

INDEX

TITLE.....	PAGE NO.
<i>List of Figures</i>	<i>i</i>
<i>List of Tables</i>	<i>vi</i>

CHAPTER 1 INTRODUCTION	1-6
References.....	5

CHAPTER 2 LITERATURE REVIEW.....	7-47
---	-------------

2.1 Cerebral ischemia	7
2.1.1 Current Research Goals and Scientific Rationale for Treatment	8
2.1.2 Current Therapies in Ischemic Stroke	8
2.1.2.1 Medical Support.....	9
2.1.2.2 Thrombolytic Therapy	9
2.1.2.3 Anticoagulation	10
2.1.2.4 Other Anticoagulants	11
2.1.2.5 Antiplatelets Agents.....	11
2.1.2.6 Neuroprotection and Current Status.....	12
2.2 Nasal Delivery system	13
2.2.1 Nasal Anatomy and Physiology	14
2.2.2.1 Advantages of Intranasal Drug Administration(45)	15
2.2.2.2 Limitations of Nasal Delivery System.....	16
2.2.3 Mechanism of Nasal Drug Absorption.....	16
2.2.4 Factors Affecting Nasal Absorption of Drug across Nasal Epithelium	16
2.2.4.1 Physiological Issue	17
2.2.4.2 Physicochemical Character of the Formulation and Drug Substance..	17
2.2.4.3 Delivery Device Related Factors	19
2.2.5 Characteristics Feature of Nasal Dosage Forms(45).....	20
2.2.6 Formulations for Nasal Drug Delivery.....	20
2.2.6.1 Nasal Drops	20
2.2.6.2 Nasal Sprays (Solution and Suspension Sprays)	20
2.2.6.3 Micro-emulsions	20

2.2.6.4 Nasal Gels.....	21
2.2.6.5 Nasal Powders	21
2.2.6.6 Nanoparticles	21
2.2.6.7 Liposomes	21
2.3 Micelles.....	21
2.3.1 Polymeric Micelles.....	23
2.3.2 Targeted Polymeric Micelles	25
2.3.3 Advantages of Polymeric Micelle as a Drug Carrier	28
2.3.4 Disadvantages of Polymeric Micelle as a Drug Carrier	30
2.3.5 Methods of Preparation of Polymeric Micelles	31
2.3.5.1 Dialysis Method.....	31
2.3.5.2 Oil-in-Water Emulsion Solvent Evaporation Method	31
2.3.5.3 Solid Dispersion Method.....	32
2.3.5.4 Microphase Separation Method.....	32
2.3.6 Targeted Polymeric Micelles	32
2.4 Drug and Polymer Profile	34
2.4.1 Nicergoline	34
2.4.2 Poloxamer F 127.....	35
2.4.3 Tocopherol Polyethylene Glycol 1000 Succinate (TPGS)	36
2.5 Research Envisaged	37
2.6 Reference.....	38
CHAPTER 3 ANALYTICAL METHOD DEVELOPMENT	48-63
3.1 Introduction	48
3.2 Materials and Instruments	49
3.2.1 Selection of Chromatographic Conditions.....	50
3.2.1.1 Column.....	50
3.2.1.2 Mobile Phase	51
3.2.1.3 Flow Rate	51
3.2.1.4 UV/Vis Detection	52
3.3 Reagents	52
3.3.1 Phosphate Buffer.....	52
3.3.2 Stock Solution of Drug.....	52

3.3.3 Standard Solutions of Drugs	52
3.4 Preparation of Calibration Curve.....	53
3.5 Method Validation	53
3.5.1 Specificity and selectivity	53
3.5.2 Accuracy	54
3.5.3 Linearity.....	54
3.5.4 Range.....	54
3.5.5 Precision	55
3.5.6 Limit of detection (LOD) and quantification (LOQ)	55
3.5.7 Robustness	56
3.5.8 Solution Stability.....	56
3.6 Result and Discussion	56
3.6.1 Method Development	56
3.6.2 Calibration Standard	57
3.6.3 Accuracy	58
3.6.4 Precision	59
3.6.5 LOD and LOQ	60
3.6.6 Robustness	60
3.6.7 Solution Stability.....	61
3.7 Conclusion.....	62
3.8 References	62

