FORMULATION AND EVALUATION OF SELF-NANO EMULSIFYING DRUG DELIVERY SYTEM LOADED WITH TETRABENAZINE FOR THE TREATMENT OF DYSTONIA

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutics

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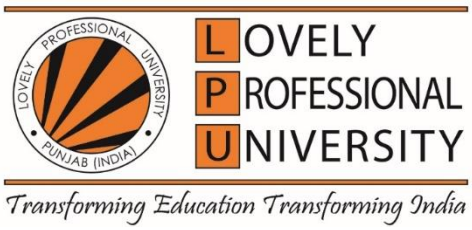

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2023

DECLARATION

I, hereby declared that the presented work in the thesis entitled **"Formulation and evaluation of self-nano emulsifying drug delivery system loaded with Tetrabenazine for the treatment of Dystonia"** in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision **Dr. Narendra Kumar Pandey,** working as **Professor**, in the **School of Pharmaceutical Sciences** of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **"Formulation and evaluation of self-nano emulsifying drug delivery system loaded with Tetrabenazine for the treatment of Dystonia"** submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in the **School of Pharmaceutical Sciences**, is a research work carried out by **Shashi, 11919680**, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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lending a helping hand during emergency and the fact that every work has thousands of

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Table of Contents

1. INTRODUCTION 1

1.1. Dystonia 1

1.2. Prevalence of dystonia 1

2. REVIEW OF LITERATURE 2

2.1. Dystonia 5

2.1.1. Signs and symptoms of dystonia 5

2.2. Classification 6

2.2.1. Clinical classification 6

2.2.1.1. Focal dystonia 6

2.2.1.2. Segmental dystonia 6

2.2.1.3. Multifocal dystonia 7

2.2.1.4. Generalized dystonia 7

2.2.2. Etiologic classification 8

2.2.2.1. Primary dystonia 8

2.2.2.2. Secondary dystonia 8

2.2.2.3. Dopa-responsive dystonia 8

2.2.3. Age onset classification 8

2.2.4. Genetic classification 9

2.3. Different type of hyperkinetic movements 9

2.3.1. Parkinson Disease 10

2.3.2. Stereotypies 10

2.3.3. Tics 10

2.3.4. Huntington 10

2.3.5. Athetosis 11

2.3.6. Myoclonus 11

2.3.7. Tremor 11

2.4. Factors responsible for Dystonia 11

2.4.1. Injury 11
2.4.2. Genetics 12
2.5. Animal models of Dystonia 16
2.5.1. Kainic acid induced models 17
2.5.2. Ouabain induced models 17
2.5.3. 3-Nitropropionic acid induced model 18
2.5.4. Muscimol and Bicculine induced model 18
2.5.6. Haloperidol induced model 18
2.6. Treatment 19
2.6.1. Oral medication 19
2.6.2. Neurotoxic protein (Botulinum toxin) 22
2.6.3. Surgery (Deep Brain Stimulation (DBS)) 24
2.7. Tetrabenazine 26
2.7.1. Mechanism of Tetrabenazine 26
2.8. Drug Profile 30
2.8.1. Tetrabenazine (TBZ) 30
2.9. SNEDDS 31
2.9.1. Mechanism of SNEDDS Formulations 31
2.9.2. Mechanism of self-emulsification 31
2.9.3. Mechanism of Drug transport to brain after oral administration through SNEDDS 31
2.9.4. Composition of SNEDDS 32
2.9.5. Advantage of formulating SNEDDS over other NDDS 34
3. RATIONALE OF THE STUDY, AIM and OBJECTIVES 37
4. MATERIALS AND METHODS 39
4.1. Materials 39
4.2. Methodology 41
4.2.1. UV-Spectrum Analysis 41
4.2.2. Analytical method development 41

4.2.3. Method validation 43
4.2.4. Characterization of active pharmaceutical ingredients (API) 45
4.2.5. Preparation of TBZ SNEDDS using pseudoternary phase diagram 48
4.2.6. Formulation optimization using DoE 50
4.2.7. Characterization of SNEDDS 51
4.2.7.1. Zeta Potential, globule size and Poly dispersity index (PDI) 51
4.2.7.2. Self-emulsification time (SEF) 52
4.2.7.3. Drug Loading 53
4.2.7.4. % Transmittance 53
4.2.7.5. Compatibility study by FTIR 54
4.2.7.6. Transmission electron microscopy (TEM) 54
4.2.7.7. Cell line toxicity study 55
4.2.8. In-Vitro Release Study 56
4.2.9. Accelerated Stability Study 56
4.2.10. In vivo study 57
4.2.11. Methods 57
4.2.12. Drug treatment schedule 61
4.2.13. Pharmacokinetic study 63
4.2.14. Behavioural 63
4.2.15. Oxidative biomarkers 64
4.2.16. Histopathology 66
5. RESULT AND DISCUSSION 66
5.1. Spectrum analysis 66
5.2. Analytical method development and validation 66
5.2.1. Selection of mobile phase for TBZ estimation 67
5.2.2. Method validation 68
5.3. Appearance 75
5.4. Melting Point Determination 75

5.4.1. Capillary fusion method 75
5.4.2. DSC 75
5.5. FTIR 76
5.6. PXRD 77
5.7. Formulation development of SNEDDS 78
5.7.1. Solubility of TBZ in various oil, surfactant and co-surfactant 78
5.8. Miscibility Study 80
5.8.1. Screening of surfactant 80
5.8.2. Screening of Co-surfactant 80
5.9. Construction of ternary phase diagram 81
5.10. Stability evaluation of selected formulations 83
5.11. Effect of pH on dilutions 84
5.12. Formulation optimization using DoE 84
5.12.1. Response analysis for optimization 86
5.13. Optimization of L-SNEDDS 91
5.14. Characterization of optimized TBZ loaded SNEDDS 93
5.14.1. Zeta potential, globule size, and poly dispersity index 93
5.14.2. Self-emulsification time 94
5.14.3. Drug loading 95
5.15. Transmission Electron Microscopy (TEM) 95
5.16. Physicochemical Characterstics 96
5.17. In vitro release study 96
5.17.1. Kinetic modelling 97
5.18. Cell line study 99
5.19. Accelerated stability study 100
5.20. In vivo study 101
5.21. Bioanalytical method development 101
5.21.1. Specificity, linearity and range 102

5.21.2. Accuracy 103
5.21.3. Precision 104
5.21.4. Stability study of plasma samples 106
5.21.5. LOD and LOQ 108
5.21.6. System suitability 108
5.22. Pharmacokinetic studies 108
5.23. Body weight 109
5.24. Behavioural 110
5.24.1 Locomotor 110
5.24.2. Muscle coordination 112
5.24.3. Catalepsy 113
5.24.4. Akinesia 115
5.25. Oxidative biomarkers 117
5.26. Histopathology study 119
6. SUMMARY, CONCLUSION 120
7. REFERENCES 126

LIST OF ABBREVATIONS

Ach	Acetyl choline
ACN	Acetonitrile
AD	Alzheimer disease
AE	Adverse effect
ANOVA	Analysis of variance
API	Active Pharmaceutical Ingredients
AUC	Area Under Curve
BA	Bioavailability
BBB	Blood-Brain Barrier
BBD	Box Behnken Design
BoNT	Botulinum toxin
BSP	Blepharospasm
CAT	Catalase Assay
CD	Cervical Dystonia
CNS	Central Nervous System
DALY's	Disability adjusted life years
DBS	Deep Brain Stimulation
DL	Drug loading
DMEM	Dulbecco's Modified Eagle Medium
DOPAC	3,4-Dihydroxyphenylacetic acid
DRD	Dopa Responsive Dystonia
DSC	Differential Scanning Calorimetry
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid
EDTA	Ethylene diamine tetraacetic acid
FDA	Food and drug administration
FTIR	Fourier Transform Infrared
GABA	Gamma-aminobutyric acid
GCH	GTP cyclohydrolase
GIT	Gastro Intestinal Tract

Gpi	Globus Pallidus Internus
GS	Globule Size
GSH	Reduced Gulathione Assay
GTP	Guanosine triphosphate
HD	Huntington's Disease
HETP	Height Equivalent Theoretical Plate
HLB	Hydrophilic Lipophilic Balance
HPLC	High performance liquid chromatography
HQC	Higher Quality Control
ICH	International Conference of Harmonisation
IP	Intraperitoneal
i.v.	Intravenous
LD	Laryngeal Dystonia
LOQ	Limit of Quantification
LQC	Lower Quality Control
MAO	Monoamine oxidases
MD	Myoclonus-dystonia
MDA	Malondialdehyde
MQC	Middle-Quality Control
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	Milli Volt
NBT	Nitroblue tetrazolium
NC	Normal Control
NE	Nano Emulsion
NMDAR	N-methyl-D-aspartate
NMS	Neuroleptic Malignant Syndrome
NPR	National patient register
NSF	N-ethylmaleimide Sensitive Fusion
O/W	Oil in Water emulsion
OD	Optical Density
ODF	Orodispersible Film

OF	Optimized formulation
PAMPA	Parallel artificial Membrane Permeability Model
PBS	Phosphate Buffer Solution
PD	Parkinson Disease
PDA	Photodioade array detector
PDI	Poly Dispersity Index
PEG	Polyethylene Glycol
P-gp	P-Glycoprotein
PXRD	Powdered X-RAY Diffraction
QoL	Quality of life
RDP	Rapid-onset dystonia-parkinsonism
RH	Relative Humidity
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
RT	Retention Time
SD	Standard Deviation
SEF	Self-Emulsification Time
SEM	Scanning Electron Microscopy
SMEDDS	Self-Micro-Emulsifying Drug Delivery System
SNEDDS	Self-Nano-Emulsifying Drug Delivery System
SOD	Superoxide Dismutase
STN	Subthalamic Nucleus
TBARS	Thiobarbituric acid reactive substances assay
TBZ	Tetrabenazine
TCA	Trichloroacetic acid
TEM	Transmission Electron Microscopy
TH	Tyrosine Hydroxylase
TST	Tail Suspension Test
USFDA	United States Food and Drug Administration
USP	United State Pharmacopeia

WHO	World Health Organization
ZP	Zeta potential

LIST OF TABLES

Table 1	:	Published clinical studies of TBZ for Dystonia	25
Table 2	:	Reported studies of Tetrabenazine with various formulations	27-28
Table 3	:	Compositions of SNEDDS	31-32
Table 4	:	Composition and salient features of SNEDDS of Neuronal Diseases	33-35
Table 5	:	List of material used in study	38-39
Table 6	:	List of equipment's utilised in the research	39-40
Table 7	:	Formulation of SNEDDS in different ratios (1:1, 1:2, 2:1)	47-48
Table 8	:	Specifications of the formulations and process factors for SNEDDS optimisation	49
Table 9	:	Study design based on Box- Behnken design	49-50
Table 10	:	Pharmacodynamics study	60
Table 11	:	System suitability results for TBZ	66
Table 12	:	Result of accuracy studies	67
Table 13	:	Results of precision studies of TBZ	67-68
Table 14	:	Robustness results of various parameters tested for TBZ	69
Table 15	:	Physical properties of the drug	73
Table 16	:	Comparison of TBZ spectra with reported spectra	74-75
Table 17	:	Comparison of TBZ-PXRD spectra with reported spectra	75
Table 18	:	Miscibility study for the screening of surfactant	78
Table 19	:	Miscibility study for the screening of co-surfactant	78
Table 20	:	Appearance of formulation of SNEDDS using oil Capryol PGMC and S_{mix} (Tween 20 and Transcutol ©P)	79-80
Table 21	:	Results of thermodynamic stability studies	81
Table 22	:	Results of effect of pH on dilution	82
Table 23	:	Box-Behnken study design was used to examine the effect of variable A-C on responses Y_1-Y_5	82
Table 24	:	Results of an ANOVA for a Box-Behnken Design	83
Table 25	:	Release kinetic of naïve, marketed and optimized formulation	94

Table 26	:	Details of storage stability study	95-96
Table 27	:	Accuracy study results	99
Table 28	:	Precision study's result	99-100
Table 29	:	Short term TBZ plasma sample stability	100-101
Table 30	:	TBZ plasma sample's Freeze thaw stability	101
Table 31	:	Long term stability for plasma samples of TBZ	101-102
Table 32	:	System suitability parameters	102
Table 33	:	Inference of histopathology of brain	112

LIST OF FIGURES

Figure 1	:	Prevalence of different form of dystonia	2
Figure 2	:	Flow chart of different kind of hyperkinetic movement	9
Figure 3	:	Mechanism of Tetrabenazine for Dystonia	25
Figure 4	:	Mechanism of SNEDDS formulations	29
Figure 5	:	Mechanism of Drug transport to brain through oral SNEDDS	31
Figure 6	:	Hypothesis of research work	36
Figure 7	:	HPLC chromatography conditions	41
Figure 8	:	Steps for preparation of dilutions	42
Figure 9	:	Steps for solubility	46
Figure 10	:	Steps for globule size, zeta potential and PDI	50
Figure 11	:	Steps for SEF	51
Figure 12	:	Steps for drug loading	52
Figure 13	:	Steps for % transmittance	52
Figure 14	:	Steps for TEM	53
Figure 15	:	Steps for dissolution study	54
Figure 16	:	Protocol for animal study	60
Figure 17	:	Steps for histopathology study	64
Figure 18	:	Absorbance of Tetrabenazine	65
Figure 19	:	Chromatogram of blank ACN and formic acid	66
Figure 20	:	Chromatogram of TBZ in ACN and 5 mM ammonium acetate (A), Chromatogram of TBZ in ACN and 0.1% glacial acetic acid (B), Chromatogram of TBZ in ACN and 0.1% orthophosphoric acid (C), Optimize chromatogram of TBZ in ACN and 0.1% formic acid (90:10) (D)	66
Figure 21	:	Calibration curve of TBZ	67
Figure 22	:	DSC thermogram of TBZ	74
Figure 23	:	FTIR spectra of TBZ	74
Figure 24	:	PXRD pattern of TBZ	75
Figure 25	:	Solubility of Tetrabenazine in various oil	76
Figure 26	:	Solubility of Tetrabenazine in various surfactant	77

Figure 27	:	Solubility of Tetrabenazine in various co-surfactant	77
Figure 28	:	Pseudo ternary phase diagram	81
Figure 29	:	Various Nano-emulsion according to DOE	83
Figure 30	:	Effect of variables on globule size	84
Figure 31	:	Effect of variables on PDI	85
Figure 32	:	Effect of variables on zeta potential	86
Figure 33	:	Effect of variables on self-emulsification time	86
Figure 34	:	Effect of variables on drug loading	87
Figure 35	:	Perturbation plot of individual factors	87-88
Figure 36	:	Overlay plot of optimized formulation for TBZ loaded SNEDDS, prepared optimized formulation, predicted and observed results of optimized formulation	88-89
Figure 37	:	Globule size and PDI of optimized formulation	90
Figure 38	:	Zeta potential of optimized formulation	90
Figure 39	:	TEM micrographs of TBZ loaded SNEDDS (A-F) Images at different magnification of optimized formulation	91
Figure 40	:	FTIR spectra of (a) TBZ, (b) surfactant, (c) oil, (d) optimized formulation, (e) co-surfactant	90
Figure 41	:	Release profile in phosphate buffer (pH 6.8) of naïve TBZ, marketed TBZ and L-SNEDDS	93
Figure 42	:	Release kinetics of naïve drug, marketed and optimized formulation	93-94
Figure 43	:	Cell line cytotoxicity of blank and TBZ-SNEDDS	95
Figure 44	:	Globule size and PDI of 6 months stored optimized formulation	96
Figure 45	:	ZP of 6 months stored optimized formulation	97
Figure 46	:	In vitro release of fresh formulation and 6 month stored formulation	97
Figure 47	:	Calibration curve of TBZ	98
Figure 48	:	(A) Blank plasma, (B) Benzoquinolizine (RT. 3.665) and TBZ (RT. 5.064) in rat plasma chromatogram	98
Figure 49	:	Concentration of TBZ in brain and plasma of Naïve and TBZ	101

		loaded SNEDDS	
Figure 50	:	Effect on bodyweight of different treatment	104
Figure 51	:	Effect on locomotor of different treatment	105
Figure 52	:	Effect on motor coordination of different	106
Figure 53	:	Effect on bar catalepsy of different treatment	107
Figure 54	:	Effect on akinesia of different treatment	109
Figure 55	:	Variation in CAT, GSH, SOD, and MDA of rats in different groups in vivo studies	110
Figure 56	:	Photomicrographs of various groups of rats	111-112
Figure 57	:	Flow chart of self-nano-emulsifying drug delivery system formulation through oral route for brain	114
Figure 58	:	Pharmaceutical challenges of tetrabenazine	115

ANNEXURES

Annexure 1: Candidacy letter of Ph.D.

Annexure 2: List of publications, patents, awards, certificates

Annexure 3: Certificate of analysis of TBZ

Abstract

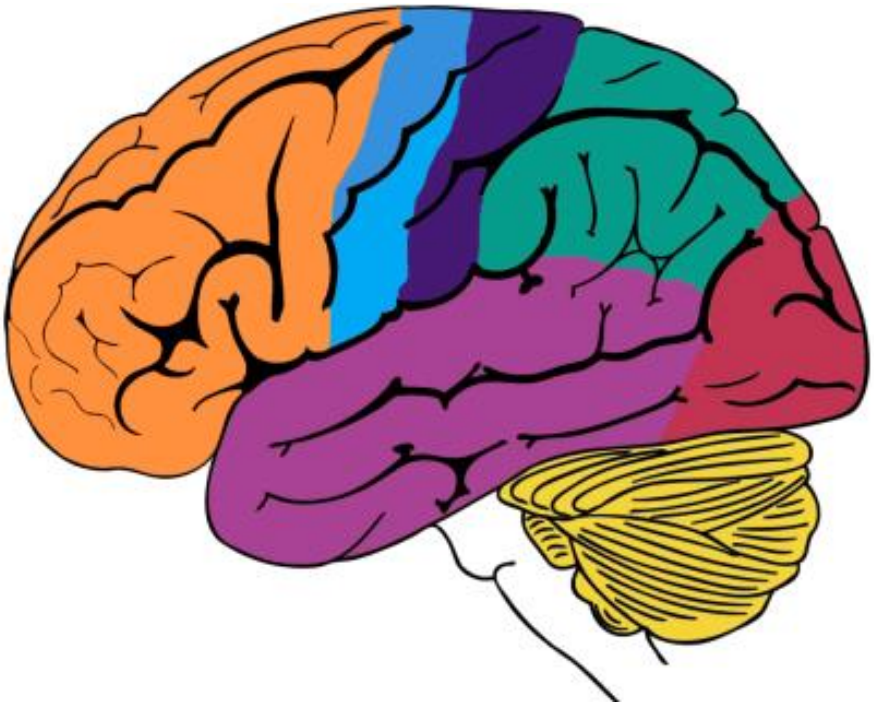
The present research work was aim to develop self-nano-emulsifying drug delivery system of tetrabenazine, characterization and its pharmacological evaluation for the treatment of haloperidol-induced dystonia in rats. Selection of excipients like oil, surfactant and co-surfactant were performed on the basis of saturation solubility at 37±5℃. The optimized mixing ratio of excipients were determined using BBD and their characterization was further processed by GS, ZP, PDI, SEF and drug loading. Further optimized formulation undergoes in vitro SH-SY 5Ycell line study, TEM and in vivo evaluation for the treatment of dystonia induced by haloperidol in rats. Finally behavioural examination oxidative biomarkers, brain permeability study and histopathological studies were performed.

