a dispersion of drugs in potassium bromide with a Shimadzu model FTIR 8400S spectrophotometer. Preparation of drug pellets-Finely grounded drug sample was mixed with potassium bromide (KBr) and pellets were prepared by applying 10 metric ton pressure using Shimadzu hydraulic press. Prepared pellets were then scanned over range of 4000-400cm-1. 4. 3. 5 Determination of absorbance maxima (λmax) of Azathioprine and Lignocaine hydrochlorideAn absorbance maximum of both drugs was determined using double beam UV-visible spectrophotometer (Shimadzu UV-1800). Preparation of Phosphate buffer saline (pH 6. 4)Accurately weighed 1. 79 g of disodium hydrogen phosphate, 1. 36 g of potassium dihydrogen phosphate and 7. 02 g of sodium chloride were dissolved in sufficient amount of distilled water to produce 1000 ml (Indian Pharmacopoeia, 2010). Determination of absorbance maxima (λmax) of Azathioprine50 mg of azathioprine was accurately weighed and dissolved in small quantity of 0. 1N NaOH (Prepared by dissolving 0. 4 g of NaOH in distilled water and final volume was made up to 100 ml with distilled water). The solution was then transferred to 50 ml volumetric flask. Final volume was made up to 50 ml with 0. 1N NaOH. From this stock, 10 µg/ml solution of azathioprine was made by diluting 1 ml of above stock solution up to 100 ml with phosphate buffer saline (pH 6. 4). 10 µg/ml solution was scanned over the range 400-200 nm on UV spectrophotometer to determine λmax of azathioprine. Determination of absorbance maxima (λmax) of Lignocaine hydrochloride50 mg of lignocaine hydrochloride was accurately weighed and dissolved in small quantity of 0. 1N NaOH. The solution was then transferred to 50 ml volumetric flask. Final

volume was made up to 50 ml with 0. 1N NaOH. From this stock, 100 µg/ml solution of lignocaine hydrochloride was made by diluting 1 ml of above stock solution up to 100 ml with phosphate buffer saline (pH 6. 4). 100 µg/ml solution was scanned over the range 400-200 nm on UV spectrophotometer to determine λmax of lignocaine hydrochloride. 4. 3. 6 Drug Excipient Compatibility Study (Physical and Chemical analysis)Compatibility study was done between pure drug, excipients and drug excipient mixture in different ratios as mentioned in table 1. Drug and each excipient was accurately weighed, thoroughly mixed and then introduced into vials. 5 ml of water for injection was added to each vial. All combination mixtures were covered with aluminium foil and divided equally in two groups i. e. porous and non porous. Both porous and non porous samples were kept a three different conditions i. e. 5±3oC (refrigerated), 25±2oC (room temperature) and 40±2oC/75±5% RH (Accelerated condition) for 30 days. Physical analysis of samples was carried out on 0th, 7th, 15th and 30th day was observing their appearance. Chemical analysis of samples was carried out by measuring Rf value of drugs using TLC technique at 0th and 30th day. 4. 4 Preparation of calibration curve of AzathioprinePreparation of stock solution of Azathioprine100 mg of azathioprine was accurately weighed and dissolved in small quantity of 0. 1N NaOH (Prepared by dissolving 0. 4 g of NaOH in distilled water and final volume was made up to 100 ml with distilled water). The solution was then transferred to 50 ml volumetric flask. Final volume was made up to 100 ml with 0. 1N NaOH. Preparation of serial dilutionsSerial dilutions were made from stock solution by diluting it with phosphate buffer saline pH 6. 4 to obtain 2, 4, 6, 8, 10, 12 µg/ml concentrations. All solutions were prepared in