CHAPTER 4 DEVELOPMENT, OPTIMIZATION AND CHARACTERIZATION OF MICELLE FORMULATION64-103

4.1 Introduction	64
4.2 Materials and Equipment	67
4.3 Method	67
4.3.1 Selection of Surfactant	67
4.3.2Preparation of Nicergoline Loaded Micelles	68
4.3.3 Separation of Un-entrapped Drug	68
4.3.4 Optimization of Process TFH Parameters	68
4.4 Optimization of Formulation Components	69
4.5 Characterization of Micelle.....	72

4.5.1 Particle Size and Zeta Potential Analysis	72
4.5.2 Zeta Potential Analysis	72
4.5.3 Drug Loading and Encapsulation Efficiency	72
4.5.4 Critical Micelle Concentration (CMC) Determination	72
4.5.5 Fourier Transform Infra-Red (FT-IR)	73
4.5.6 Differential Scanning Calorimetric (DSC)	73
4.5.7 Morphological Analysis of Micelle.....	73
4.6 In Vitro Diffusion Studies	73
4.7 Nasal Toxicity Studies	76
4.8 Result and Discussion	76
4.8.1 Feasibility Trial	76
4.8.2 Impact of Process Parameters on Micelle Formulation.....	77
4.8.2.1 Speed of Rotation.....	77
4.8.2.2 Hydration Volume	77
4.8.2.3 Hydration Time.....	78
4.8.2.4 Temperature for Removal of Organic Solvent	78
4.8.2.5 Vacuum	78
4.8.3 Optimisation of components of micelle formulation	79
4.8.3.1 Percent Drug entrapment.....	79
4.8.3.2 Particle size.....	84
4.8.3.3 Selection of Optimized Formulation.....	89
4.8.3.4 Point Prediction and Confirmation	90
4.8.4 Micelle Characterisation.....	91
4.8.4.1 Particle Size and Zeta Potential	91
4.8.4.2 Morphological Analysis of Micelle	92
4.8.4.3 CMC Determination.....	92
4.8.4.4 DSC Thermogram.....	93
4.8.4.5 FTIR	94
4.8.5 In vitro Diffusion Studies	95
4.9 Conclusion.....	99
4.10 Reference.....	99

CHAPTER 5 FUNCTIONALIZATION OF MICELLE FORMULATION..... 103-111

5.1 Introduction	104
5.2 Materials and Methods.....	105
5.3 Preparation of Functionalized Micelle.....	105
5.3.1 Introduction.....	105
5.3.2 Identification of Maleimide Functional Group of DSPE-mPEG2000-maleimide	105
5.3.3 Confirmation of DSPE-mPEG2000-Maleimide Insertion in Micelle.....	106
5.4. Results and Discussion	107
5.4.1. Preparation of Functionalized Micelle	107
5.4.2 Confirmation of Presence of Maleimide Groups over Micelle.....	108
5.4.3 Calculation of the Free Sulfhydryl Concentration	108
5.5 Reference.....	110

CHAPTER 6 PREPARATION OF PEPTIDE CONJUGATED MICELLE 112-124

6.1 Introduction	112
6.2 Materials and Methods.....	115
6.2.1 Ellman’s assay to confirm the presence of –SH groups on CLEVSRKNC peptide	115
6.2.2 Preparation of Peptide Conjugated Micelle.....	115
6.2.3 Characterization of Peptide Conjugated Micelle	116
6.2.3.1 Determination of Sulfhydryl Groups on Peptide.....	116
6.2.3.2 SDS-PAGE Analysis of Peptide Conjugated Micelles.....	117
6.2.4 Silver Staining Protocol.....	119
6.2.5 Determination Peptide Concentration in Unknown Solution and Micelle Surface	120
6.2.5.1 Isolation of Conjugate from Reaction Mixer	120
6.3 Results and Discussion	120
6.3.1 SDS-PAGE Analysis of Peptide Conjugated Micelles	120
6.3.2 Preparation of Peptide Conjugated Micelle	123
6.4 Reference.....	124