Optimized formulation composed of capryol PGMC, tween 20, transcutol© P as oil, surfactant, co-surfactant in the ratio of 240.68 µL, 506.85 µL, 252.47 µL respectively. The prepared formulation containing TBZ showed 142.6±0.98 nm, 0.136±0.01, -19.6±0.23mV, 10.46±0.67 and 72.42±0.53 mg/mL as globule size (GS), poly dispersity index (PDI), zeta potential (ZP), self-emulsification time (SEF) and drug loading (DL) respectively. Stability study was no significant changes in accelerated testing study as well as effect of pH on dilution. Haloperidol induction successfully develop dystonia in rats and behavioural study like rota rod test, actophotometer test, akinesia and bar catalepsy test were improved significantly from day 14th only to the group of rats received low and high doses of TBZ-SNEDDS while naïve TBZ were producing its effect in very slowest manner. Improvement ever recorded at the end of experimental week. In support of this finding pharmacokinetic study was performed between blood and brain and results were highly interesting when TBZ-SNEDDS provides more delivery to the brain. Significant ameliorations were also observed in TBZ-SNEDDS against oxidative stress. Histopathological study also supported the in vivo results when more were recorded in TBZ-SNEDDS treated group of rats.

The present investigation provides first report of TBZ-SNEDDS which seems to be promising alternative for improving bioavailability of TBZ as well as successful in providing effective treatment approach to treat dystonia.

Keywords: tetrabenazine; bioavailability; permeability; self-nano-emulsifying drug delivery system; behavioural; solubility

CHAPTER 1



INTRODUCTION

1. INTRODUCTION

1.1. DYSTONIA

Dystonia is a neurological movement disorder marked by discontinuous, irregular, and rhythmic contraction of muscles resulting in patterned, stereotypical, repetitive, and abnormal nature of movements of individuals. Dystonia includes overflow activation of extraneous muscle leads to blepharospasm (BSP), abnormal postures, and involuntary contraction that interfere with blinking of eyelids or larynx [1]. One of the most impairing movement disorders with chronic long-term effects like orthopedic complications, fixed posture, and severe pain. An untreated extreme form of dystonia can bring medical emergency with compromised breathing, muscle breakdown, myoglobinuria, and hyperthermia [2]. The seriousness and involvement of muscles in clinical signs of dystonia. Dystonic movements are slow, but more often may be jerky and quick. Sometimes it appears like tremor in selective individuals. Initially it can be focal, eventually it becomes generalized and spread to other body parts [3].

1.2. PREVALENCE OF DYSTONIA

Dystonia prevalence depends on the specific type of dystonia and the population being studied. Overall, dystonia is estimated to affect 1% of the worldwide population [4]. Commonest type of dystonia is primary dystonia, which has an estimated prevalence of 30 to 50 cases per 1 Lakh people [5]. Primary dystonia is typically categorized by age of onset and the affected body region, such as cervical dystonia (CD) (affecting the neck), BSP (affecting the eyelids), and writer's cramp (affecting the hand). Secondary dystonia, which is caused by another underlying condition or factor, such as medication side effects or brain injury, has a lower prevalence, estimated to be around 5 to 7 cases per 1 Lakh people [6].

In 2019, according to Sweden’s national patient register (NPR), 4239 patients (31.6% male) with primary dystonia were identified [7]. Another study it was reported that, oromandibular dystonia in Kyoto was approximated at 9.8 per 1 Lakh persons [8]. Oromandibular dystonia may not be as rare as previously considered and common as

CD or BSP. Prevalence study in patients of various kinds of tremor in patients estimated that dystonic tremor appears to be reoccurring feature in CD patients i.e. 58.3% and tremor associated with dystonia seems less common (11.7%) [9].

Cervical dystonia is the commonest form, which affects approximately 60 to 90 people per 1 Lakh [10]. Another kind of dystonia, such as BSP, spasmodic dysphonia, and writer's cramp have lower prevalence rates, ranging from 5 to 50 cases per 1 Lakh individuals [11]. Dystonia can occur in individuals of any age, although onset is most common in middle age. Dystonia is also more commonly diagnosed in women than in men [12].

It is worth noting that due to the heterogeneity of dystonia, accurate prevalence estimates can be challenging. Additionally, because dystonia is a rare disease, more research is required to understand its prevalence and how it affects different populations.

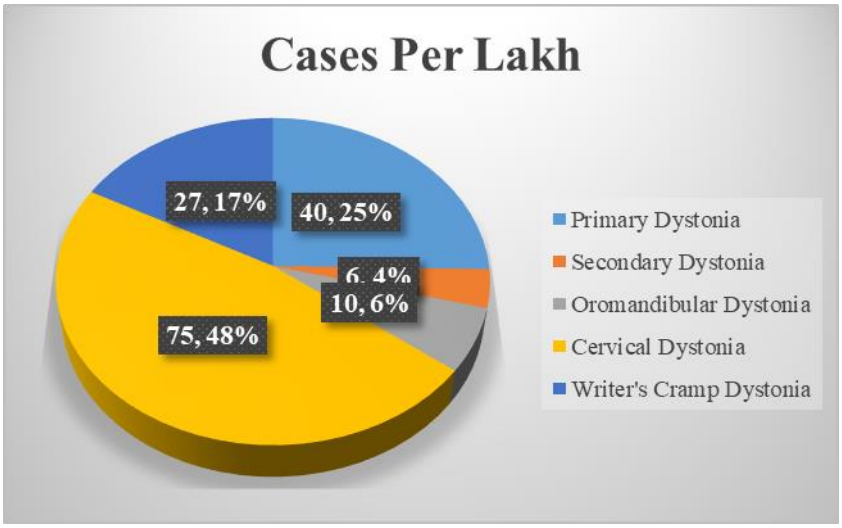


Figure 1: Prevalence of different form of dystonia

There are few drugs which are present for the treatment of hyperkinetic movement disease like dystonia, chorea, tics, and dyskinesia. Drugs present for the cure of hyperkinetic movement disorder have so many negative effects such as tardive dyskinesia movement [13].

Focal Hand Dystonia has been treated using oral drugs like trihexyphenidyl, atropine, chlorpheniramine, levodopa, baclofen and benzotropine. Prescribed tricyclic

antidepressants, anticholinergics, benzodiazepines, antipsychotics, and levodopa to patients had negative effects and were ineffective. Lang et al. looked at the effectiveness of treatment with intravenous (i.v.) atropine, chlorpheniramine, and benztropine in the treatment of focal dystonia, [14].

Administration of Botulinum toxin (BoNT) of A type via i.v. route are often given 3–4 times year at set intervals, with each injection based on the patient's medical histories, after careful examination, and localization of the affected muscles, and with electromyography assistance [15]. Patients' dissatisfaction with BoNT treatment may be due to the fact that they actually experienced a lack of efficacy or it did not function as they had anticipated. Poor technique may be the cause of ineffectiveness [16]. BoNT had no considerable impact to the explanation of disability in CD but may be made a less impact on the reason for impairment. When the effect BoNT worn out, dystonia intensity and pain levels at its highest [17]. BoNT cause flaccid paralysis by interfering with vesicle fusion and neurotransmission release in neuronal cells [18,19]. It is also noted that Deep brain stimulation (DBS) surgery is linked to increases in children as mechanical malfunction and followed by revision in surgical and the rate of infection is overall 10% [20].

Tetrabenazine is an orphan drug and was first introduced as antipsychotic drug in 1960's but didn't give beneficial effects [21]. It was taken off the market in 1966 because it was ineffective as a strong neuroleptic [13]. It gained FDA approval in 2008 for the treatment of chorea associated with HD and other hyperkinetic movement disorders [22–25]. The central nervous system consequences of TBZ are its negative effects, such as depression, somnolence, sedation, fatigue, akathisia, and insomnia [26,27]. TBZ belongs to BCS class IV (low solubility and low permeability), tablet formulation (marketed) is available in the dose of 12.5 mg and 25 mg [22,28] and mainly excreted by urine [22].

Tetrabenazine has the benefit of not causing tardive dyskinesia, which makes it superior to other antidopaminergic medications [29]. With outstanding outcomes in the subgroup Huntington disease, which showed considerable better tolerability profile, there is strong evidence that TBZ, single or combined with other antidyskinetic drugs, demonstrated effective and well tolerated in the management of

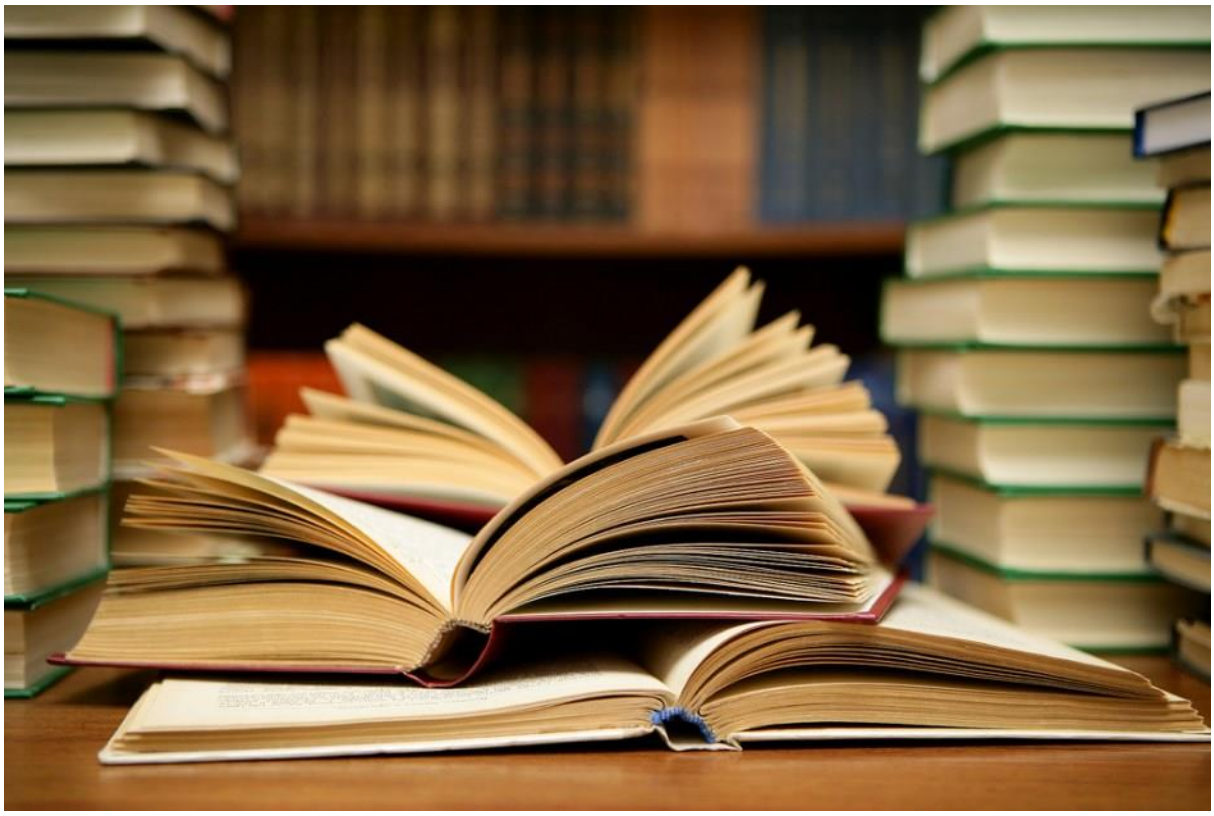
a wide spectrum of hyperkinetic movement disorders [30]. Double-blind, placebo-controlled experiment are supported by a long-term evaluation of tetrabenazine in individuals with hyperkinetic movement disorders who have not responded well to conventional treatments. In the treatment of movement disorders, particularly tardive dystonia and Huntington's disease, tetrabenazine is an effective and secure medication [31].

Tetrabenazine (TBZ) having very low BA, low solubility and it possess first pass metabolism by Cytochrome P-450. So couldn't get maximum effect while treating with it. There are so many techniques to overcome such problems like co-solvency, solid dispersion, nano-suspension, complexion, chemical modification and nano-ionization. But these methods have some limitation of stability and toxicity [32].

In past it has been observed that SNEDDS reported most effective due to simplicity of preparation, scalability, better drug loading, lower toxicity and stability. It enhances the stability, solubility, dissolution, and hence BA of the drug [33,34]. It protect the drug from proteolysis [35]. It increases the membrane fluidity, facilitating transcellular transport and inhibition of "P-gp and cytochrome P450 to increase intracellular concentration" [36]. It increases the drug absorption and easily pass the gastrointestinal tract (GIT) [37]. Ease of administration as compare to solid [38]. SNEDDS is suitable for the delivery of lipophilic drugs (i.e. BCS class II and IV) such as depagliflozin [39], darunavir [40], docetaxel [41], glibenclamide [42], lopinavir [43], paclitaxel [44] etc.

To overcome the limitations of TBZ, the present investigation is being proposed to prepare SNEDDS of TBZ for the treatment of Dystonia in Haloperidol induced rat model. This investigation predominantly focuses on development, optimization, characterization, and animal study to investigate the therapeutic effect of TBZ loaded SNEDDS in the treatment of dystonia.

CHAPTER 2



LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Dystonia

Abnormal postures or movements that are carried prolonged or intermittent contractions of muscle describe as Dystonia [1]. Despite early clear descriptions of dystonia, it took more than 50 years before physicians accepted that this strange condition was due to brain disorder [45]. Dystonia also affects behaviourally. Because of excessive movements cramp appears such as writer's and other occupational cramp [46]. The anatomical basis for dystonia is still debated. There is still no recognised anatomical cause of dystonia. In general, idiopathic or genetic dystonia patients' neuropathological examinations have not found any obvious macroscopic or microscopic level changes in the brain. [47].

2.1.1. Signs and symptoms of dystonia

Pigeon-toed walking and inverted feet are typically the first symptoms in patients before other symptoms appear [48]. Continuous muscle contractions can force the boot and limbs into fixed positions. Two primary (dystonic postures and movements) and two additional "(gestes antagonistes or tricks, mirror dystonia, and overflow dystonia)" physical symptoms of dystonia can all be recognised [49].

Seventy percent of the patients experience generalisation, but normally the cranial area is spared. Dystonia has two kinds of symptoms i.e., motor and non-motor. They include sleep, sensory problems, autonomic function, discomfort, and neuropsychiatric and cognitive issues. It has been demonstrated in numerous studies that NMS are more significant determinants of quality of life (QoL) in movement disorders including PD and dystonia than motor symptoms [50–52]. "Difficulty performing" is a common way for patients to describe their earliest dystonia symptoms. Initial complaints most frequently related to loss of control of embouchure, lip tiredness, lip tremor, and uncontrollable facial motions [53].

It takes a peripheral trigger for motor symptoms to manifest. The overuse and incorrect use of the limb may function as triggers. In those who are vulnerable, peripheral damage may also reveal dystonia. This is likely due to a change in

peripheral somatosensory input that results in a central restructuring of the somatosensory system [54]. Walking difficulty or balancing problem, feeling unwarrantedly anxious, fearful, or nervous, having unpleasant sensations like tingling, numbness, or pins & needles. Eating challenges, such as chewing or swallowing issues [55].

2.2. CLASSIFICATION

Involuntary muscular spasms that cause repetitive or aberrant postures and twisting motions characteristics in movement disorder dystonia. Dystonia can affect any parts of the body, and there are several categorization systems that are used to narrate divergent types of dystonia [56]. Here are some of the most used classification systems:

2.2.1. Clinical classification: This classification system is based on the location of the dystonia symptoms. There are several types of clinical classification such as

2.2.1.1. Focal dystonia

Affects only one part of the body, such as the neck (CD), face (cranial dystonia), or hand (writer's cramp). Focal dystonia, also known as task-specific dystonia. It is a neurological condition that affects specific muscles or muscle groups in the body causing involuntary muscles contraction, Spasms, or abnormal postures during certain activities or movement. The condition is usually a task specific, meaning it occurs only during certain activities such as writing playing an instrument or typing. One of the most common types of focal dystonia is called ‘‘writer’s cramp’’ which affects the muscles of the hand and wrist, making it difficult for people to write or perform other motor tasks. Other forms of focal dystonia include CD, which act on neck muscles, and BSP, which affects the muscles around the eyes and can cause excessive blinking or involuntary eyelid closure. It is thought that a mix of genetic and environmental factors is responsible for focal dystonia, and the exact mechanism underlying this condition are not exactly known. However, it is believed to be related to changes in the way the brain process and control movement [57].

2.2.1.2. Segmental dystonia

Affects two or more adjacent body parts, such as the neck and arm. Segmental dystonia, also known as focal dystonia, which is a movement disorder that affects a particular part or segmental of the body. It is a type of dystonia, which us a neurological condition that cause involuntary muscle contraction that lead to abnormal postures and movements.

Any part of the body can be affected by segmental dystonia, but the most common areas affected are neck (CD), face (cranial dystonia), hand, and vocal cords (spasmodic dystonia). The symptoms of segmental dystonia vary depending on the area of the body that is affected. Common symptoms include involuntary movement, muscle spasm, abnormal posture, and pain [58].