triplicate. UV absorbance of these all dilutions were observed at 280 nm. Calibration plot was obtained by plotting value of absorbance against concentration. Regression coefficient and slope were also calculated. 4. 5 Method Validation for Azathioprine in pH 6. 4 phosphate buffer saline4. 5. 1 Linearity and RangeLinearity is the ability of the method to elicit the results of test samples that are directly proportional to analyte concentration within a given range. Range is the interval between the upper and lower levels of analytes that can be determined with accuracy, precision and linearity. Linearity had been accessed by calibration curve in which solutions of 2, 4, 6, 8, 10 and 12 µg/ml respectively were prepared in triplicate. Regression method was used to calculate linearity. The range of analytical procedure was given by interval between upper and lower concentration of azathioprine in the solution. 4. 5. 2 AccuracyIt is the closeness to the true value. It is the percent of analyte recovered by assay from a known added amount. This study was done by recovery studies. Standard samples of 4, 6 and 10 µg/ml were prepared in triplicate. UV absorbance was noted. Percentage mean recovery, standard deviation and percentage RSD was then calculated. 4. 5. 3 PrecisionIt is the degree of repeatability of analytical method under normal condition. The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurement obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. 4, 6 and 10 µg/ml dilutions were made in triplicate from stock solution of azathioprine and their U. V. absorbance were observed at 280 nm at three different time points of same day (intraday precision) and at three different time points on different days (interday precision). From

these all values of U. V. absorbance of dilutions their respective S. D., % R. S. D and % mean recovery was calculated. 4. 5. 4 RobustnessIt is the measure of capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during normal usage. 6 µg/ml dilution was prepared by three different analysts and their U. V. absorbance observed at 280 nm. From the values of U. V. absorbance of dilutions their respective S. D. and % R. S. D was calculated4. 5. 6 Limit of Detection (LOD) and Limit of Quantification (LOQ)Limit of detection is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. LOQ is defined as the lowest amount of analyte that can be determined quantitatively. LOD and LOQ were determined by standard deviation of the intercept of linear regression equation (σ) and slope of regression equation (S). Equations (1) and (2) for LOD and LOQ respectively are as follow. LOD= 3. 3 σ/S (1)LOQ= 10 σ/S (2)4. 6 Simultaneous Estimation of Azathioprine and Lignocaine hydrochlorideSimultaneous equation method was used for the simultaneous estimation of azathioprine and lignocaine hydrochloride in saline phosphate buffer (pH 6. 4). Procedure-Calibration plot of both drugs was plotted in phosphate buffer saline pH 6. 4 using sodium hydroxide as a co-solvent to know the value of concentrations of both drugs which had absorbance in the range (0. 2-0. 8). UV absorbance of selected concentrations of both drugs were observed at 262 (λ1) and 280 nm (λ2) for determination of absorptivities of both drugs at λ1 and λ2. Mixtures of both drugs were prepared in different ratios and UV absorbance of each mixture was observed at 262 and 280 nm. Concentration of each drug in mixture

calculated using following simultaneous equations-At λ1A1 = ax1bcx + ay1bcy (3)At λ2A2 = ax2bcx + ay2bcy (4)Where, ax1 and ax2 are absorptivities of drug X i. e. lignocaine hydrochloride at λ1 and λ2 respectively. ay1 and ay2 are absorptivities of drug Y i. e. Azathioprine at λ1 and λ2 respectively. A1 and A2 are absorbance of diluted samples at λ1 and λ2 respectively. cx and cy are concentrations of drug X and Y respectively in diluted samples. 4. 7 Validation of Simultaneous Estimation MethodFollowing parameters had been calculated for validation of simultaneous estimation method. 4. 7. 1 Linearity and RangeLinearity had been accessed by calibration curve of both drugs. Solutions of 2, 4, 6, 8, 10 and 12 µg/ml concentration were prepared for azathioprine and 100, 200, 300, 400 and 500 µg/ml concentration were prepared for lignocaine hydrochloride in triplicate in phosphate buffer saline pH 6. 4 using sodium hydroxide as a co-solvent. The range of analytical procedure was given by interval between upper and lower concentration of azathioprine and lignocaine hydrochloride in the solution. 4. 7. 2 AccuracyIt is the closeness to the true value. This study was done by recovery studies. Mixture of both drugs was prepared in different ratio i. e. azathioprine: lignocaine hydrochloride (1: 1, 2: 1 and 3: 1). Each ratio was prepared in triplicate. UV absorbance of each ratio was noted at 262 and 280 nm. Actual concentration of both drugs present in mixture was determined using simultaneous equation method. Percentage mean recovery, standard deviation and percentage RSD was then calculated for each ratio. 4. 7. 3 PrecisionIt is the degree of repeatability of analytical method under normal condition. U. V. absorbance of each ratio i. e. 1: 1, 2: 1 and 3: 1 was observed at 262 and 280 nm at three different time points of