CHAPTER 7 IN VITRO CYTOTOXICITY STUDIES 125-143

7.1 Effect of formulations on viability of nasal and neuronal cells	125
7.1.1 Introduction.....	125
7.1.1.1 RPMI-2650 Cell line.....	126
7.1.1.2 SH-SY5Y Cell line	127
7.1.1.3 MTT Assay.....	127
7.1.2 Cell line subculture.....	129
7.1.2.1 Protocol for RPMI 2650 cell line subculture.....	129
7.1.2.2 Protocol for SH SY5Y cell line sub-culture (6, 7).....	130
7.1.2.3 Protocol for MTT assay	130
7.1.3 Results and discussion.....	132
7.1.4 Conclusions.....	135
7.2 Effect of formulations on H ₂ O ₂ induced oxidative stress.....	136
7.2.1 Introduction.....	136
7.2.2 Determination of IC 50 value of H ₂ O ₂ induced oxidative stress	136
7.2.3 Neuroprotective effect of Nicergoline micelle in H ₂ O ₂ induced oxidative stress	136
7.2.4 Results and discussion.....	137
7.2.5 Conclusion	140
7.3 References	141

CHAPTER 8 PRESERVATIVE EFFICACY STUDY 144-152

8.1 Introduction	144
8.2 Preparation of inoculums	145
8.3 Method	146
8.3.1 Criteria of Acceptance	146
8.3.2 Formulations Tested	147
8.4 Results of Microbial challenge Test	147
8.5 Result and discussion.....	151
8.6 References	152

CHAPTER 9 STABILITY STUDIES..... 153-157

9.1 Introduction	153
9.2 Method For Stability Study and Characterization Parameters	154
9.2.1 Dilution stability and Uniformity in dispersion	154
9.2.2 Assay and Drug retention	154
9.2.3 Micelle Size and Zeta Potential	154
9.2.4 pH of formulation.....	155
9.2.5 % Transmittance.....	155
9.2.6 Stirring.....	155
9.3 Results and Discussion	155
9.3.1 Dilution stability and Uniformity in dispersion.....	155
Assay, Drug retention, Size, pH, Size, Zeta Potential	156
9.4 Conclusion.....	157
9.5 Reference.....	157

CHAPTER 10 MICELLE NEUROPROTECTIVE AND TARGETING EFFICIENCY

..... 158-169

10.1 Introduction	158
10.2 Methods.....	159
10.2.1 Induction of transient global ischemia in brain.....	159
10.2.2 Procedure for Sham Surgery	160
10.2.3 Evaluation of anti-ischemic activity	160
10.2.4 Histological estimation of brain infarct volume	161
10.2.5 Preparation of TTC.....	161
10.2.6 Estimation of brain glutathione.....	161
10.2.7 Method to prepare brain homogenate.....	162
10.2.8 Preparation of coumarin loaded peptide conjugated TPGS/PF 127 micelles .163	
10.2.8.1 Targeting efficiency of peptide conjugated micelle.....	163
10.3 Statistical Analysis.....	163
10.4 Results.....	164
10.5 Discussion	166
10.6 Conclusion.....	167
10.7 References	167

CHAPTER 11 DEVELOPMENT, OPTIMIZATION AND CHARACTERIZATION OF NASAL SPRAY	170-220
11.1 Introduction	170
11.2 Material and Methods	171
11.3 Optimization of Spray parameters	171
11.3.1 Factors responsible for variation in spray parameters	171
11.3.2 Determination of Droplet Size Distribution (D10, D50, D90)	177
11.3.2.1 Statistical Analysis of Response 1 (D90)	177
11.3.2.2 Statistical Analysis of Response 1 (D50)	183
11.3.2.3. Statistical Analysis of Response D10	186
11.3.2.4 Impact of distance on Droplet size distribution	191
11.3.2.5 Impact of Force on droplet size distribution	192
11.3.3 Statistical Analysis of Response 2 (Shot weight)	193
11.3.4. Statistical Analysis of Response 3 (Span)	204
11.3.5 Statistical Analysis of Response 4 (Stable Phase)	209
11.4 Selection of Optimum Processing Parameters	215
11.5 Desirability Plot for Selection of Optimum Process Parameters	216
11.6 Point Prediction and Confirmation	217
11.7 Conclusion	217
11.8 References	218
CHAPTER 12 SUMMARY AND CONCLUSION	221-236