2.2.1.3. Multifocal dystonia

Affects two or more nonadjacent body parts. Multifocal dystonia is a type of dystonia that affects multiple body parts simultaneously or sequentially. In multifocal dystonia, the contraction can affect different body parts, such as arms, leg, neck, face, and trunk. The symptoms can vary from person to person, and the severity of the symptoms can also vary. The causes of multifocal dystonia are still in doubt, but it is believed connected to abnormalities of brain's basal ganglia, which are responsible for controlling movement [59].

2.2.1.4. Generalized dystonia

Affects the entire body**.** It is a neurological condition marked by uncontrollable muscular contractions that originate twisting, repetitive movements and abnormal postures throughout the body. Generalized dystonia is a rare condition that can occur at any age, however it typically starts during puberty or childhood. It can affect various part of body including face, jaw, and tongue. Some cases of generalized dystonia may be inherited, while others may be caused by brain injuries, infection, and medication [60].

2.2.2. Etiologic classification

This classification system is based on the underlying cause of the dystonia. Some examples of etiologic classifications include:

2.2.2.1. Primary dystonia

No known cause or underlying neurological condition. It is called 'primary' because it is not caused by an underlying neurological condition or injury. The symptoms of primary dystonia can be mild or severe, and can progress over time [61].

2.2.2.2. Secondary dystonia

Caused by an underlying neurological condition, such as PD, Huntington's disease, or stroke. Secondary dystonia is caused by an underlying medical condition or an external factor such as trauma, infection, medication, or other neurological disorders. Unlike primary dystonia, which is a genetic condition, secondary dystonia can develop at any stage and often has a sudden onset [62].

2.2.2.3. Dopa-responsive dystonia (DRD)

Treatment with levodopa gave good response in DRD, which is for PD. DRD is a rare genetic disorder that affects the way dopamine is used by the body. Dopamine is a neurotransmitter that plays a critical role in controlling movement and muscle activity. DRD is caused by mutation in the GCH 1 gene, which described how to create an enzyme called GTP cyclohydolase1. This enzyme is important for the production of tetrahydrobiopterin (BH4), which is essential for the production of dopamine. DRD is usually diagnosed in childhood or early adulthood, and treatment involves taking medication that increase dopamine level in brain. Most common medication used to treat DRD is Levodopa. Brief description about this treatment is discussed in treatment of dystonia below [63].

2.2.3. Age onset classification

This classification system is according to the age at which the dystonia symptoms first appear [1]. There are several types of age of onset classification includes.

- *Early onset dystonia*: Symptoms appear before the age of 26.
- *Adult-onset dystonia*: Symptoms appear between the age of 26 and 60.
- *Late onset dystonia*: Symptoms appear after the age of 60.

2.2.4. Genetic classification

This classification system is according the genetic cause of dystonia [64].

- *DYT1 dystonia*: Due to mutation in DYT1 gene [65].
- *DYT6 dystonia*: Due to mutation in DYT6 gene [65].
- *DYT5 dystonia*: Due to mutation in GCH1 gene [65].

2.3. Different type of hyperkinetic movements

Any unintentional excessive movement is considered hyperkinetic. A taxonomy of paediatric motor disorders includes hyperkinetic movements in addition to hypertonia and adverse symptoms. Atypically increased resistance to externally induced movement is known as hypertonia. Insufficient muscle activation or poor regulation of muscle action are negative symptoms [66].

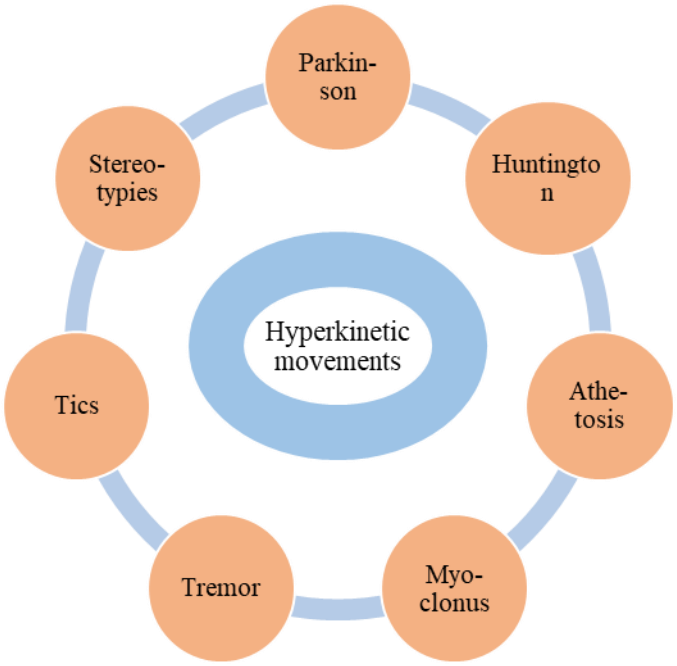


Figure 2: Flow chart of different kind of hyperkinetic movement

2.3.1. Parkinson Disease

This is the 2nd most common Neuronal disease after Alzheimer disease (AD) [67]. It occurs as a result of dopamine neurons in "substantia nigra pars compacta" and gathering of misfolded α-synuclein which is present in intracytoplasmic inclusion called Lewy bodies. On diagnosis, a substantial part of "dopaminergic neurons in substantia nigra pars compacta" has already lost [68]. It occurs rarely before the age of 50 but the people are prone to be affected after 60 years of age [69].

2.3.2. Stereotypies

Flapping of hand/arm, finger wiggling, rotating hand are some symptoms of stereotypies. Most of the physicians were confused between seizures and stereotypies. This is mostly happened in the upper part of the body [70]. This is of two types primary and secondary. The exact pathophysiology of this disorder is still unknown. According to expertise suggestions such abnormalities in behaviours entail neurological interrelated the neocortex with basal ganglia and striatum. Till date no pharmacological therapy is available for stereotypies [71]. Many medicines are in use for stereotypies and related problems, but use of these medications is inadequate and because of adverse effects their use is limited. This disorder mostly occur to people who had family history of maternal or paternal [72].

2.3.3. Tics

Tics is motor movement disorder, which is rapid, repetitive, arrhythmic, and unexpected. This is common in childhood and uncommon in adults [73]. Prevalence rate of tics is 21%. Exact mode of disorder is still unknown but autoimmunity or genetic factor both are responsible for tics [74].

2.3.4. Huntington

Huntington's disease is a genetic neurological disorder which is genetic by an autosomal dominant trait because of trinucleotide which is expanded and repeat in a gene on chromosome 4p16.3 [75]. Onset of Huntington's disease is usually during 4th or 5th decade of life [76]. It is originate by CAG repeat enlargement in the gene encoding the protein huntingtin [77]. The indication of Huntington's disease develop progressively and characterized by both behavioural and motor disturbances which usually appear in older age [78].

2.3.5. Athetosis

Athetosis is derived from Greek word which means slow, this disorder is slow as compare to chorea. The prevalence of this disorder is less than 1%. Because of slowness the motion is toss and turn [79].

2.3.6. Myoclonus

Myclonus is involuntary movement which occur burst or silencing of muscular activity which is known as positive or negative respectively [80]. It may be present at any time while working or rest position. Its onset is very rapid and for short duration [81].

2.3.7. Tremor

Tremor covers most of the hyperkinetic movement disorder which are of two types i.e., essential and Parkinson tremor [82]. Many preclinical studies are going on and some are published. DBS is the best treatment for tremor. Increased number of brain stem lewy bodies in 25% patients and in 75% patients change in cerebellar which is in purkinje cell loss, basket cell changes, and axonal torpedoes [83]. It affects 4% of the population above 65 years [84].

2.4. Factors responsible for Dystonia

The results support the hypothesis that the aetiology of adult-onset dystonia may involve both hereditary and environmental variables [85].

i) **Injury**
ii) **Genetics**

2.4.1. Injury

A number of movement problems, such as tremor, dystonia, paroxysmal dystonia, parkinsonism, and tics, have been linked to even modest head injuries [86]. Hemidystonia and torticollis are two dystonia distributions that occur after a head injury. The findings that infant males experience craniocerebral trauma 70% more commonly than infant females (30%) are in good agreement with the preponderance of males [87]. Accidents involving cars frequently result in soft tissue damage, particularly to the neck. The key finding is that all surgical procedures and car accidents requiring hospitalisation were substantial environmental risk factors for the onset of CD.[88]

Compared to control participants, a number of environmental factors connected to primary adult-onset focal dystonias. There is evidence linking BSP, laryngeal "dystonia, CD, and upper limb dystonia to, respectively, eye conditions, sore throats, idiopathic scoliosis, and repetitive upper limb motor" activity.

2.4.2. Genetics

2.4.2.1. TOR1A

TOR1 gene is accountable for the majority of early-start of isolated dystonia cases. Mutation in this gene and deletion of GAG cause DYT1 dystonia [89,90]. The TOR1A gene is vital in making of protein called Torsin A [91]. Torsin-1a controls the links between the proteins that bind the nucleus to the cytoskeletal system within the nuclear envelope lumen network, which is necessary for the normal development and functioning of nerve cells in the brain. According to reports, the polytopic membrane-bound proteins the dopamine transporter and others are inhibited by torsin-1a [61].

A patient with atypical early-onset dystonia has a TOR1A mutation that is projected to result in the deletion of "21 amino acids, with six amino acids at the carboxy terminus below the glutamic acid deletion". This novel mutation, which was only discovered in one patient with dystonia and neither controls nor those with early-onset Parkinsonism, would eliminate a protein's expected casein kinase 2 phosphorylation [92,93]. This gene test is suggested to the patient under the age of 30.

2.4.2.2. TAF1

TAF1 is well stand for TATA box binding protein associated factor 1 which is compose of 43 exon in which 38 exon is well known and coded with TAF1 and remaining 5 (d1-d5) is still unknown [94,95]. This factor is responsible for DYT3/X-linked dystonia Parkinsonism (XDP), this disease locus of DYT3 gene containing TAF1, formerly known as $TAF_{11}250$, was located at xq1301. According to a series of linkage analysis the biggest component of transcription factor 11D is TAF1 which is necessary for the regulation of RNA polymerase II dependent gene transcription [96]. The gene known as TNF134', one of the annoated TAF isoforms. In XDP/DYT3 brain 40 fold reduction of TAF1 present as compare to healthy brain [97]. This factor can

be checked by RT-PCR and c-DNA technique [95]. This disease is mainly spread in Philippine islands and also diagnosed in Canada and U.S [98].

2.4.2.3. TUBB4A

TUBB4 is first introduced in 1985, which is responsible for DYT4 or whispering dystonia [99]. The basal ganglia and cerebellum of the brain contain this protein [100]. These regions help control movement. This disorder start showing its symptoms in the 2nd to 3rd decade of life [101]. DYT4 was first elucidate by Parker in large Australia kindred in 2013 [102,103]. The β-tubulin 4a (TUBB4A) gene's E410K mutation was discovered through exome sequencing which is in the C-terminal domain. Gene mutation of TUBB4A causes dystonia and by replacing the amino acid arginine with the amino acid glycine at position 2 (R2G), this disrupts the structure of the β-tubulin resulting in the hindrance in the formation of microtubules [104].

Protein models for the wild type and variant primary sequences were built to evaluate the pathogenicity of the mutation [105]. Major Cytoskeletal elements called tubulin networks carryout fundamental tasks to sustain all morphologies and changes like differentiation, proliferation and mutation [106]. In MRI patient with DYT4 showing normal image [102].

2.4.2.4. GCH1

The GCH 1 gene is first discovered by Segawa in 1976, found on chromosomes 14q22.1 to q22.2, is responsible for the DRD known as “autosomal dominant (AD) GTP cyclohydrolase I (GCH 1) deficiency, also known as Segawa disease” [107,108]. Hereditary progressive basal ganglia disease is the name of this condition [109]. Men are less likely to be affected than women. There are one to two people with DYT-5 dystonia for every two million people. The GCH1 gene has an exon 1 deletion that caused a frame shift and the premature stop codon p.W53X [110]. Revealed connection to chromosome 14's long arm at the beginning and later shown that this was brought on by a heterozygous GCH1 gene mutation, which is located on 14q22.1–q22.2 [111]. Mutation at position T106I in exon 1 of GCH1, which is connected to the enzyme's helix-turn helix shape [112]. This gene is very variable,

and the GCH1 coding area has seen the identification of more than 100 distinct mutations thus far. This can be confirmed by biochemical test [113].

Studies on animals have revealed that nigrostriatal neurons in rats and mice had lower levels of GCH1 protein and mRNA expression than other monoaminergic neurons. In humans, reports of similar low GCH1 mRNA expression in nigrostriatal neurons have been made [114].

2.4.2.5. *TH*

The TH gene directs the production of the tyrosine hydroxylase enzyme, which is critical in normal functioning of nervous system. This enzyme participate in the initial phase of the production of a catecholamines are a class of hormones. Tyrosine hydroxylase converts tyrosine to a catecholamine called dopamine which is neurotransmitter as explaines above in GCH context. Dopamine produces norepinephrine and associates with the autonomic nervous system that controls involuntary process such as blood pressure and body temperature. More than two dozen alterations in the TH gene has been diagnosed to be causing DRD.

2.4.2.6. *THAP1*

The THAP1 "[Thanatos-associated (THAP) domain-containing apoptosis-associated protein]" mutations that cause DYT6. An "N-terminal DNA-binding domain (DBD), a nuclear localization signal, and a coiled-coiled domain" towards the terminus-C are all features of the zinc-finger transcription factor Thap1 [115,116]. In 1997 DYT6 was first introduced in Amish Mennonite families [117]. THAP1 has a biological purpose, mainly in neurons, but there is also a neuron-specific THAP1-like immune reactive species that binds DNA [118]. The protein's N-terminus contains a nuclear localization and a DNA-binding THAP domain and a zone rich in prolines, define THAP1's 213 amino acid length [119]. Without appearing to have any impact on oligodendrocyte progenitor cells, deficiency of THAP1 slows the development of oligodendrocytes into myelin-producing cells. Studies using chromatin immunoprecipitation and sequencing show a striking cobinding of THAP1 and YY1, transcription factors necessary for OL lineage development, on DNA [120].

2.4.2.7. SGCE

The initial discovery of M-D (Myoclonus-dystonia) causing mutations in SGCE was made by Zimprich et al., between 30 and 50% of M-D cases have SGCE gene mutations located at chromosome 7q21.3 [121]. M-D is thought to be caused by more than 50 distinct mutations, the majority of which result in loss of function due to frame shift and protein truncation before the transmembrane region [122]. SGCE is inherited from the mother. M-D has been causally linked to a wide variety of nonsense, missense, indel, and big deletion mutations in SGCE [123]. Given that almost half of DYT11 patients report improved motor symptoms after taking alcohol, A different name for DYT11 is alcohol-responsive dystonia. Surprisingly, alcohol seems to offer these individuals greater more effective than any pharmacological treatments, which have usually proven useless or unpleasant to take [124]. Other genes associated with this condition, such as those for the CACNA1B calcium channel gene and 17 gene containing KCTD17 potassium channel tetramerization domain, provide additional hints about the cause of DYT11/SGCE myoclonus dystonia. These both genes again suggest that myoclonus dystonia has a primary neurophysiological origin [125].

2.4.2.8. ***ATP1A3***

Other name for this is RDP, DYT12 ATPase, and MGC13276 etc. which occur at the age of 4 to 59 years [126]. ATP1A3 gene is necessary for making the α-3 subunit of Na^+/K^+ ATPase (sodium pump) [127]. This protein utilize energy (ATP) from a molecule to transport ions in and out of the cells. Sodium pump, primarily found in the nerve cells (neurons) are critical in their functioning (regulating the electrical activity of neurons; signalling process that controls muscle movements) ATPase alterations in the ATP1A3 gene have been pointed out in the rapid-onset dystonia-parkinsonism [128,129]. Most of these mutation cause changes in the proteins structure, reducing its activity and making it unstable [130]. There are no signs of DYT 12 dystonia until they are brought on by a physiological stressor (such as a fever), which commonly occurs in late adolescence or early adulthood. Following this trigger, indications start to show up between minutes to days and then become permanent [131,132]. Symptoms typically have between head to tail, with the face

being affected first, followed by the arms and subsequently the legs [133–135]. After this trigger, symptoms start to show up between minutes to days and then become permanent. Three families that share linkage to the same region on chromosome 19q13, known as DYT12, have been described so far [133].

2.4.2.9. PRKRA

In 2008, Camargos et al. published the first description of dystonia-parkinsonism associated with DYT-PRKRA (DYT16), along with the discovery of a homozygous PRKRA variation [136]. This is present in chromosome 2q31.2 [137]. PRKRA is mandatory gene for the production of protein called "PACT". This protein is critical when a cell reacts to another stress (free radicals, experiencing a viruses, and toxic substances) [138]. When the cell is in stress, this protein activates PKR protein, which deactivates the e1F2α protein. This protects the cell from the damage [139]. Several viruses aim for PACT inactivation in order to replicate effectively since PACT is necessary for IFN synthesis in after affects of viral infection [140]. 4.9% of the solitary occurrences of dystonia were caused by PRKRA mutations [141]. The only effective intervention in DYT-PRKRA might be globus pallidus internus (GPi)-DBS [142].

2.4.2.10. SLC2A1

Also known by other names such as DYT18, and GLUT etc, SLC2A1 gene is necessary for the production of a "protein called glucose transporter protein type 1 (GLUT1)". This protein caries glucose which can be used as fuel, into blood cells or other types of cells. Across the blood-brain barrier (BBB), glucose serves as the brains main energy source. This barrier functions as border between blood capillaries and brain tissues; protecting the brain's delicate nerve tissue by prohibiting other molecules from entering into brain. More than 150 SLC2A1 gene mutations with deficiency of GLUT1 have been discovered causing frequent seizures.

2.5. Animal models of Dystonia

As functional illnesses, idiopathic dystonia's require animal models to study the neurological foundation of disease aetiology [143]. Animal models are employed in

the development of innovative therapies as well as the identification of pathogenesis or pathophysiology-related pathways [144]. Because they offer experimental methods for illuminating the implements behind this movement condition, dystonia animal models are of great attentiveness. Dystonia is uncommon in rodents, with only a few well characterized inherited models [145]. Over the decade, number of animal models for dystonia explored. Major 2 categories are there for animal models of dystonia that are: etiologic or symptomatic [144]. It can be seen in mice, rats, and hamsters. The rat model's dystonia is persistent and only abating when the animal is at rest [146]. The choice of an appropriate animal model is compound, and variety of factors like should be taken into considerations, (a) convenience, (b) background knowledge of biological properties, (c) transferability of information, (d) appropriateness, (e) ethical consideration, (f) adaptability to experimental manipulation, and (g) genetic uniformity of organisms where applicable [147].