same day (intraday precision) and at three different time points on different days (interday precision). From these all values of U. V. absorbance of different ratio of mixture at λ1 and λ2, S. D., percentage R. S. D and percentage mean recovery of both drugs were calculated. 4. 7. 4 RobustnessIt is the measure of capacity to remain unaffected by small, but deliberate variations in the method parameters and provides an indication of its reliability during normal usage. 2: 1 mixture was prepared by three different analysts and U. V. absorbance was observed at 262 and 280 nm. From the values of U. V. absorbance, respective S. D. and % R. S. D of both drugs was calculated. 4. 8 Formulation of Dummy Nano-elastic vesiclesThin film hydration method was used for the preparation of elastic vesicles (Cevc and Blume, 2001; Singh et al., 2009; Trotta et al., 2002). Phospholipid and surfactant were accurately weighed and dissolved in chloroform: methanol mixture in 250 ml round bottom flask. Solvent was evaporated using rotary vacuum evaporator at 50oC (under reduced pressure) and 100 rpm to obtain a thin lipid layer. This layer was kept overnight in vacuum oven at 60oC for removal of residual amount of moisture present in lipid layer. Lipid film was then hydrated at 60oC and 60 rpm for one hour to obtain transferosomal suspension. This suspension was then kept at room temperature for two hours for complete swelling of phospholipid. Resulting solution was then sonicated using bath sonicator for 30 minutes for further size reduction of vesicles.

Table 4. 3

Components used in preparation of dummy Nano-elastic vesicles (Gavali et al., 2011)

S. No.

Class

Chemical Name

Use

1PhospholipidSoya lecithinVesicle forming component2SurfactantSpan 60, Span 80For providing flexibility3AlcoholMethanolAs a solvent4Buffering agentPhosphate buffer saline pH 6. 4As hydration media4. 9 Formulation of Drug loaded Nano-elastic vesiclesSame procedure was followed as that was used for preparation of dummy nano-elastic vesicles. Drug was dissolved in aqueous phase for its incorporation in dummy nano-elastic vesicles. Composition of different vesicles described in table 4. 4.

Table 4. 4

Composition of prepared Nano-elastic vesicles

S. No.

Formulation code

Soya lecithin (mg)

Span 60

(mg)

Span 80

(mg)

Drug (Azathioprine)

(mg)