List of Figures

Figure 1.1 Cerebral ischemia cascade events; PARP: Poly A Ribose Polymerase, iNOS: inducible nitric oxide synthase.....	3
Figure 2.1 Self assemble structure of block copolymer micelles.	24
Figure 3.1 Structure of Nicergoline	48
Figure 3.2 Calibration curve of Nicergoline.	58
Figure 3.3 Chromatogram of calibration curve.....	58
Figure 4.1 Impact of poloxamer F127 concentration on drug entrapment.	81
Figure 4.2 Impact of TPGS concentration on drug entrapment.	81
Figure 4.3 Impact of drug concentration on drug entrapment.	81
Figure 4.4 Combined impact of PF and TPGS concentration on drug entrapment.	82
Figure 4.5 Combined impact of PF and nicergoline concentration on drug entrapment.	82
Figure 4.6 Combined impact of TPGS and drug concentration on drug entrapment.	82
Figure 4.7 Impact of PF on micelle particle size.	86
Figure 4.8 Impact of TPGS on micelle particle size.....	86
Figure 4.9 Impact of drug on micelle particle size.	87
Figure 4.10 Combined impact of PF and TPGS on micelle particle size.	87
Figure 4.11 Combined impact of PF and nicergoline on micelle particle size.	87
Figure 4.12 Combined impact of TPGS and nicergoline on micelle particle size.....	88
Figure 4.13 Desirability plot for selection of parameters.	90
Figure 4.14 TEM images of nicergoline micelle.	92
Figure 4.15 CMC determination of micelle	93
Figure 4.16 DSC Thermogram	94
Figure 4.17 FTIR spectra of physical mixture, blank micelle, Nicergoline loaded micelle and Nicergoline.	95
Figure 4.18 In vitro diffusion of Nicergoline.....	96
Figure 4.19 Nasal Toxicity in excised sheep nasal mucosa (A) IPA treatment 1 Hr (B) IPA treatment 2 hrs (C) PBS Treatment 1 Hr (D) PBS treatment 2 Hrs (E) Treatment with	

Drug loaded micelle formulation 1 Hr	(F) Drug loaded micelle formulation 2 Hrs	
(G) Treatment with Peptide conjugated Micelle 1 Hr	(H) Treatment with Peptide conjugated Micelle 2 Hrs	98
Figure 5.1 Chemical structure of DSPE-mPEG2000-Maleimide		107
Figure 5.2 FTIR spectrum of DSPE-mPEG2000-Maleimide		107
Figure 6.1 Mechanism of silver staining of separated proteins on SDS-PAGE gel		114
Figure 6.2 Confirmation of CLEVSRKNC peptide and peptide conjugated micelles		122
Figure 7.1 Reduction of MTT using mitochondrial reductase		128
Figure 7.2 Effect of different formulations on cell viability of RPMI 2650 cell line		133
Figure 7.3 Effect of different formulations on cell viability of SHSY5Y cell line		134
Figure 7.4 H ₂ O ₂ -induced cytotoxicity on SH SY5Y cells		137
Figure 7.5 Effect of NCG 0.2 μ M on cell viability		138
Figure 7.6 Effect of NCG 0.4 μ M on cell viability		139
Figure 7.7 Effect of NCG 0.6 μ M on cell viability		139
Figure 7.8 Effect of NCG 0.8 μ M on cell viability		140
Figure 8.1 Sub-culture of test microorganism		146
Figure 8.2 Colony count of control sample vs preservative at T – 0 time point		150
Figure 8.3 Comparison of control sample vs preservative at T – 7 time point		150
Figure 8.4 Comparison of control sample Vs preservative at T – 14 time point		151
Figure 8.5 Comparison of control sample Vs preservative at T – 28 time point		151
Figure 9.1 PF 127/TPGS micelle particle size at 25 °C and 100 rpm shaking (A: Before incubation; B: after 24 h incubation; C: after 48 h incubation)		156
Figure 10.1 TTC stained brain slices A Sham control (Group I), B Ischemic control (Group II), C NGM (Group III), D NGPC (Group IV)		164
Figure 10.2 Effect of NG on infarct volume		165