2.5.1. Kainic acid induced models

In 2002 Pizoli and collaborator developed kainate model. In this model the injection is giving in cerebellar by drilling into the skull of rat/mouse. Hamilton syringe used to deliver the dose of kainic acid. This model is dose (25 µg/mL to 100 µg/mL) as well as time dependent [145]. As a strong agonist at AMPA and kainate sensitive glutamate receptors, this is likely to cause dystonia by overexciting glutamatergic receptors. signalling within the cerebellum [144]. If NBQX and kainic acid administered together prevents dystonia. This indicating that activation of glutamate signalling in excess amount within cerebellum is required to produce dystonia [145].

2.5.2. Ouabain induced models

Comparing the two neuronal a1 isoforms, ouabain is 1000 times more selective for the mutant isoform. The key element of RDP needed to be replicated was dysfunction of the sodium pump in both the cerebellum and basal ganglia [148]. Dystonia is brought on by abnormal cerebellar activity, which also negatively impacts basal ganglia function. Guide cannula were stereotactically inserted into specific mouse brain areas, and low doses of ouabain were either continuously or physically perfused to partly block Na pumps [149]. Ouabain’s cerebellar perfusion caused ataxia and unmistakably dystonic-like postures in a time-concentration-dependent way.

Alternatively Childhood hemiplegia and other movement problems that are linked. [150].

Average duration of 39 min. ($\alpha_3^{+/D8014}$) and 0.07 min $\alpha_3^{+/D801N}$ RDP- severe and persistent /AHC recurring spells 10mm ouabain was directly dissolved into appropriate external solution (water) [151].

2.5.3. 3-Nitropropionic acid induced model

Low and high dose of 3-nitropropionic acid in mice model is 340 and 560 mg/Kg in a week. High dose divided into 60-80 mg/Kg per day by subacute injection [152]. At low dose less than 40 mg/Kg no significant or behaviour sign pop up. The dose increase of 40–60 mg/Kg resulted in worsening of the impairment [153].

In monkey model 5 mg/Kg drug administered by intramuscular injection and dose increases by 1 mg/Kg up to 8mg/kg [154].

2.5.4. Muscimol and Bicculine induced model

Ketamine (5 mg/kg, rompin 1 mg/kg) is administered under general anaesthesia to a stainless steel [155]. A stylet-containing cannula guide was introduced into the brain and fixed to the skull using acrylic cement and dental steel screws. Over the right GPi and left SNr, a cannula guide was carried by each animal. Bicuculline injection into the right GPi resulted in a sharp decline during locomotion and significant akinesia [156]. Ten injections are done at the greatest volumes. After 4 of the 9 injections, abnormal posture was seen in the contralateral hemi body, primarily in the lower limb, at lower volumes 2 to 5 [157].

In mutant hamsters, muscimol demonstrated a fast-acting antidystonic effect in the first hour of the three-hour observation period at a higher dose of 25 ng/hemisphere and considerably lengthened the latency to commencement of dystonia. Bicuculline dose in hamster are 5 ng/hemisphere [157].

2.5.6. Haloperidol induced model

Haloperidol can use to induce dystonia in monkey, rat, and mice with different dose range. Haloperidol belongs to antipsychotic drug which is used to treat schizophrenia

is potent D_2 receptor antagonist [158,159]. But this is weak muscarinic antagonist. But the exact mechanism is still unknown. Haloperidol can be administered through peritoneal or muscarinic route. Dose of haloperidol should be in limit because highest dose is not inducing disease [160]. Reported dose for monkey (*Cebus apella monkey*) is 0.03-10 mg/Kg (i.m.) [158], for mice 0.25 mg/Kg (i.p.) [161], and for rat 1 mg/Kg (i.p.) [159,162].

2.6. TREATMENT

Dystonia is a neurological disorder characterized by involuntary contraction of muscles that cause abnormal postures and twisting movements. The treatment of dystonia can be challenging and often involves a combination of approaches [163]. There has been prominent progress in understanding this disease leads to precise beneficial symptomatic treatments. Age, disease history, underlying aetiology, therapeutic objectives, and dystonia's impact on daily chores and QoL should be taken into mind when select a dystonia therapy [164]. Despite of the severity dystonia, the available treatments are moderately effective. So therapeutic strategy needs to be customised for individual patients [165]. Here are some of the treatments that may be used to manage dystonia:

2.6.1. Oral medication

Oral medications are used as add on therapy to get additional relief in symptoms. Yet, youngsters might be able to handle heavy amount with considerable satisfaction [166]. Systemic adverse effects are preventing adults from using oral medications. To reduce side effects, combination of medications should be started at a low dose and gradually increased over several weeks for desired result [167].

2.6.1.1. Anticholinergic

Anticholinergic medications are frequently used to treat dystonia. These drugs work by blocking the action of acetylcholine (Ach), a neurotransmitter that is involved in muscle control [168]. It is reported that trihexyphenidyl (Artane), benztropine (Cogentin), procyclidine (Kemadrin) can be effective in reducing symptoms of dystonia like muscle spasm and involuntary movements [169]. However, some side

effects like constipation, urinary retention, blurred vision, dry mouth, and cognitive changes also have identified [170]. The potential benefit of trihexyphenidyl was studied in a double-blind clinical intervention study involving 31 dystonic subjects in which a 30 mg maximum tolerability dose was taken. After follow up of 2.4 years, 42% patient showed significant effect [171]. Trihexyphenidyl is a muscarinic Ach antagonist, which daily doses are 24 mg and 41 mg for adult and children respectively. Adults are more prone to the side effects caused by trihexyphenidyl, so the dose is less as compared to children [172]. Other anticholinergic drugs such as atropine, biperiden, scopolamine, and orphenadrine have been used in treatment of dystonia [173]. As mentioned earlier that, this class of medication comes with some side effects, however these unwanted effects can be nullified by other medications like pilocarpine for blurred vision, pyridostigmine for constipation and artificial saliva for dry mouth [174].

2.6.1.2. Baclofen

Baclofen is a presynaptic gamma aminobutyric acid that is frequently in use to treat spasticity in conditions such as multiple sclerosis, spinal cord injuries, and cerebral palsy [175]. It works by activating GABA-B receptors in the CNS, which leads to decreased excitability of nerve cells and reduced muscle tone [176]. In dystonia baclofen can also be used to reduce muscle spasm and improve motor function. It is particularly effective in treating focal dystonia, which affect only specific part of body like neck, hand, etc. Baclofen can be administered orally or via intrathecal pump, which delivers the medication directly to the spinal cord [177]. However, like all medication baclofen also have side effects including nausea, weakness, dizziness, and drowsiness [178]. For improved results Trihexyphenidyl with baclofen may be used as an adjunctive therapy [179]. Similarly, combined medication of valproic acid and baclofen used for additional benefit [180]. As per reports older patients can tolerate high dose of baclofen as compared to trihexyphenidyl. An average daily dose of this drug ranges between 60 to 120 mg [181].

2.6.1.3. Benzodiazepines

Benzodiazepines are a class of medications that act on the CNS by enhancing the activity of gamma-aminobutyric acid, a neurotransmitter that inhibits the activity of neurons [182]. They are commonly used in the treatment of anxiety disorders, insomnia, and seizures. This class include drugs like alprazolam, chlordiazepoxide, clonazepam, and diazepam [183].

In dystonia, benzodiazepines may be used as treatment option, although their use is not considered as first line therapy. These drugs can help to reduce muscle tone and relieve dystonic spasms, making them potentially useful in managing certain forms of dystonia, like cervical and BSP [51]. However, use of benzodiazepines in dystonia treatment is limited as a result of their potential for addiction, tolerance, and adverse effects such dizziness, sedation, and impaired coordination [184]. There is also potential negative interaction with alcohol. Abrupt withdrawal can cause seizures. In addition, benzodiazepines may exacerbate other dystonic symptoms such as tremors [185]. Therefore, these drugs are typically used as an adjunct therapy in combination with other medications such as BoNT injection or anticholinergic drugs [186]. Effective oral daily dose ranges from 30 to 120 mg of 3 to 4 doses [187]. Overall, the option of treatment depends on the type and severity of dystonia, as well as individual patient factors and preferences.

2.6.1.4. Dopaminergic

Dopaminergic drugs, which affects the level of the neurotransmitter dopamine in the brain, have been used in the treatment of dystonia [188]. In DRD, dopamine deficiency induces dystonia. DRD is characterized by childhood onset dystonia developing in legs [189]. In such case, treatment with a dopamine precursor named levodopa can be effective. Levodopa is converted to dopamine in the brain, which helps to reduce muscle contraction and improve motor control [190]. However, some dystonia's are not responsive to dopaminergic drugs, and in some cases, they can worsen the symptoms. Spasmodic torticollis, commonly known as CD induce due to the use of dopamine antagonists, which block dopamine activity in the brain and can be effective in reducing symptoms [191]. It has been reported that mutation in the

GTP cyclohydrolase 1 (GCH1) gene on chromosome 14q results into DRD [192]. Generally dopaminergic therapy involve combination of decarboxylase inhibitor carbidopa/levodopa with an average daily dose of 25/100 mg [193]. Unlike Parkinson's disease, the subject with dystonia may develop side effects like hallucination, confusion, nausea, orthostasis, and constipation [194]. Sepiaterin deficiency, "6-pyruvol-tetrahydropterin synthase deficiency, aromatic amino acid decarboxylase" deficit, and tyrosine hydroxylase deficiency are other dopamine metabolism disorders that can result in dystonia [195].

Tetrabenazine (TBZ) is a dopamine depleting drug was approved by USFDA in 2008 for treatment of Huntington's disease [196]. This agent depletes dopamine, serotonin, and norepinephrine in CNS by inhibiting vesicular monoamine transporter type 2. It has mild D2 receptor blocking activity and has proven to be effective against movement disorder like dystonia [13]. In a double blind, crossover study, TBZ revealed significantly effective in different kinds of dystonia. In this study researcher evaluated the tolerability of TBZ in a patient group including 132 dystonia patients. At the end of the study it was observed that 69.5% of patient had satisfactory symptomatic recovery with a daily dose of 50-75 mg [197]. Moreover, tardive dystonic patients got excellent benefit from this TBZ therapy. Generally dose of the drug started from 12.5 mg and maximize upto 25-100 mg with considering the unwanted effects such as akathisia, depression, anxiety, insomnia, drowsiness, and PD [198] [199]. Sometimes to counteract the side effects an adjunct therapy with lithium can be prescribed to patients for better result [200]. Another dopamine depleting agent named Reserpine also found effective against tardive dystonia and the mechanism of action is quite familiar with mentioned drug TBZ [201]. However, due to serious peripheral side effects like GIT upset, migraine, etc. TBZ is preferred over all oral medication.

2.6.2. Neurotoxic protein (Botulinum toxin)

Botulinum toxin, also known as BoNT, is a neurotoxic protein generated by "bacterium *Clostridium botulinum"* and related species [202]. It is one of the most lethal substances known and can cause muscular and respiratory paralysis [203]. It exists in seven stereotypes such as toxin "A, B, C, D, E, F, G" [204]. The toxin is a

dipeptide with a light chain of 50 kDa and heavy chain of 100 kDa that blocks the Ach release at nerve terminal. Both heavy and light chain prevent Ach release with their specific mechanism of action. The presynaptic cholinergic vesicle must fuse with the plasma membrane for Ach to be released into the synaptic cleft. To do this, soluble "N-ethylmaleimide-sensitive factor attachment receptor proteins" are cleaved by the light chain. For entrance into the synaptic bulb, the heavy chain attaches to peripheral cholinergic nerve terminals [205].

Total 4 toxins formulations are approved by FDA and readily available in market e.g. 3 type A (NT 201, Dysport, Botox) and one type B formulation (Myobloc) [206]. All these mentioned formulations are not equivalent but quite similar on their properties and dosing frequency. Type A formulations are generally crystalline so before use they are diluted in normal saline. Unlikely type B toxins are available in liquid form [207].

It is reported that BoNT has become primary choice for treating different types of dystonia including lingual, CD, spasmodic, oromandibular, BSP, and segmental [186]. Similar to other medication BoNT also exhibit side effects based on their site of injection e.g., dysphagia after neck injection, diplopia after eyelid injection, weakness after intramuscular, etc. However, some general side effects involve with this toxin are flu, dry mouth, constipation, local bruising, etc [208].

Despite of all side effects, patients immunized with BoNT may develop resistance and neutralizing antibodies in long term use. Medication repetition in every three months may reduce the generation of neutralizing antibodies. Similarly keep switching between stereotypes also easily nullify the resistance produce by specific touse [209]. Keeping this in consideration physician should select adequate dose and medication. A double blind, placebo, cross over study on BSP found that phytase and zinc supplement significantly increase the duration of positive impact of toxin [210]. It has been shown in an experimental study that, Trans-X, a topically applied medication contain NSF (N-ethylmaleimide sensitive fusion) protein can effectively induce paralysis and proven as a safer alternative to BoNT [18]. The use of BoNT is strictly restricted in pregnancy and lactation. Additionally neuromuscular diseases like myasthenia gravis is a relative contraindication [211].

2.6.3. Surgery (Deep Brain Stimulation (DBS))

DBS is a type of neurosurgery that involves inserting electrodes into specific parts of the brain [20] and using electrical stimulation to treat movement disorders like PD, dystonia, and tremors [212]. DBS was first approved as a Humanitarian Exemption Device for dystonia by USFDA in 2003 [213]. Before DBS introduced, pallidotomy and thalamotomy like ablative procedures were used to treat movement disorders like dystonia. DBS has several advantages over other ablative procedures such as reversible, adjustable, and non-destructive nature. The risk of long-term speech, swallowing, or cognitive impairments associated with bilateral ablative procedures can be avoided by performing it bilaterally carefully [214]. Individuals who have undergone optimum medical diagnosis of dystonia and impaired after receiving the best medical care should be prescribed for DBS. The GPi area has identified as a prospective target for DBS. Several of the motor symptoms linked to advanced movement disorder can be treated using the well-established, secure, and efficient DBS of the GPi [215].

A blinded clinical assessment trial with 40 patients found that, bilateral GPi stimulation significantly alleviate symptoms compared to sham treatment. All patients thereafter finished a 6-month open-label extension of their active treatment. Between 3 and 5 years, further follow-up revealed a considerable improvement in dystonia [216].

Prolonged disease duration adversely linked with response magnitude, although age and disease severity did not, according to a retrospective research examining predictors of response to stimulation in CD. This implies that earlier DBS might produce better outcomes in patients with severe CD [217].

Another study of twenty eight patients with bilateral GPi DBS and idiopathic CD found no association between baseline severity, disease duration, or age onset. DBS or any other therapy would be expected to have a poor in patients with contractures [218]. Individuals with generalised dystonia were treated with bilateral GPi DBS in a blind assessment. There was a major clinical progress in the treatment of dystonia at

12 months juxtapose to the patient's pre-surgical baseline. A three-year follow-up research showed that this benefit was sustained [219].

It is reported that, primary dystonia responds better to GPi DBS than secondary dystonia. Secondary dystonia respond to DBS include post-infarct hemidystonia, X-linked dystonia Parkinsonism, brain iron accumulation associated neurodegeneration, and DRD [220].

It has been proven that patients with CD who do not benefit from oral medication and BoNT treatment can get better result from bilateral GPi DBS [221].

The effectiveness of bilateral pallidal stimulation was assessed in a prospective, multi-centre pilot trial of age 33 years in 13 patients. These included patients with severe dystonia-choreathetosis, improved by 24.4% [222].

Some studies found that, depression prior to surgery leads to suicidal attempts followed by pallidal DBS for dystonia [223].

While DBS has been shown significant improvement in patients who have primary dystonia but, there are potentially side effects that could happen, as reported in a long-term research on DBS. Speaking abnormalities "(dysarthria, dysphonia, and stuttering), paresthesias, perioral tingling, and lack of coordination" few side effects of GPi stimulation that can be harmful. Additionally, hardware related side effects include electrode damage, lead fracture/misplacement, electrode erosion, implanted pulse generator dysfunction, hematoma, or infection [215].

A major issue comes with DBS is the faster depletion of battery in the patients who use high efficiency configuration. For some patients, rechargeable neurostimulators may be a viable choice because they don't require regular battery replacement. Recharging may be necessary every few days for several hours, depending on the intensity of the stimulation [224].

Primary and tardive dystonia have been studied using the "subthalamic nucleus (STN)" as an alternative effective target. STN DBS has been connected to several of advantages, comprising the absence of bradykinesia as a side effect, instant symptom relief following programming, and low settings resulting in longer battery life [225].

2.7. Tetrabenazine

2.7.1. Mechanism of Tetrabenazine

TBZ acts on VMAT2 on presynaptic cleft and less effective on D2 receptor on postsynaptic cleft.