Hydration Media

CHCl3 : CH3OH

1F1283. 33

-

1525A*80: 202F2283. 33

-

1525B*80: 203F3316. 675

-

25A*80: 204F4300. 0010

-

25A*80: 205F5283. 3315

-

25A*80: 206F6283. 3315

-

25B*80: 207F7266. 6720

-

25A*80: 208F8250. 0025

-

25A*80: 20A*- NaOH + Phosphate buffer saline pH 6. 4, B*- NaOH + Ethanol (7%)4. 10 Characterization of prepared Nano elastic vesicle (Transferosome®)4. 10. 1 Optical microscopyEight formulations prepared were examined using optical microscope before sonication to observe shape of prepared vesicles. A drop of prepared formulation was placed on glass slide and cover slip was carefully placed over it. Sample was observed at 1000x using immersion oil. 4. 10. 2 Drug Entrapment efficiencyUltracentrifugation method was used for studying entrapment efficiency. 10 ml of transferosomal suspension was centrifuged at 35, 000 rpm (205, 432g) for 50 minutes for separation of free drug. Supernatant was removed and formed pellet was washed with triple distilled water to remove free drug from formed pellet. Pellet was then dissolved in phosphate buffer saline pH 6. 4. These vesicles were the digested with the help of triton X-100 (0. 1% v/v) and resulting solution was then spectrophotometrically analyzed at 280 nm. Entrapment efficiency was calculated using following equation- Entrapment efficiency (%) × 1004. 10. 3 Vesicle size and size

distributionVesicle size and size distribution was determined using Malvern ZetasizerTM (Malvern Instruments Ltd., UK) which works on dynamic light scattering (DLS) method. Vesicle suspension was suitably diluted to prevent multi scattering phenomena. The PDI was observed to determine width of particle size distribution. 4. 10. 4 Determination of zeta potential of optimized nanoelastic vesiclesZeta potential of optimized formulation was determined using Malvern ZetasizerTM. Sample was introduced into zeta cuvette after proper dilution. An average of 20 zeta counts was used for determination of zeta potential of optimized formulation (Aggarwal and Goindi, 2012). 4. 10. 5 Morphological study of optimized nanoelastic vesiclesThe optimized formulation had been evaluated for morphology by Transmission electron microscopy (FEI Technai F 20). A drop of sample was placed on carbon coated copper grid to form a thin film on the grid. Nanoelastic vesicles were negatively stained with phosphotungstic acid (1% w/v). Excess stain was drained off with filter paper and sample was examined in TEM at 120 kV. 4. 11 Preparation of topical hydrogel containing Azathioprine (H1)Procedure25 mg of carbopol 934 was accurately weighed and dispersed in distilled water. Transferosomal suspension equivalent to 50 mg of pure drug was added to above solution. Preservatives were added in final volume of solution was made up to 5 ml with distilled water. Resulting solution was left for 2 hours at room temperature for swelling of carbopol 934. Triethanolamine was added dropwise to maintain the pH and consistency of the gel. Composition of topical hydrogel H1 had been summarized in table 4. 5. 4. 12 Preparation of topical hydrogel containing Azathioprine and lignocaine hydrochloride (H2)Procedure25 mg of carbopol

934 was accurately weighed and dispersed in distilled water. Transferosomal suspension equivalent to 50 mg of pure drug was added to above solution.

Table 4. 5

Composition of Topical Hydrogel

Component

Topical Hydrogel

H 1

H 2

Azathioprinetransferosomal suspensionEquivalent to 1 % w/wof pure drugEquivalent to 1 % w/wof pure drugLignocaine hydrochloride

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2 % w/wCarbopol 9340. 5 % w/w0. 5 % w/wButylated hydroxylanisole0. 02 % w/w0. 02 % w/wMethyl paraben0. 2 % w/w0. 2 % w/wPropyl paraben0. 02 % w/w0. 02 % w/wTriethanolamineq. s. q. s. Distilled waterq. s. q. s. 100 mg of lignocaine hydrochloride and preservatives were added in final volume of solution was made upto 5 ml with distilled water. Resulting solution was left for 2 hours at room temperature for swelling of carbopol 934. Triethanolamine was added dropwise to maintain the pH and consistency of the gel. Composition of topical hydrogel H2 had been summarized in table 4. 5. 4. 13 Evaluation of topical hydrogelPrepared topical hydrogels were examined for determination of following parameters. 4. 13. 1. AppearancePrepared hydrogels were visually examined for clarity, color and presence of any unwanted particles. 4. 13. 2 pHpH of prepared hydrogels