Figure 10.3 Effect of NG on brain edema.....	165
Figure 10.4 Effect of NG on brain glutathione (GSH)	165
Figure 10.5 A) Brain section of rat treated with MGM B) Brain section of rat treated with NGPC.....	166
Figure 11.1 One factor response plots A. Distance Vs D90 B. Angle Vs D90 C. Force Vs D90	180
Figure 11.2 Two factor response surface plots A. Force x distance Vs D90 B. Angle x Distance Vs D90	181
Figure 11.3 Pattern of plume determined by TLC method.....	182
Figure 11.4 Phases of nasal spray development	182
Figure 11.5 One factor response plots A. Distance Vs D50 B. Force Vs D50 C. Angle Vs D50	185
Figure 11.6 Two factor response surface plots A. Force x distance Vs D50 B. Angle x Distance Vs D50	186
Figure 11.7 One factor response plots A. Distance Vs D10 B. Force Vs D10 C. Angle Vs D10	189
Figure 11.8 Two factor response surface plots A. Force x distance Vs D10 B. Angle x Distance Vs D10	190
Figure 11.9 Plot showing impact of distance on DSD.....	191
Figure 11.10 Plot showing impact of Force on DSD.....	192
Figure 11.11 One factor response plots A. Distance Vs Shot weight B. Angle Vs Shot weight C. Force Vs Shot weight.....	196
Figure 11.12 Two factor response surface plots A. Angle x distance Vs Shot weight B. Angle x Force Vs Shot weight.....	197
Figure 11.13 A) Conventional Nasal Spray B) Modified Nasal Spray.....	198
Figure 11.14 Number of sprays Vs shot weight	201
Figure 11.15 Impact of resting time on shot weight	202
Figure 11.16 Plot showing effect of stroke length on shot weight	204
Figure 11.17 One factor response plots A. Distance Vs Span B. Force Vs Span C. Angle Vs Span.....	207
Figure 11.18 Response surface plot showing effect of Angle and Force on Span	208
Figure 11.19 One factor response plots A. Distance Vs Stable Phase B. Force Vs Stable Phase C. Angle Vs Stable Phase D. Force Vs Stable Phase	212

Figure 11.20 Two factor response surface plots A. Angle x distance Vs Stable phase B. Force time x Force Vs Stable Phase.....	213
Figure 11.21 Qt diagram	214
Figure 11.22 Plot showing effect of force on stages of spray development	215
Figure 11.23 Desirability plot for selecting optimum parameters	216

List of Tables

Table 3.1 Materials and equipments	50
Table 3.2 Final chromatographic conditions	52
Table 3.3 Peak area for different concentration of nicergoline	57
Table 3.4 Accuracy data of developed method.....	58
Table 3.5 Standard addition data of developed method.....	59
Table 3.6 Data of system precision and method precision study.....	59
Table 3.7 Results of intermediate precision.....	60
Table 3.8 LOD and LOQ determination	60
Table 3.9 Data of robustness of developed method	61
Table 3.10 Data of ruggedness of developed method.....	61
Table 3.11 Data of solution stability study	62
Table 4.1 List of materials	67
Table 4.2 List of equipment	67
Table 4.3 Process parameters for micelle formation	69
Table 4.4 Various factors and responses used in optimization	69
Table 4.5 Coded and actual values of process parameters.....	70
Table 4.6 Design matrix.....	71
Table 4.7 Results of initial feasibility trails	76
Table 4.8 ANOVA analysis of different models	79
Table 4.9 ANOVA table for response surface quadratic model	79
Table 4.10 Summary of ANOVA results of quadratic model	80
Table 4.11 ANOVA analysis of different models	84
Table 4.12 ANOVA for response surface quadratic model	85
Table 4.13 Summary of ANOVA results of quadratic model	85
Table 4.14 Constraints Applied for Selection of Optimized Batch	89
Table 4.15 Formulation parameters based on desirability	90
Table 4.16 Experimental confirmation of predicted results.....	91
Table 4.17 Formulation optimization by % drug entrapment.....	91
Table 4.18 In vitro drug release kinetics.....	97