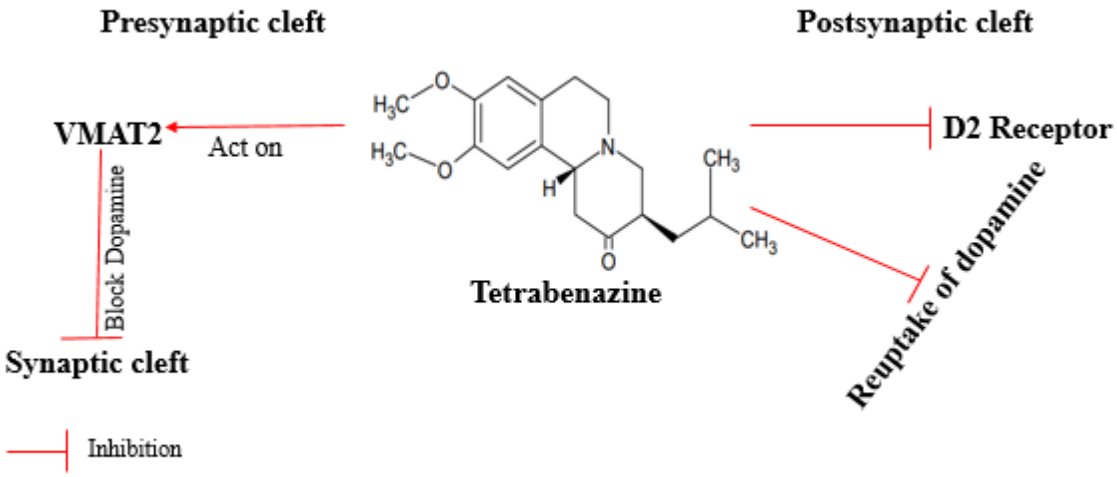


Figure 3: Mechanism of Tetrabenazine for Dystonia

Table 1: Published clinical studies of TBZ for Dystonia

Study	Design	Average dose of TBZ (mg/day)	Total patient	Outcomes	Interpretation	Reference
Jankovic, 1981	Double blind, randomized, placebo and controlled	162.5	12	11 (92%)	Useful and effective	[226]
Marsden, 1984	Retrospective (P & A)	90/75 (adult/paediatric)	14	10 (71%)	Useful and effective	[227]
Jankovic and Orman,1988	Retrospective (P & A)	85-155.4	124	94 (76%)	Useful and effective	[30]
Jankovic and Beach, 1997	Retrospective (P & A)	112-125	190	132 (60%)	Useful and effective	[200]
Kenney et	Retrospective	No data	No data	68%	Useful and	[228]

al., 2007	(P & A)				effective	
Swash et al., 1972	Single blind (P & A)	128	8	3 (38%)	Not effective	[229]
Asher and Aminoff, 1981	Crossover, single blind, placebo	175	8	2 (25%)	Not effective	[230]
Paleacu et al., 2004	Retrospective	No data	41	20 (49%)	Not effective	[231]

*P & A- Paediatric and Adult

Joseph Jankovic reported in 1981 double blind crossover studies which was showing effective results for hyperkinetic disorder. Drowsiness, insomnia, restlessness, and tremor was some common adverse effect (AE). In this 0-4 rating scale was considered 0 was showing absent, 1 was for hardly noticeable, 2 for moderate, 3 for severe and 4 for very severe. Total of 12 dystonic patient were treated with TBZ and showed very impactful result i.e., 92% which conclude effective and useful for dystonia [226].

Marsden et al., in 1984 reported retrospective studies of 14 dystonia patient included paediatric and adult treated with TBZ in the mean dose range of 90/75 mg/d. Out of 14 patients 10 patients were treated well and gave good response with less side effects [227].

Jankovic et al., reported double-blind, placebo study of 217 patients of different kind of involuntary movement disorder. After the prolonged study of TBZ in 217 patients conducted, scale of 1-5 were considered for their response. Total of 124 patients of different kind of dystonia in which one patient gave scale 1 response, 32 out of 42 patients gave scale 2 response 43 out of 53 gave 3^{rd} number of response, 20 out of 25 gave 4^{th} response and 4 out of 5 scale gave 5^{th} rating of scale. Parkinsonism, drowsiness, depression, and nervousness was found common adverse effect. So through this study one can conclude that TBZ is efficacious and safe for the treatment of involuntary/hyperkinetic movement. Treatment was started from the initial dose of 25 mg/d up to 100 mg/d and higher dose was showed AE [30].

Jankovic et al., in 1997 reported another study of 526 patients, treated with TBZ in the period of 15 years. 126 patients were exclude because of lack of efficacy, side effects. Responses of 400 patients were considered as mentioned scale in previous studies.

Initial TBZ dose was taken 25 mg/d until a total dose of 150-200 mg/d. Total of 190 patient were considered for dystonia in which 132 patients were treated and showed good efficacy and safe [200].

Kenney et al., reported study of TBZ on various hyperkinetic movement disorder at Baylor College of Medicine Parkinson's Disease Centre and Movement Disorders Clinic. The study overall of 448 patients of different hyperkinetic movement disorders such as Dyskinesia (132 patients), Chorea (98 Patients), Tics (92 Patients), and Myoclonus (19) treated with TBZ in the time duration of Jan 1997 to Jan 2004. Males were 42% in the study. Patients were regularly checked after every 3 to 6 months and their responses were taken by the rating scale i.e., 1 to 5 in this 1 was for better improvement, 2 was for good improvement, 3 was for mild, 4 was for poor response, and 5 was for worsening. Adverse effects were also checked during the study by open ended questions and drowsiness, Parkinsonism, depression, and akethisia were common AE [228].

Some other preclinical studies were also reported like Swash et al., 1972 presented study of 8 patient in which 3 responded well with the mean dose of 128 mg/d, Asher and Aminoff, 1981 reported treatment of dystonia with TBZ (mean dose 175 mg/d) in 8 patient in which only 2 gave good results/response, Paleacu et al., 2004 presented study of 41 patient who were suffered with dystonia treated with TBZ treated 20 patients i.e. 49%. So these studies reported TBZ for Dystonia disorder was not very much effective [229–231].

Most cases of myoclonic dystonia is occur due to deletion or mutation in SGCE. This kind of dystonia is more severe as compare to normal dystonia because it causes functional disabilities. In this deep stimulation therapy is chosen as compare to oral therapy because in oral therapy gives poor response to the patient. Tetrabenazine is used for the treatment of the subject and showed mild to moderate response in myoclonic dystonia. So before going to deep brain stimulation a therapeutic trial of tetrabenazine should be consider in patient [232].

Scharder et al., reported use of TBZ in Huntington's disease so the data available is also about Huntington's disease. Few cases are present for dystonia and tardive dyskinesia with the TBZ. Dystonia treated with TBZ gives most promising effect in displaying predominant phasic, repetitive movement but not so much effective in the

treatment of tonic movement and also in depression which occur most commonly while treating dystonia [233].

Table 2: Reported studies of Tetrabenazine with various formulations

Sr. No	Formulation	Route	Compositions	No. of animals	Dose	Results	Reference
1.	Orodispersible film (ODF) tablet for paediatric	Oral	Polymer + TBZ + citric acid + Glycerol + sorbitol	NA	NA	Enhance BA	[234]
2.	Nanoemulsion	Intranasal	Capmul MCM + Tween 80 + Transcutol© P	12 (Wistar rat)	1.25 mg/day	Higher AUC and C_{max} (7 folds)	[235]
3.	Nanoacervates	Oral	Chitosan + glacial acetic acid + sodium hydroxide	NA	NA	Sustained release	[236]

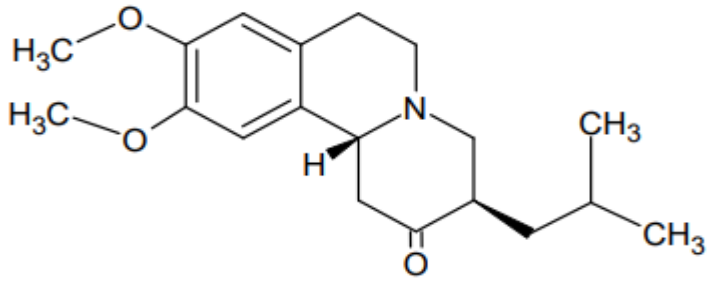
Arora et al., 2016 formulate TBZ loaded nano-emulsion for intra-nasal route with capmul MCM, transcutol© P, tween 80 as oil, co-surfactant, and surfactant to overcome the low aqueous solubility, and extensive first pass metabolism. Different characterization such as GS, % transmittance, PDI, ex-vivo and in-vitro studies was performed for optimization. In-vivo studies conducted on Wistar albino rats which provide higher AUC and C_{max} in brain as compare to suspension of TBZ by i.v. route (intravenous route) [235].

Senta loys et al., 2016 formulated ODF of TBZ to treat hyperkinetic movement in paediatric patients. They used different polymer (4 in number) and solvent casting/evaporation method to prepare ODF. Different characterization of ODF was performed such as FTIR, DSC, in vitro and morphological by polarized light microscopy [237].

Haseena begum et al., 2021 prepared nanocoacervates of TBZ for the enhancement of solubility and permeability. These nanocoacervates prepared with chitosan as polymer and coacervates technique. Formulation was characterized by ZP, GS, encapsulation efficiency, PDI, scanning electron microscopy, and in vitro release kinetic. 2^4 full factorial design was used to optimize the formulation. Drug release was found up to 12 hours [236].

2.8. Drug Profile

2.8.1. Tetrabenazine (TBZ)

Characteristics	Description
Drug Name	Tetrabenazine
Formula	$C_{19}H_{27}NO_3$
Category	Hyperkinetic movement
Molecular weight	317.42
Synonyms	Nitoman, Xenazine
IUPAC Name	9,10-dimethoxy-3-(2-methylpropyl) 1H,2H,3H,4H,6H,7H,11bH-pyrido[2,1-a]isoquinolin-2-one
Chemical structure	
Water Solubility	Sparingly soluble (343.5mg/L)
Melting Point	126℃
Log P	2.66
PKa	6.51
Absorption	75%
Protein binding	82-88%
Half-life	10hours

2.9. SNEDDS

2.9.1. Mechanism of SNEDDS Formulations

The isotropic mixtures were prepared (1 mL) by mixing the amount of co-surfactant, surfactant and oil and then vortexed for 15 minutes. The prepared isotropic mixture (1mL) was diluted in five hundred milliliter of distilled water to form SNEDDS as shown in Figure 1. The diluted mixture stirred at 700-800 rpm for 7 min. The temperature of the solution was maintained at 37± 0.2°C.

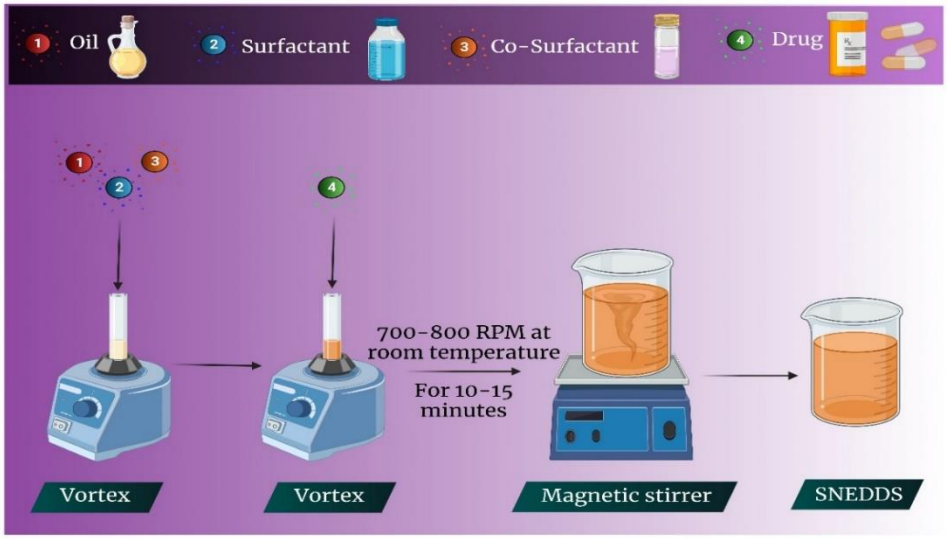


Figure 4: Mechanism of SNEDDS formulations

2.9.2. Mechanism of self-emulsification

Self-emulsification process happen when there is change in energy i.e. entropy. When emulsion formation occur free energy release which used in direct function of energy which form new surface between both phases i.e. oil and water [238].

2.9.3. Mechanism of Drug transport to brain after oral administration through SNEDDS

SNEDDS are the pre-concentrated form of the NEs. The optimized formulation will be an efficient carrier of the hydrophobic drugs across the biological membranes through the passive diffusion mechanism. On reducing the hepatic first pass metabolism, p-gp efflux using SNEDDS as carrier BA of the hydrophobic drugs can be increased. When the formulation enters into the GIT, agitation is provided by means of gastric motility to the formulation which makes it to convert into NEs. These nanosized globules which are less than 200nm can offer efficient permeability across the membranes as depicted in Figure 3. Thus, through lymphatic absorption

route hydrophobic drugs which are soluble in oil forms globules with the help of surfactant and co-surfactant and serves as a sustained delivery depots offering efficient permeability across the blood brain barrier and produce neuroprotective activity (Figure 5).

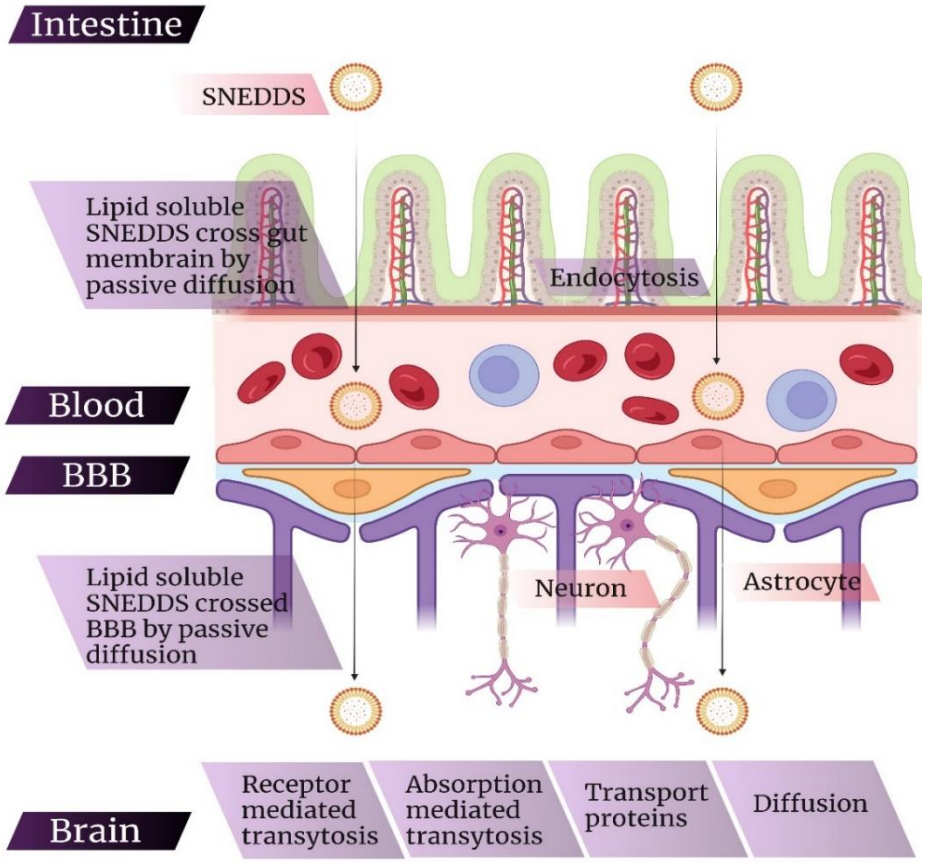


Figure 5: Mechanism of Drug transport to brain through oral SNEDDS

2.9.4. Composition of SNEDDS

The SNEDDS formulations are done with the help of oils, surfactants and co-surfactant and their HLB values which play a significant part in in making the formulation as a transparent homogeneous phase is depicted in Table 3.

Table 3: Compositions of SNEDDS

Lipid	**HLB**	**Surfactant**	**HLB**	**Co-Surfactant**	**HLB**
Castor oil	10.5	Brij-30	9.7	Plurol Oleique CC497	6
Corn oil	9	Labrafil M 2125 CS	4	Caprol 6G20	-
Olive oil	8	Labrafill M 1944 CS	4	Hodag PGO-62	-
Peanut oil	6	Labrasol	14	Span 80	4.3
Cotton seed oil	7.5	Labrafac CM	>10	Lauroglycol 90	5

Canola oil	7	Pluronic F127	18-23	Lauroglycol FCC	4
Rapeseed oil	-	Labrafil WL 2609	6-7	HCO-60	14
Coconut oil	-	Lecithin	4-9	SLS	40
Capmul MCM	5.5-6	Tween 85	15	Poloxyl 35	-
Capryol MCM	-	Tween 80	15	Pluronic L44	12-18
Capryol PGMC	5	Tween 65	17	Transcutol© P	4.2
Capryol 90	6	Tween 20	17	Capmul MCM-C8	5-6
Phosal 53 MCT	-	Simulsol 1292	17	Labrafill 1944	4
DHA oil	-	Cerex ELS 250	17	Arlasolve DMI	-
Glycerol monooleate	-	Tagat TO	17	Pluronic L64	12-18
Sefsol-218	-	Cremophor EL	12	Akoline MCM	5-6
Eucalyptus	-	Cremophor ELP	14	Lutrol F 68	29
Soyabean oil	-	Macrogolglycerol hydroxyl stearate	-	Carbitol	4.2
Palm oil	-	Brij 97	12.4	Transcutol© HP	4
Palm kernel oil	-	Cremophor RH 40	13	Labrasol	-
Cocoa butter	-	Span 80	4.3	PEG 200	8.1
Lard	-	TPGS	13	PEG 400	11.5
Tallow	-	HCO 40	13	Di-ethyl glycol mono ethyl ether	14-16
Lauroglycol	-	Pluronic L-64	13	Polyglycerol-3-dioleate	-
Isopropyl myristate	8-9	Peceol	3-4	Capryol 90	6
Linseed oil	-	Emulphor EI-620	12-15	Soya Phosphatidycholine	5.0
Captex triacetin	-	Sorbitan Oleate	-	Imuntor 988	13-15
Captex 500	-	Kolliphor RH 40	-	Propionic Acid	-
Black seed oil	-	Solutol HS15	-	Cremophor EL	12-14
Labrafil M1944 CS	-	Labrafac lipophile WL 1349	1	Miglyol N812	-
Oleoyl polyoxyl-6 glycerides	-	Transcutol© HP	4	Glycofurol	-
Sefsol	-	Triton X-100	13.5	Transcutol©	-
Vitamin E	-	Diethyl Glycol monoethyl ether	9.7	Lutrol E 400	-

Crodamol GTCC	-	PEG 200	8	Glycerol	7
Rose Oil	-	PEG 400	-		
Labrafil	-				
Capmul MCM EPC8	-				
Acconon E	-				
Oleic acid	-				
Cinnamon oil	-				
Triglyceride	-				
Captex 355	-				
Maisine 35-1	-				

2.9.5. Advantage of formulating SNEDDS over other NDDS

2.9.5.1. Enhanced bioavailability

SNEDDS enhanced BA as compare to other oral route. As the BA increase the drug is more effective and can target the specific site. By increasing BA SNEDDS can cross so many barriers which is present in our body. So as compare to other formulations it enhances more BA. R. N. Vanani et al., reported increasing BA of sunitinib (anticancer) when loaded in SNEDDS, AUC and C_{max} of the drug increased by 1.45 and 1.24 which has shown enhancement in the BA of the drug [239].

2.9.5.2. Economical

As stated in numerous papers, SNEDDS increases the BA of a medicine. As the BA of a drug increases, the quantity of the drug in a single dose decreases, making SNEDDS formulation cost effective. SNEDDS are cost effective since they do not require advanced manufacturing facilities.