were examined using digital pH meter. 4. 13. 3 ViscosityViscosity of prepared hydrogel was examined using digital Brookfield viscometer at 3 rpm using spindle S-63. 4. 13. 4 Drug contentDrug content was measured using flask shake method. Prepared hydrogel was dissolved in saline phosphate buffer pH 6. 4 using sodium hydroxide as co-solvent in 100 ml volumetric flask. Content of volumetric flask were shaken for 2 hr. on mechanical shaker for complete dissolution of drug. Solution was sufficiently diluted and UV absorbance was noted for determination of drug content in hydrogels. 4. 14 In vitro drug release study of topical hydrogel H1 and H2In vitro release study of topical hydrogel was carried out by using Franz diffusion cell having receptor compartment of 25 ml capacity and effective diffusion area of 2. 54 cm2. Cellophane membrane was mounted on diffusion cell assembly having phosphate buffer saline pH 6. 4 in its receptor compartment. Content of receptor compartment was stirred at 100 rpm using magnetic stirrer and temperature was maintained at 32 ± 0. 5oC during whole study time period using external water jacket. 1 g of gel formulation was placed in donor compartment and aliquot of 2 ml was withdrawn at regular time intervals i. e. 15, 30, 45, 60, 90, 120, 240, 480, 720 and 1440 min. through sampling port of receptor compartment. 2 ml fresh diffusion media was added at each withdrawal point to sink condition maintain in receptor compartment. Samples were analyzed spectrophotometrically for determination of amount of drug diffused through cellophane membrane. 4. 15 Interpretation of release mechanism of Drugs from topical hydrogel H1and H2In vitro drug release data was analyzed for determination of type of release model followed. Release data was studied

for different kinetic equations i. e. zero order, first order, Higuchi, Hixson-Crowell and Korsmeyer- Peppas model equations. Dash et al., 2010 described equation for different release models. They are as follow-4. 15. 1 Zero order modelThis model is generally used to describe drug dissolution profile of those dosage forms from which drug is slowly released. It can be represented by the equation: Q0 - Qt = K0twhere, Qt is the amount of drug dissolved in time t, Q0 is the initial amount of drug in the solution, andK0 is the zero order release constant which is expressed in units of concentration/time. For determination of the release kinetics, graph was plotted between cumulative amount of drug released and time. 4. 15. 2 First order modelThe release of the drug which follows first order kinetics can be determined using following equation-: log C = log C0 - Kt / 2. 303where, C0 is the initial concentration of drug, C is the concentration of drug present at time t, K is the first order rate constant, For determination of the release kinetics, graph was plotted between log cumulative percentageof drug remaining and time. Slope of graph is equal to –K/2. 303, thus value of rate constant can be calculated from the slope. 4. 15. 3. Higuchi modelThis model is used to describe drug release from a matrix system. Earlier it was used only for planar systems, but later it was extended to geometrics and porous systems. The release of the drug which follows Higuchi model can be determined using following equation-: Q = KH × t1/2where, Q is the amount of drug released in time t per unit area, andKH is the Higuchi dissolution constant. For determination of the release kinetics, graph was plotted between cumulative percentage drug release and square root of time. 4. 15. 4 Hixson-Crowell modelHixson and Crowell showed that regular area of

particle is proportional to the cubic root of its volume. They had derived following equation for determination of release profile of drug from pharmaceutical dosage forms-W0 1/3 - Wt 1/3 = κ twhere, W0 is the initial amount of drug present in dosage form, Wt is amount of drug remained in the dosage form at time t, andκ (kappa) is a constant which incorporates the surface and volume relation. For determination of the release kinetics, graph was plotted between cube root of drug percentage remaining in dosage form and time. 4. 15. 5 Korsmeyer-Peppas modelKorsmeyer and Peppas described a simple mathematical equation for determination of drug release from a polymeric system. For determination of mechanism of drug release, first 60% drug release data was filled in following Korsmeyer-Peppas model equationMt / M∞ = K tnwhere, Mt / M∞ is a fraction of drug released from dosage form at time t, k is the release rate constant andn is the release exponent. For determination of the release kinetics, graph was plotted between log cumulative percentage drug release and log time.