Table 5.1 Estimated cysteine (thiol) concentrations using Elman’s assay	110
Table 6.1 Composition of resolving gel.....	118
Table 6.2 Composition of stacking gel	118
Table 6.3 Sample loading buffer composition.....	119
Table 6.4 Comparison Functionalized micelles and peptide conjugated micelles	123
Table 7.1: Material and Equipment list.....	129
Table 7.2 Relative cell viability of different formulations in RPMI 2650 cell line.....	132
Table 7.3: Relative Cell viability of different formulations in SHSY5Y cell line	133
Table 7.4 Hydrogen Peroxide Toxicity Study	137
Table 7.5 Data of Neuroprotective study	138
Table 8.1 Acceptance criteria for preservative effectiveness testing as per USP.....	147
Table 8.2 Test formulations	147
Table 8.3 Results of microbial challenge to Formulation 1 with ought preservative	147
Table 8.4 Log reduction in microbes after microbial challenge to NS 1 without preservative ..	148
Table 8.5 Results of microbial challenge to NS 2 with 0.025% w/v preservative	148
Table 8.6 Log reduction in microbes after microbial challenge to NS 2 with 0.025% w/v preservative	148
Table 8.7 Results of microbial challenge to NS 3 with 0.05% w/v preservative	149
Table 8.8 Log reduction in microbes after microbial challenge to NS 3 with 0.05% w/v preservative	149
Table 8.9 Results of microbial challenge to NS 4 with 0.1% w/v preservative	149
Table 8.10 Log reduction in microbes after microbial challenge to Formulation 4 with 0.1% w/v preservative	150

Table 9.1 Dilution stability of micelle	155
Table 9.2 Stability compilation of micelle formulation	156
Table 10.1 Brain infarct volume, ratio of hemisphere areas, glutathione estimated for anti- ischemic activity	164
Table 11.1 Various Factors and Responses Used in Optimization	171
Table 11.2 Coded and Actual Values of Process Parameters	172
Table 11.3 Design matrix.....	173
Table 11.4 ANOVA Analysis of Different Models for Response 1 - D90.....	177
Table 11.5 Factors Selected on Basis of Stepwise Regression Analysis D90	178
Table 11.6 ANOVA Table for Response Surface Reduced 2FI Model Response 1 - D90	178
Table 11.7 Summary of ANOVA Results of Reduced 2FI Model Response 1-D90	179
Table 11.8 ANOVA Analysis of Different Models (D50)	183
Table 11.9 Factors Selected on Basis of Stepwise Regression Analysis (D50)	183
Table 11.10 ANOVA for Response Surface Reduced 2FI Model (D50)	184
Table 11.11 Summary of ANOVA results of 2FI model (D50)	184
Table 11.12 ANOVA Analysis of Different Models (D10)	187
Table 11.13 Factors Selected on Basis of Stepwise Regression Analysis (D10)	187
Table 11.14 ANOVA table for Response Surface Reduced 2FI Model (D10)	188
Table 11.15 Summary of ANOVA results of reduced quadratic model.....	188
Table 11.16 ANOVA Analysis of Different Models on response-2 Shot weight	193
Table 11.17 Factors Selected on Basis of Stepwise Regression Analysis on Shot weight.....	194
Table 11.18 ANOVA table for Response Surface Reduced Quadratic ModelResponse 2 -Shot weight	194
Table 11.19 Summary of ANOVA Results of Reduced Quadratic Model Response 2-Shot weight.....	195
Table 11.20 Effect of priming on shot weight	199
Table 11.21 Number of sprays and their shot weight	200
Table 11.22 Effect of Resting Time on Priming, Repriming.....	202
Table 11.23 Effect of temperature cycle on DSD	203

Table 11.24 ANOVA analysis of Different Models Response 3 (Span)	204
Table 11.25 Factors Selected on Basis of Stepwise Regression Analysis Response 3 (Span)	205
Table 11.26 ANOVA Table for Response Surface Reduced Quadratic Model	
Response 3 (Span)	205
Table 11.27 Summary of ANOVA Results of Reduced Quadratic Model response 3 – Span....	206
Table 11.28 ANOVA Analysis of Different Models on Response 4 (Stable Phase).....	209
Table 11.29 Factors Selected on Basis of Stepwise Regression Analysis	209
Table 11.30 ANOVA table for Response Surface Reduced 2FI Model (Stable Phase)	211
Table 11.31 Summary of ANOVA Results of Reduced 2FI Model (Stable Phase)	211
Table 11.32 Constraints Applied for Selection of Optimized Batch	216
Table 11.33 Selected Process Parameters based on Desirability	216
Table 11.34 Confirmation of Optimum Processing Parameters	217