2.9.5.3. Easy to Prepare

Because of the processes utilized in their manufacturing, SNEDDS are simple to create and scale up. SNEDDS requires relatively basic and cost-effective production facilities for large-scale manufacturing, such as a simple mixer with a volumetric liquid loading apparatus and an agitator [240].

2.9.5.4. Stability

Enhancer Supersaturated SNEDDS, cemented SNEDDS, controlled release solid SNEDDS, and mucus permeable SNEDDS are some of the options. Solidification is used to increase drug stability and avoid medication-excipient interactions. While in

storage, SNEDDS become more stable. As A. Alghananim reported S-SNEDDS of deferasirox, which shown stable SNEDDS because after keeping the formulations in different stressed conditions SNEDDS were isotropic single phase emulsion [241].

Table 4: Composition and salient features of SNEDDS of Neuronal Diseases

S. No	Disease	Drug	Compositions of SNEDDS			Observation	References
			Oil	**Surfactant**	**Co-Surfactant**		
1	**Parkinson Disease**	Fisetin	Castor oil /laurogly col	Tween80	Transcutol© P	Better oral BA and increase neuroprotective effect	[242] [243]
		Hisperidine extract	Isopropyl Myristate	Tween 80	PEG 200	Improve MAO B inhibiting effect, decrease in IC_{50} and prevent Neuronal disease	[244]
		Rutin	Sefsol/Vit E	Solutol HS	Transcutol© P	Faster and better absorption	[245]
2	**Alzheimer Disease**	Vinpocetine	Maisine 35-1	Cremphor EL	Transcutol© P	Enhanced solubility	[246]
		Ginsenoside Rg1	Isopropyl myristate /capryol	Tween 80	Transcutol©	Improve brain biodistribution, higher efficiency	[247]
		Luteolin	Crodamol GTCC	Cremphor EL	PEG 400	Increased aqueous solubility and poor BA	[248]
3	**Antiepileptic**	Lamotrigine	Rose oil	Cremphor EL	PEG400/Tween 80	enhanced the solubility and dissolution, increased BA	[249]
		Zaleplon	Labrafil/capryol	Labrasol	Transcutol©	↑ed C_{max} 1.25 folds	[118]
4	**Schizophernia**	Lurasidone Hcl	Capmul MCM EP C8	Cremphor EL	Transcutol© HP	Enhanced BA, increased intestinal absorption	[250]
			Capmul MCM	Tween 80	Glycerol	Enhanced BA, reduce food effect on drug absorptions	[251]
		Ziprasidone	Capmul MCM	Labrasol	PEG 400	↑ed $AUC_{0-\infty}$ 1.6 and 2.8 folds in fed and fasted state	[252]

5	**Antipsychotic**	Phenothiazine	Linseed oil/olive oil	Tween 85	Ethanol	Phenothiazines incorporated into SNEDDS leads to enhanced uptake by plasma derived chylomicrons, compared to naked phenothiazines in solution	[253]
		Quetiapine	Olive oil	Span 80	PEG 400	Thermodynamically stable and chemical intact over a period	[254]
		Olanzapine	Acconon E	Cremophor EL		Compatibility with all excipients, better release and better pharmacodynamic efficacy, no harmful effect on organs	[255]
			Capryol PGMC	Tween 60	Lutrol E 400	Physical stability	[256]
			Capryol 90	Brij 97	Ethanol	It increased by 1.2 fold compare to marketed formulation and 1.6 fold compared to pure drug suspension.	[257]
		Amisulpride	Caproyl 90	Cremophor RH 40	Transcutol© HP	Solubility increased and permeability increased	[258]
		Chloropromazine	captex, triacetin, linseed oil, and olive oil	Tween 85	Ethanol	↑ed 6.5 fold BA ↑ed 3.6 folds in C_{max} ↑ed ~2-6.5 folds in AUC_{0-24} ↑ed 3.2 folds permeation	[259]
6	**Neuroprotective**	Akebia saponin D	Castor Oil	Cremphor EL	Polyoxyl 35	↑ed permeability and 4.3 folds BA	[260]

CHAPTER 3

RATIONALE OF THE STUDY

3. RATIONALE OF THE STUDY

Non-communicable disease are leading cause of mortality and disability in the world, increasing due to changes in the epidemiology and demographics that are happening in both developed and emerging nations [261]. In 2006, WHO insistence the significance of neurodegenerative disorders for public health, and with an approximately that these disorders were responsible for 6.3% of the world's disability adjusted life years (DALY's) [262].

In Europe estimate for brain disorders of 296 billion corresponded to 24%. [263]. In China if all neuronal disorder cases try to get white collar therapy the possible economic load would be $484.1 billion in 2013 as compare to 2005 which was $149.6 billion [264]. From stage I to stage IV, the average yearly cost of hospital and residential care increased from £99.40 to £12,566. The average yearly cost per person in stage I was £2250, while in stage IV it increased to £89,760 [265].

Tetrabenazine's potential as a treatment for dystonia is severely constrained by its poor solubility in water and low oral absorption. Due to involvement of SNEDDS in boosting oral BA of weakly water-soluble drug by increasing their aqueous solubility and expanding their GI permeability, liquid SNEDDS have been offered as a solution to these problems. Additionally, the SNEDDS formulation will shield the drugs against GIT breakdown [37]. It also protect drug from P-gp and cytochrome P450 enzymes to increase intracellular concentration [36].

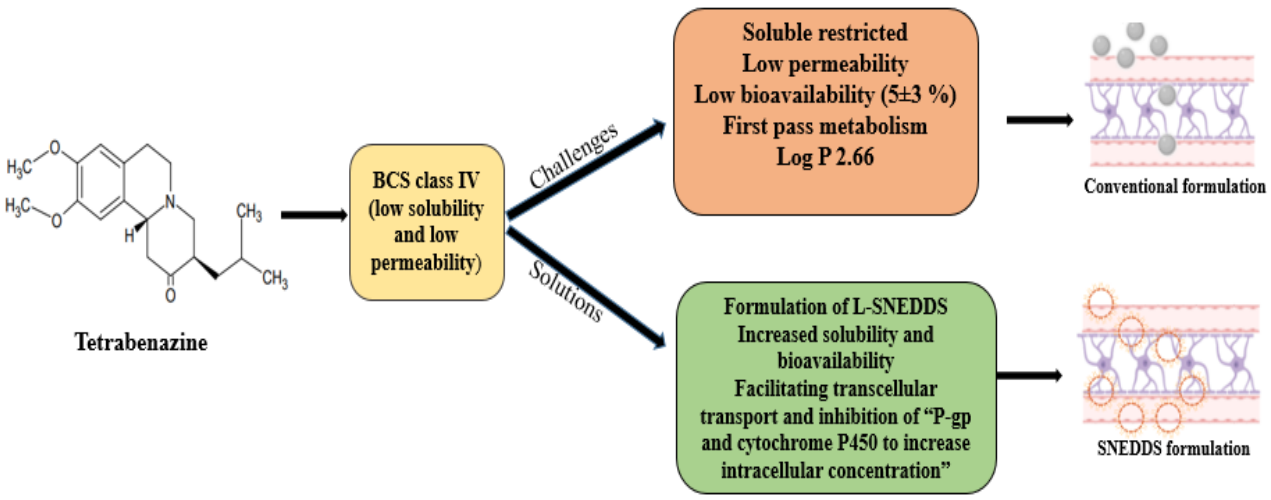


Figure 6: Hypothesis of research work

Aim

Formulation and evaluation of self-nano emulsifying drug delivery system loaded with Tetrabenazine for the treatment of Dystonia

Objectives

The objective of proposed study are:

- Formulation, development and optimization of SNEDDS loaded with Tetrabenazine.
- Characterization and evaluation of optimized formulation.
- Stability study of optimized formulation.
- In-vitro studies.
- Pharmacokinetics and pharmacodynamics evaluation of optimized formulation.

CHAPTER 4

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. Materials

The list of various materials and equipment's used are mentioned in Table 1 and 2 respectively.

Table 5: List of material used in study

Chemicals	Manufacturers
Tetrabenazine	Synnat Pharma Pvt. Ltd., India
Haloperidol	Sigma Alderich
Acetonitrile HPLC grade	Merck, Mumbai, India
Olive oil	Central drug house Pvt. Ltd, New Delhi, India
Cotton seed oil	Central drug house Pvt. Ltd, New Delhi, India
Peanut oil	Central drug house Pvt. Ltd, New Delhi, India
Paraffin oil	Central drug house Pvt. Ltd, New Delhi, India
Potassium dihydrogen ortho phosphate	Central drug house Pvt. Ltd, New Delhi, India
EDTA	Central drug house Pvt. Ltd, New Delhi, India
Formaldehyde	Central drug house Pvt. Ltd, New Delhi, India
Sodium hydroxide	Central drug house Pvt. Ltd, New Delhi, India
Milipore water	Central drug house Pvt. Ltd, New Delhi, India
Hydrochloric acid	Central drug house Pvt. Ltd, New Delhi, India
Eucalyptus oil	Central drug house Pvt. Ltd, New Delhi, India
Capryol MCM	M/S Abitec Corp., Ohio
Tween 20	Lobachemie Pvt. Ltd., Mumbai, India
Iso propyl myristate	Lobachemie Pvt. Ltd., Mumbai, India
Tween 60	Lobachemie Pvt. Ltd., Mumbai, India
PEG 400	Lobachemie Pvt. Ltd., Mumbai, India
Tween 80	Lobachemie Pvt. Ltd., Mumbai, India
Ammonium acetate	Lobachemie Pvt. Ltd., Mumbai, India
Acetonitrile	Lobachemie Pvt. Ltd., Mumbai, India
Formic acid	Lobachemie Pvt. Ltd., Mumbai, India
Orthophosphoric acid	Lobachemie Pvt. Ltd., Mumbai, India
Triethyl amine	Lobachemie Pvt. Ltd., Mumbai, India
Span 80	Lobachemie Pvt. Ltd., Mumbai, India
Labrasol	Gattefosse Pvt. Ltd., Mumbai, India
Labrafac WL 1349	Gattefosse Pvt. Ltd., Mumbai, India
Labrafil M 1944 CS	Gattefosse Pvt. Ltd., Mumbai, India

Capryol PGMC	Gattefosse Pvt. Ltd., Mumbai, India
Labrafac PG	Gattefosse Pvt. Ltd., Mumbai, India
Peceol	Gattefosse Pvt. Ltd., Mumbai, India
Capryol 90	Gattefosse Pvt. Ltd., Mumbai, India
Lauroglycol 90	Gattefosse Pvt. Ltd., Mumbai, India
Lauroglycol FCC	Gattefosse Pvt. Ltd., Mumbai, India
Transcutol© P	Gattefosse Pvt. Ltd., Mumbai, India
Transcutol© HP	Gattefosse Pvt. Ltd., Mumbai, India
Plurol Oleique	Gattefosse Pvt. Ltd., Mumbai, India
Maisin CC	Gattefosse Pvt. Ltd., Mumbai, India
Milipore water	Bio-Age Equipment Ltd., Mohali, India
Methanol	Molychem, Mumbai, India

Table 6: List of equipment's utilised in the research

Equipment's	**Model/Manufacturer**
Electronic weighing balance	CY360, Shimadzu Co. Ltd., Kyoto, Japan
pH meter	Labtronic LT10, India
HPLC	1. HPLC LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan 2. Autosampler HPLC LC-20AD, Shimadzu Co. Ltd., Kyoto, Japan
Magnetic Stirrer	Remi 5MLH, Vasai, Mumbai, India
Water bath shaker	Labfit, India
Centrifuge	REMI RM-12C, Remi Elektrotechnic. Ltd, Vasai, India, Mumbai, India
Vortex	REMI CM101, Delhi, India
UV visible spectrophotometer	UV-1800, Shimadzu Co. Ltd., Kyoto, Japan
Dissolution apparatus	DS 8000 (Manual) Lab India, Mumbai, India
Melting point apparatus	Popular, India
Particle size analyser	Malvern Zetasizer, Nano ZS90, UK
Transmission electron microscopy	TEM-2100 plus Electron microscope, Jeol, Japan
FTIR	PerkinElmer, USA
XRD	Bruker D8 Advance, USA
DSC	1. DSC Q200 TA, Universal instrument, Bangalore, India 2. DSC 6000 PerkinElmer, USA
0.45 µm syringe filter	Merck, Germany
0.25 µm syringe filter	Merck, Germany
Stability chamber	Remi C4M 10Plus, India

Hot air oven	Navyug India Q5247
Homogenizer	Glass-Teflon potter homogenizer, Thomas Scientific, USA
Bath sonicator	Loba Life, Lobachemie, Mumbai, India
Millipore vacuum filter	Sigma-Aldrich Chemicals Private Limited, Bangalore, India
Ultra-low temperature freezer	BIOEVOPEAK INC, USA

4.2. Methodology

4.2.1. UV-Spectrum Analysis

For spectrum analysis, 10 µg/mL of TBZ solution was prepared using acetonitrile (ACN) and water (2:8) solution was scanned using UV- spectrophotometer at wavelength ranging from 200-800 nm. During analysis water and ACN (9:1) was used as blank.

4.2.2. Analytical method development

HPLC analysis was carried out using HPLC (instrument from Shimadzu Japan) equipped with a pumping system of LC-20 AD series, a PDA detector (SPDM20A; Shimadzu, Japan), manual Rheodyne injector capacity 20 µL. LC Solutions software was used for data processing and interpretation. Sonicator was employed to degas the air bubbles in the mobile phase. Calibrated pH meter was used to measure the pH of prepared formic acid. For estimation of the drug, the stationary phase was a C-18 reversed phase column (C18, 250 mm × 4.6 mm, 5 µm) was used. Number of trails were made using acetonitrile, - 5 mM ammonium acetate; acetonitrile- 0.1% glacial acetic acid; acetonitrile-0.1% ortho-phosphoric acid and acetonitrile-0.1% formic acid as a mobile phase by varying their pH and ratio of mobile phase. Final mobile phase composed of formic acid (pH 3.2) and acetonitrile (10:90 v/v). The flow rate kept 1 mL/min. The ambient temperature in the column was 25 ºC. The eluent's detection wavelength was 283 nm.

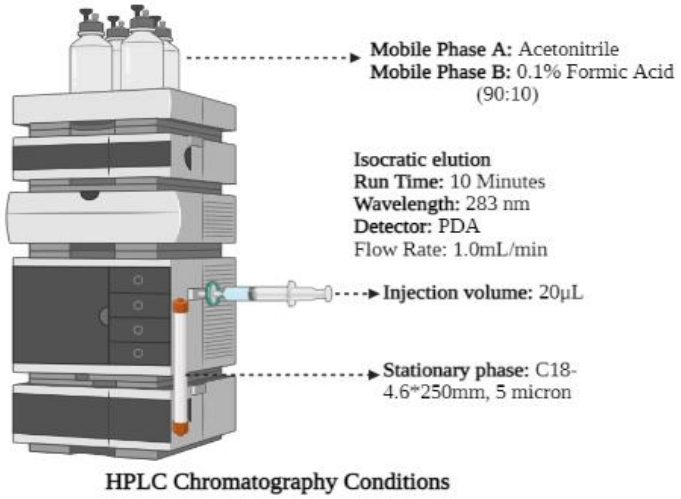


Figure 7: HPLC-chromatography conditions

4.2.2.1. Preparation of formic acid pH 3.2

In a 100 mL volumetric flask formic acid (100 µL) was taken and the volume was made up using triple distilled water. The pH of the formic acid solution was adjusted to 3.2 using triethyl amine. This prepared solution is filtered through a 0.45 µm membrane filter and sonicated for 10 min to remove the air bubbles.

4.2.2.2. Mobile phase composition

The mobile phase was incorporated of 90 parts of ACN and 10 parts of formic acid with a pH of 3.2 and filtered through a 0.45 µm membrane filter and was ultrasonicated for 10 min to degas the mobile phase and remove the air bubbles.

4.2.2.3. Preparation of Standard Stock Solution

Ten milligrams of TBZ was dissolved in 2 mL ACN and made up the volume up to 10 mL with distilled water (1000 µg/mL). Serial dilutions were performed by taking 1 mL of the above solution and making it up to 10 mL resulting in a solution of 100 µg/mL, which on further dilution yield a solution of 10 µg/mL. From the above solution, dilutions were performed to get final concentrations of 2, 4, 6, and 8 µg/mL [266,267].

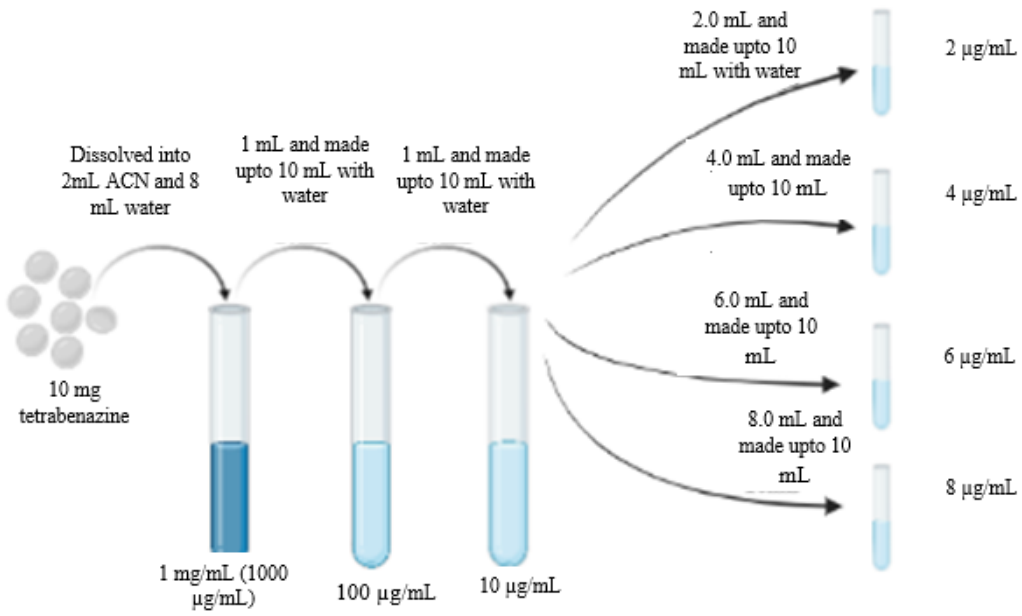


Figure 8: Steps for preparation of dilutions

4.2.3. Method validation

The developed method was validated as per the "International Conference on Harmonization (ICH) Tripartite Guideline Q2 (R1) for linearity, accuracy, precision, and specificity" [268].

4.2.3.1. System suitability

Tailing factor, height equivalent to theoretical plate (HETP), and peak purity index [269], were calculated by injecting system suitability sample i.e., 10 µg/mL of TBZ (6 times) in HPLC system [270].

4.2.3.2. Preparation of quality control standards

Standards for quality control at three separate levels were created, i.e., lower quality control (LQC), medium quality control (MQC), and higher quality control (HQC) of the calibration curve [271]. Hence the center value 6 µg/mL (100%) was kept as MQC level, 80% of 6 µg/mL was kept as LQC (4.8 µg/mL), and 120% of 6 µg/mL was kept as HQC (7.2 µg/mL). All these three concentrations were prepared in the same mobile phase.

4.2.3.3. Linearity and range

Analytical method's range is the gap between the samples lowest and the highest concentrations of analyte for which an acceptable level of precision is present in the analytical process. Linearity was evaluated by analysing a series of various concentrations (2-10 µg/mL) of TBZ. Five concentrations (2, 4, 6, 8, 10 µg/mL) of TBZ were injected six times each.

4.2.3.4. Accuracy

The quality and applicability of the developed method were checked by performing the recovery analysis of the drug from the standard solutions. All standard solution were injected six times and mean response was recorded [272]. % recovery was calculated from given equation [273].

$$\text{Percentage recovery} = \frac{\text{Actual concentration recovered}}{\text{Theoretical concentration}} \times 100$$

4.2.3.5. Precision Studies

Precision studies were performed in two parts: repeatability and intermediate precision. In repeatability, On the same day, under the identical conditions, six injections of each of the standard solutions had been given (intra-day). For the intermediate precision, an inter-day study was completed by injecting six times of standard samples for three consecutive days, and for inter-analyst study, three different analysts of the same laboratory injected six times of standard solution, which were prepared by other analysts under the identical experimental conditions. The %RSD was determined using mean of responses.

4.2.3.6. Robustness

The proposed method's robustness is an indication of its ability to remain unaffected by modest but deliberate changes in chromatographic settings, which was investigated by testing the impact of small alterations in terms of variation in the mobile phase pH (3.0, 3.2, and 3.4), flow rate (0.8, 1.0, and 1.2 mL/min), the ratio of mobile phase ACN: Formic acid (88:12, 90:10, and 92:08). MQC was injected for six times and the effect on the "peak area, recovery, and retention time were recorded".

4.2.3.7. Estimation of Limit of detection (LOD) and limit of quantification (LOQ)

These limits can be measured by three methods, i.e., visual evaluation, S/N ratio approach and SD of the slope and response. The LOD and the LOQ were determined by the standard deviation (SD) of the slope and response method by using formula provided below [274].

$$LOD = 3.3\ \sigma/S$$

$$LOQ = 10\ \sigma/S$$

Where sigma (σ) and S used for SD of slope and slope of the calibration curve respectively.

4.2.4. Characterization of active pharmaceutical ingredients (API)

4.2.4.1. Appearance

One gram of the drug was spread over a watch glass. The sample was physically observed for colour and odour and also scanned for the presence of any foreign particles/matter.

4.2.4.2. Melting point

Melting point of the drug was measured by capillary fusion method and Differential Scanning calorimetry (DSC).

4.2.4.2.1. Capillary fusion method

Drug's melting point was measured using melting point apparatus. In which a capillary (10-15 cm and 1 mm thick internal diameter) was used. One side of the capillary tube was closed by using a flame and the sample was filled in capillary from another end. To check the melting point capillary and thermometer were placed at the given position provided in the apparatus at their respective place. Heated slowly, evenly and observed from the window to check at which drug starts to melt and got completely melted [275].

4.2.4.2.2. DSC

DSC, was utilised to examine the thermal behaviour of the drug. In an aluminium pan drug (2 mg) was filled, ruffled, and sealed. The aluminium pan was heated up in the temperature range of 10-160℃/min at a heat up range of 5℃/min in the nitrogen environment 20 mL/min and for reference empty pan was used [237].

4.2.4.3. Fourier-transform infrared spectroscopy (FTIR)

FTIR (Perkin Elmer,USA) was used to check the purity and structure of TBZ were analysed using FTIR. It is one of the best technique to check the sample which ensures traceablity through various validation protocols. This is also useful to know the compatibility of drug and excipients. To obtain FTIR spectra API and selected excipients were analysed in the scanning range of 4000 cm^{-1} - 400 cm^{-1} and the results were compared with the standard [236].

4.2.4.4 Powdered X-ray diffractometer (PXRD)

PXRD is a technique that is opted for characteristics of a drug such as crystal lattice arrangement, and degree of crystallinity. PXRD of the drug was recorded by using Bruker D8 to discover PXRD with a Ni-filtered Cu-K radiation at a voltage of 40kV and a current of 30mA. The diffractogram was obtained by using a speed of 0.02º/min from 6-50℃ [237].

4.2.4.5. Solubility study of TBZ in various oil, surfactant, and co-surfactant

For the choice of suitable formulation components for the preparation of TBZ-loaded SNEDDS, the solubility study of TBZ was carried out in oils (olive oil, eucalyptus oil, cotton seed oil, capmul MCM, peanut oil, paraffin oil, caproyl 90, isopropyl myristate, and caproyl PGMC), surfactants (tween 20, 60, 80, span 80, labrafil M 1944 CS, labrasol, labrafac WL 1349, labrafac PG and peceol) and co-surfactant (Transcutol© P, PEG 400, lauroglycol 90, lauroglycol FCC, Transcutol© HP, plurol oleique and maisin CC) respectively. One milliliter of oil, surfactants and co-surfactant were taken in a glass vial. An excess amount of the drug was added to the above-mentioned oil, surfactant, and co-surfactant and then vortexed (Cyclo Mixer, REMI, India) for 10 min. A water bath shaker was used for mechanical shaking for all

prepared samples for 72 hours at 25 ± 1ºC to maintain equilibrium [276]. The samples were centrifuged after 72 h in (REMI CM-12 PLUS, India) at 10000 g for 20 min for removal of any solid drug from the saturated solution. The clear supernatants were collected, diluted with an appropriate solvent, and using HPLC the concentration of drug was determined [245].

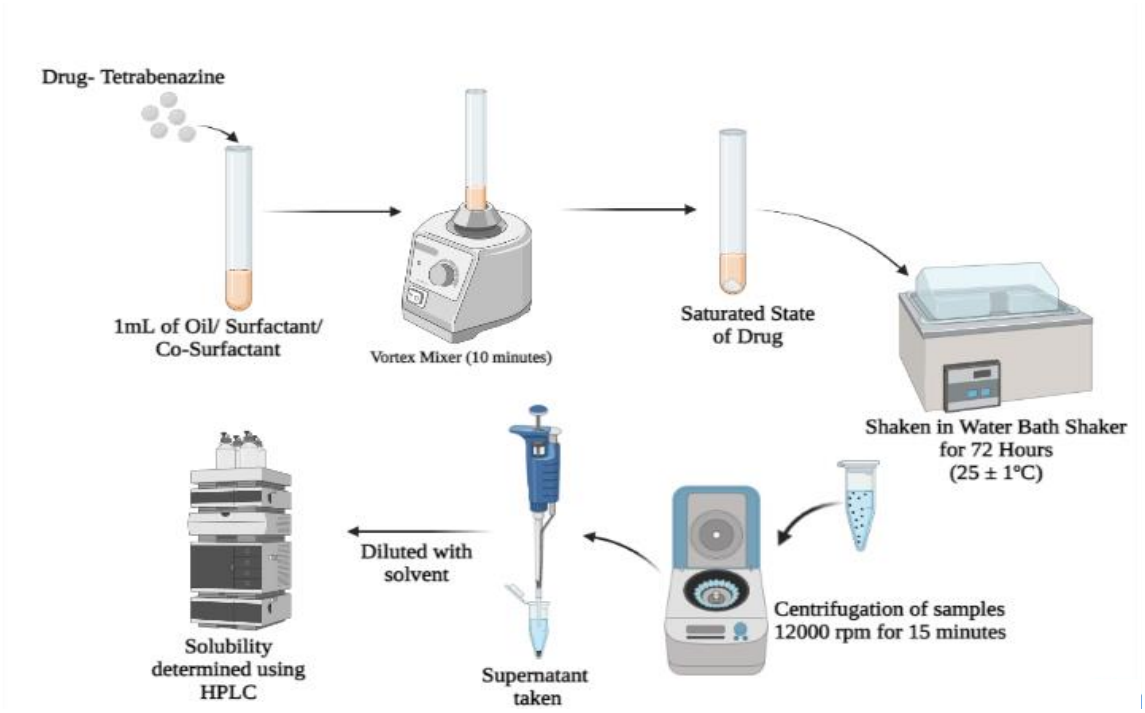


Figure 9: Steps for solubility

4.2.4.6. Miscibility study

Based on solubilizing ability lipophilic TBZ, capryol PGMC was selected as an oil phase. For surfactant screening, two of the best solubilizing surfactants [Labrafil M1944 CS and Tween 20 (0.5 mL)] were combined with 0.5 mL of capryol PGMC and stirred at 50 °C for two minutes. This combination was put into a conical flask with a stopper and diluted with 50 mL of water. Multiple simultaneous inversions of the flask were performed to create a clear, fine emulsion. The count of flask inversion was noted. The emulsions that formed was allowed to stand for two hours. Using UV visible spectrophotometer %age transmittance of produced emulsions was measured at 283 nm [276].

4.2.4.7. Co-surfactant

The effectiveness of co-surfactants, emulsification was evaluated using the same process as mentioned in 4.2.4.6. Before transferring the mixture to a stoppered conical flask, several minor adjustments were made 1mL of capryol PGMC, 0.5mL of surfactant Tween 20, and 0.5mL of co-surfactants (Transcutol© P, Lauroglycol 90) were mixed [276].

4.2.5. Preparation of TBZ SNEDDS using pseudoternary phase diagram

Twenty seven prototypes formulations were developed by combination of capryol PGMC and combination of Tween 20 and Transcutol© P as S_{mix} in the ratio of 1:9 to 9:1, whereas internal ratio of S_{mix} was varied from 1:1, 2:1, and 1:2.

The prepared isotropic mixture were diluted in 500 mL distilled water to evaluate their quality based on level of transparency; precipitation of drug; phase separation and rate of emulsification [40]. "Pseudo ternary phase diagram" was constructed using "Triplot software (4.1.2)", wherein, the obtained emulsion was categorized "as transparent (SNEDDS), translucent (SMEDDS), opaque (emulsion) and phase separation". The formulation that falls under the SNEDDS region was chosen and will be evaluated and considered for evaluation.

Table 7: Formulation of SNEDDS in different ratios (1:1, 1:2, 2:1)

Formulations	**Oil (Capryol PGMC) (µL)**	**Smix (Tween 20:Transcutol© P) (µL) (1:1)**
F1	100	450:450
F2	200	400:400
F3	300	350:350
F4	400	300:300
F5	500	250:250
F6	600	200:200
F7	700	150:150
F8	800	100:100
F9	900	50:50

Formulations	Oil (Capryol PGMC) (µL)	Smix (Tween 20:Transcutol© P) (µL) (1:2)
F10	100	300:600
F11	200	270:530
F12	300	230:470
F13	400	200:400
F14	500	170:330
F15	600	130:270
F16	700	100:200
F17	800	70:130
F18	900	30:70

Formulations	Oil (Capryol PGMC) (µL)	Smix (Tween 20:Transcutol© P) (µL) (2:1)
F19	100	600:300
F20	200	530:270
F21	300	470:230
F22	400	400:200
F23	500	330:170
F24	600	270:130
F25	700	200:100
F26	800	130:70
F27	900	70:30

4.2.5.1. Construction of pseudo ternary phase diagram

Prepared 27 prototype, using triplot 4.1.2 (Todd Thompson software) formulations were considered as nano emulsion if the solution appeared as clear transparent with a bluish appearance, SMEDDS if they were translucent in appearance, and creaming/cracking in emulsion were inferred as phase separation.

4.2.5.2. Stability evaluation of selected formulations

The stability of the selected SNEDDS were evaluated using three parameter; centrifugation, temperature change, and cloud point. To execute heating cooling cycle of the formulations [277], kept them in the temperature of 4 ℃ and 45 ℃ for 24 hours at each temperature in three cycle. Centrifugation study of diluted SNEDDS was done at 9625g for 15 min to check any cracking, creaming and drug precipitation. Freeze

thaw was done in three cycle in which one cycle was for 48 hours and formulation was kept in -21 ℃ and 25 ℃. Cloud point was measured to put selected formulations in water bath, and noted down the temperature at which formulation showed cloudy appearance. Cloud point should be >37℃ and it was observed manually [278,279].

4.2.5.3. Effect of pH on dilution

It is crucial for the SNEDDS that, once entering the GIT, neither their phase nor their size change due to the altering pH and volume of the GI fluids from the stomach to the colon. Optimised batches of SNEDDS were diluted 10, 100, and 500 times in the buffer (HCl and phosphate) with pH 1.2, 6.8, and 7.4 in order to assess the change in volume and pH [280].

4.2.6. Formulation optimization using DoE

Box Behnken design was used to optimize SNEDDS based formulation and below mentioned table showed design level of independent factors.

Table 8: Specifications of the formulations and process factors for SNEDDS optimisation

Independent factors	Design level		
Uncoded	Coded	Uncoded	Level
Oil	A	100	-1
		200	0
		300	+1
Surfactant	B	230	-1
		470	0
		600	+1
Co-surfactant	C	230	-1
		350	0
		530	+1

*+1 – Higher value; 0 – Medium Value; -1 – Lower value

BBD was applied to the present study with 3 independent variables and 5 dependent variables which provides 17 runs with a combination of 17 different formulations. The independent variables were concentration of oil (A), surfactant (B) and co-surfactant (C) on 3 levels low, medium and high which were indicated as -1, 0 and +1 respectively. For all the 17 batches dependent variables were GS (Y_1), PDI (Y_2), ZP

(Y_3), SEF (Y_4) and DL (Y_5). The model was evaluated for importance and looked for to fit in the design space. To assess the impact of various concentrations on the SNEDDS formulations, 2-D and 3-D contour plots were created. The polynomial equation derived from this is used to evaluate how dependent variables are influenced by independent variables. The entire model's optimised region is identified using the overlay plot that was produced [276].

Table 9: Study design based on Box- Behnken design

Run	Oil concentration (µl)	Surfactant concentration (µl)	Co-surf concentration (µl)
1	300 (+1)	230 (-1)	380 (0)
2	200 (0)	230 (-1)	230 (-1)
3	100 (-1)	600 (+1)	380 (0)
4	100 (-1)	230 (-1)	380 (0)
5	200 (0)	415 (0)	380 (0)
6	200 (0)	230 (-1)	530 (+1)
7	300 (+1)	600 (+1)	380 (0)
8	300 (+1)	415 (0)	230 (-1)
9	200 (0)	415 (0)	380 (0)
10	200 (0)	600 (+1)	230 (-1)
11	200 (0)	415 (0)	380 (0)
12	200 (0)	600 (+1)	530 (+1)
13	200 (0)	415 (0)	380 (0)
14	300 (+1)	415 (0)	530 (+1)
15	100 (-1)	415 (0)	230 (-1)
16	100 (-1)	415 (0)	530 (+1)
17	200 (0)	415 (0)	380 (0)

4.2.7. Characterization of SNEDDS

4.2.7.1. Zeta Potential, globule size and Poly dispersity index (PDI)

The GS, PDI and ZP of the trial batches, OF were checked using Malvern Zetasizer (Malvern Instruments, Worcestershire, UK). Pre-concentrate of formulations batches were diluted with distilled water and filtered using 0.25 µm syringe filter at room temperature. These all studies was performed in triplicate.

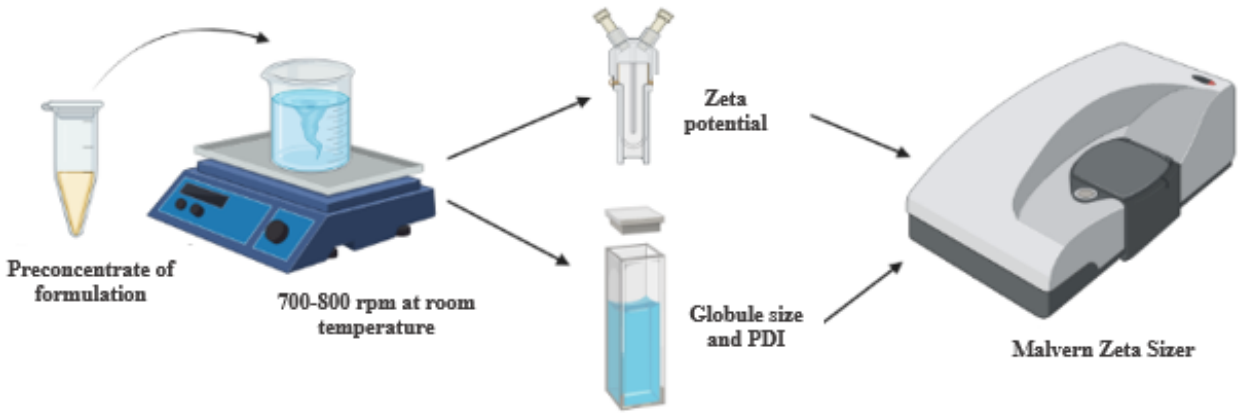


Figure 10: Steps for globule size, zeta potential and PDI

4.2.7.2. Self-emulsification time (SEF)

To assess the self-emulsification time of the trial and optimised batch, dispersibility investigations were carried out. Pre-concentrate of SNEDDS were adding drop at a time in distilled water (500 mL) on magnetic stirrer with 700-800 rpm at room temperature (25 ± 2 ℃). By visual observation the process of self-emulsification was observed until bluish, clear, transparent nano formulation was obtained. The time point at which this nano-formulation obtained was noted down with the help of stopwatch. After this these formulations were kept at room temperature to check any precipitation of the API in the formulation by visual observation [281].

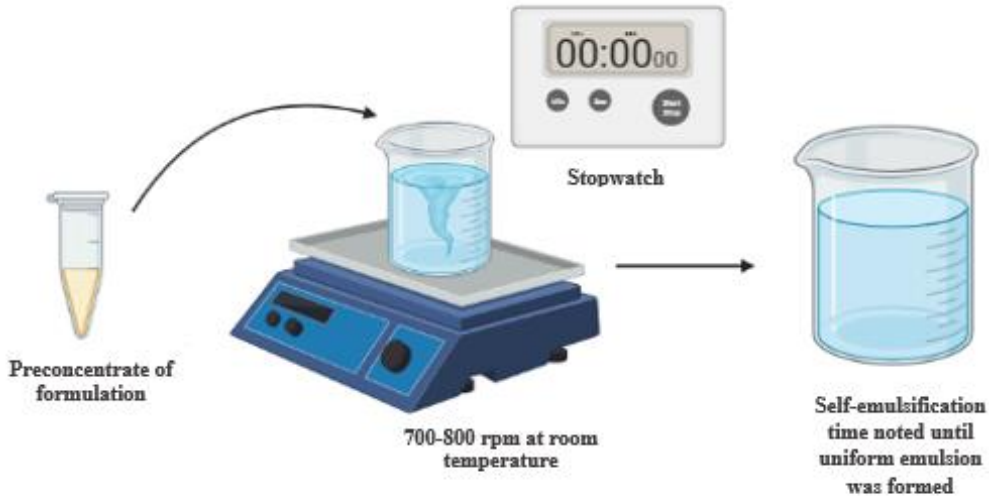


Figure 11: Steps for SEF

4.2.7.3. Drug Loading

Drug loading of L-SNEDDS loaded with TBZ was measured by HPLC at 283nm. Preconcentrate of L-SNEDDS loaded with TBZ (12.5 mg) was added to 500 mL of distilled water at 37±0.5℃. Five millilitre of the formulation was withdrawn and centrifuge it at 11200*g* for 15min for separation of undissolved drug. Supernatant was removed and used for quantification of the drug. The sample was injected in HPLC and area was recorded. The %age drug loading was calculated by formula provided below.

$$\% \text{ drug loading} = \frac{\text{Concentration of drug quantified in SNEDDS}}{\text{Total amount of formulation}} \times 100$$

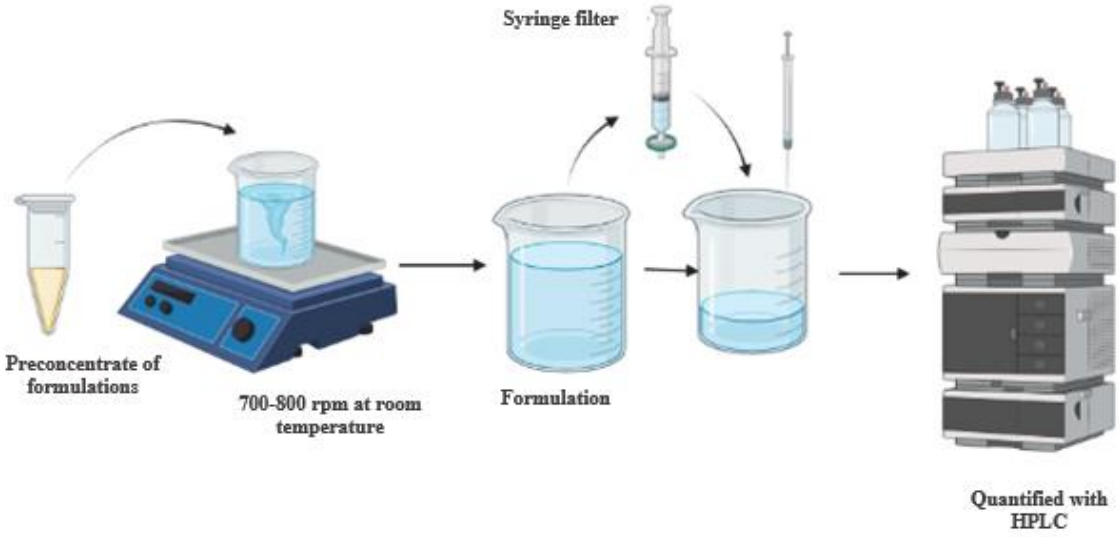


Figure 12: Steps for drug loading

4.2.7.4. % Transmittance

The SNEDDS's percentage transmittance provided insight into the compositional characteristics, including as homogeneity and droplet size. One millilitre sample of each formulation was diluted 10 times with distilled water to determine the percentage transmittance. The % transmittance were checked using UV visible spectrophotometer against water as blank [259].

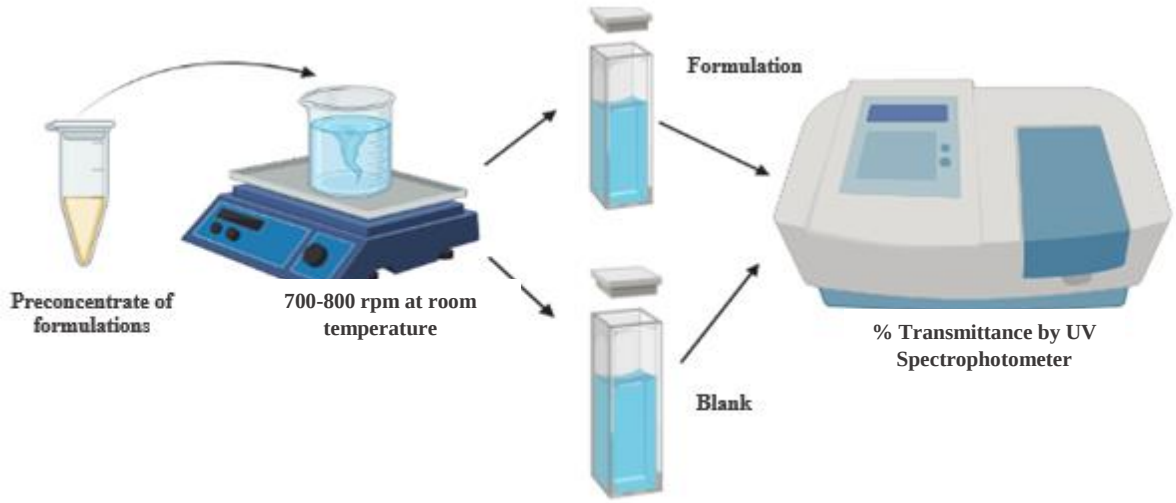


Figure 13: Steps for % transmittance

4.2.7.5. Compatibility study by FTIR

FTIR spectroscopy was used for physicochemical compatibility between Naïve TBZ, oil, surfactant, co-surfactant and TBZ-loaded-SNEDDS. By employing an FTIR KBr pellet technique, the spectra were captured between the wavelengths of 4000 and 400 cm^{-1}. The spectra acquired for Naïve TBZ, oil, L-SNEDDS, surfactant, and co-surfactant were compared [282,283].

4.2.7.6. Transmission electron microscopy (TEM)

The morphology of OF of TBZ loaded SNEDDS was examined using high resolution-TEM. Over a copper grid, a drop of the sample (diluted SNEDDS in distilled water using magnetic stirrer with 700-800 rpm) was applied and removed the extra sample by filter paper. The grid was stained with 1% phosphotungistic solution and dried. After this grid were kept under electron microscope to examine the morphology of particles [284,285].

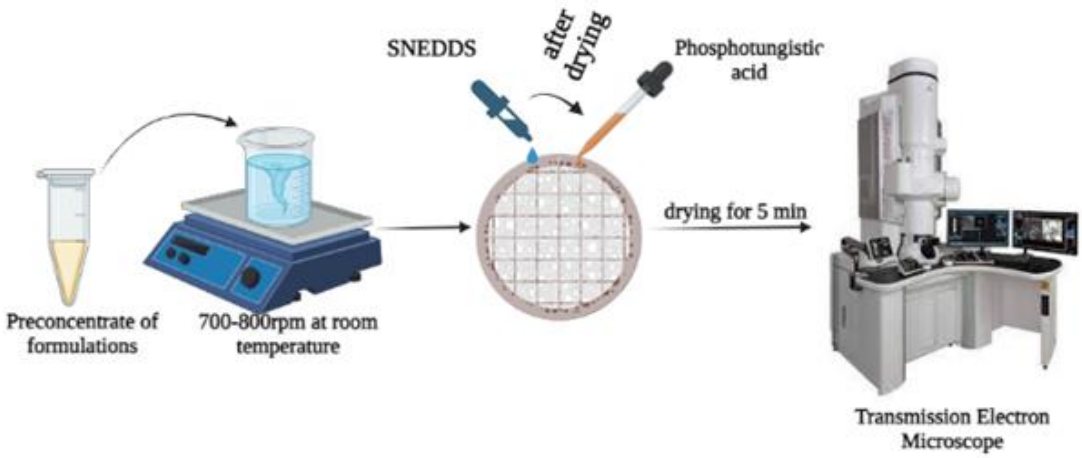


Figure 14: Steps for TEM

4.2.7.7. Cell line toxicity study

SHSY-5Y cell lines were used to conduct MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This study were conducted in three consecutive days, first day of study cells were cultured in DMEM "(Dulbecco's Modified Eagle Medium)" media till it got merging more than 70%. The cells were trypisinised and then transferred into 15 mL falcon and then centrifuged it for 5min at 1000rpm. After that, clear supernatant was removed, and the cell pellet was re-suspended in the fresh media. Pipetted out 10µL from that for the cell counting purpose. According to the counting, approx.-10,000 cells per well was seeded in triplicates (100µL per well) on 96 well plate and incubated it for 24hr at 37℃ and 5% CO_2. The media was removed from each well, and the sample was added according to the concentration to each well. Incubated it for 24 hours at 37℃ and 5% CO_2. In Day 3 10µL MTT was added in each well and incubated it for 3hrs. After that, each and every well were emptied and 100µL DMSO was added and placed it on the rocker shaker for 20minutes. The absorbance was observed at 570nm [286,287].

$$\% \text{ cell Viability} = \frac{\text{Mean OD of test group x 100}}{\text{Mean OD of control group}}$$

*OD = Optical density.

4.2.8. In-Vitro Release Study

Naïve, marketed and OF of TBZ-SNEDDS containing TBZ were considered using a USP class II dissolution device for an in vitro release investigation in USP type II dissolution apparatus with 900 mL [288] of phosphate buffer pH 6.8 maintained at 37 ± 0.5 °C, at a stirring speed of 50 rpm. Naïve, marketed and OF of TBZ-SNEDDS were measured and add in basket and put through a dissolution device. At different time interval of 5, 10, 15, 20, 30, 45 and 60 min 5 mL of aliquotes were taken and filtered with 0.25 µm syringe filter. Centrifuged the filtered solution for 15 min at 9864 *g*. Supernatant was taken out with micropipette and analysed in HPLC at 283nm for calculating release of TBZ [242,289].

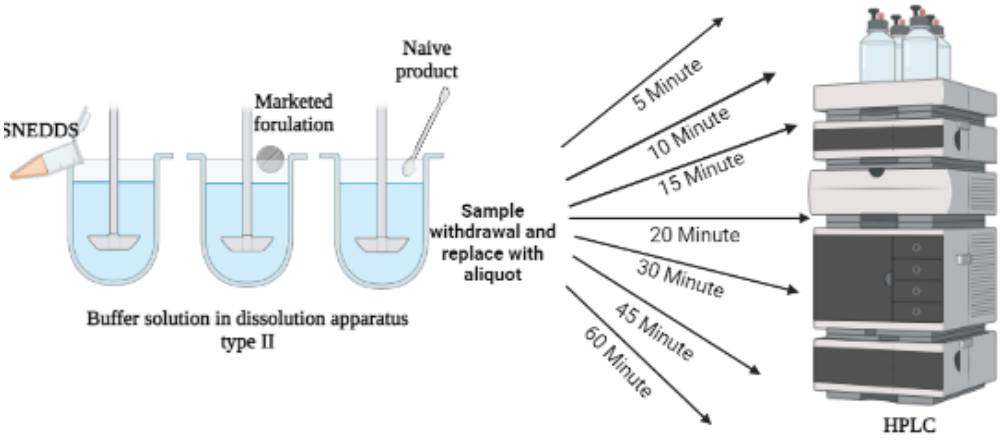


Figure 15: Steps for Dissolution study

4.2.8.1. Release kinetics of the SNEEDS formulations

From the obtained in vitro release data of TBZ SNEDDS were applied in different kinetic models like First order, Higuchi, Zero order, Korsemeyer Peppas and Hixon Crowell models. Noted down the value of correlation coefficient (R^2) and mechanism, which model was showing highest R^2 value was consider [290].

4.2.9. Accelerated Stability Study

Stability of a subset of TBZ-loaded SNEDDSs kept at ambient temperature (25±2°C) was examined for 6 months at 40 °C ± 2 °C/ 75 % RH ± 5 % RH. The samples were

assessed for any changes physically and/or self-emulsification effectiveness at 1, 2, 3, and 6 months [291]. Emulsification time, mean GS, PDI, ZP, and drug loading upon 500-fold dilution with distilled water were checked at every time gap. Every study was carried out in triplicate [292,293].

4.2.10. In vivo study

Six female "Sprague Dawley rats" were obtained for this study from the Panjab University's Central Animal Home in Chandigarh, India. Rats were between 200 and 250 g in weight and between 11 and 12 weeks old. The rats were housed at 25 ± 2°C, 55 ± 10% relative humidity, and 12:12 light:dark cycled husk-lined polypropylene cages. Rats provided with unrestricted access to water as well as a standard pellet diet. The procedure of study was authorized by the "Lovely Professional University School of Pharmaceutical Sciences' Institutional Animal Ethics Committee (Protocol no: LPU/IAEC/2022/05)".

4.2.11. Methods

4.2.11.1. Chromatographic Conditions

HPLC system "(Shimadzu LC-20AD, Japan) carry photodiode array detector (SPD-M20A) and a Rheodyne injector with 20 µL loop was used for injecting samples. A C18 reverse-phase column (Nucleodur C18, 250 mm x 4.6 mm i.d., 5μ, Macherey Nagel)" was used for the elution of plasma components. LC solution software was used for operating the entire system. The used mobile phase was ACN and 0.1% formic acid was used in the ratio of 90:10 v/v. The drug was detected using a 283 nm wavelength and a flow rate of 1 mL/min. Different mobile phase compositions, including acetonitrile-0.1% orthophosphoric acid, acetonitrile-water, and 0.1% glacial acetic acid -acetonitrile in various ratios and pH levels, were used to analyse TBZ.

4.2.11.2. Blood collection and plasma extraction

The blood was collected using the orbital sinus technique with the help of a capillary tube. In this rat is handled with thumb and forefinger to stretch the area around eyes. Clean the eyes with the help of cotton and water. Using a capillary tube and "ethylene diamine tetra acetic acid (EDTA)" crystals in radioimmunoassay (RIA) vials [294].

Once, the sinus is punctured, blood comes out via capillary and is collected in EDTA (ethylene diamine tetra-acetic acid) vials. Centrifuged these vials for 15 min at 2000 rpm with the help of micro-pippete plasma was withdrawn and kept for further studies at -20 °C [36,37].

4.2.11.3. Preparation of blank plasma

One millilitre plasma was taken in eppendorf and add 2 mL of ACN. The mixture was vortexed for about 5 min for precipitation of proteins present in plasma [295]. The clear supernatant was collected in separate eppendorf and was further centrifuged at 2000*g* for 15 min. After this, the obtained clear supernatant was transferred in a 100 ml volumetric flask and made up the volume.

4.2.11.4. Standard stock solution preparation

TBZ (10mg) was added in 10 mL of ACN to get the conc. of 1000 µg/mL (solution A). From solution A, 1 mL of aliquots was taken out and diluted with ACN upto 10 mL to get 100 µg/mL (solution B). Further 1 mL aliquots withdrawn from solution B and diluted upto 10 mL with ACN to achieve the conc. of 10 µg/mL (solution C). From solution C 1 mL of aliquots was withdrawn and make up the volume with ACN upto 10 mL to achieve the conc. of 1000 ng/mL (solution D). From above solution 1 mL of sample was removed and diluted upto 10 mL with ACN to get the conc. of 100 ng/mL (Solution E) [296].

4.2.11.5. Preparation of internal standard (IS)

To prepare the dilution, benzoquinolizine 10 mg/mL was employed as the IS. In 100 mL volumetric flask add weighed amount in ACN. After sonication of 10 minutes volume was made up to obtain 100 µg/mL concentration [296].

4.2.11.6. Specificity study

Blank plasma and TBZ samples were injected on HPLC containing acetonitrile: 0.1% formic acid (90:10 v/v) in order to verify procedures specificity. These were analysed at 283 nm to identify any interference between the drugs and plasma peaks [296].

4.2.11.7. Development of calibration curve

Aliquots of "0.5, 1.0, 1.5, 2.0, and 2.5 mL" of sample D were put into individual 10 mL flasks and addition of plasma (0.1 mL) was done in above solution. One millilitre of ACN was added in every sample mentioned above and sonicated for 15 minutes to precipitate and denature plasma protein. The entire sample was then centrifuged for 30 minutes at 4 °C using an eppendorf at 10,000 rpm. The clear supernatant was collected, and the volume was made up to 10 mL to obtain TBZ concentrations of "50, 100, 150, 200, and 250 ng/mL" and benzoquinolizine concentrations of 10 µg/mL was prepared as an internal standard. These samples were injected into HPLC for TBZ and benzoquinolizine assessment [296].

4.2.11.8. Method validation

The created approach was validated in accordance with the ICH M10 standard. To evaluate the system's performance, measurements of the tailing factor, theoretical plate, and HETP, LOQ, and LOD were checked [296].

4.2.11.9. Linearity and range

Plotting the "concentration on the X axis and the mean peak area on the Y axis" resulted in the calibration curve. The slope, SD of the response (sigma), SD of the intercept at the y-intercept, and regression coefficient (r^2) were estimated by the calibration data [35,38].

4.2.11.10. Accuracy

To assess the accuracy of the procedure, a complete drug recovery from standard samples was assessed. Standard solution i.e., LQC, 80%, MQC, 100%, and HQC, 120% at the midrange value of 100 ng/mL were the three concentration levels of the procedure that were employed to create the samples. To create these concentrations, aliquots of solution D in the quantities of 1.2, 1.5, and 1.8 mL were put into separate 10 mL volumetric flasks. Afterwards, 0.1 mL of plasma and 1 mL of solution E were added. The component were sonicated and centrifuged for 30 min at 10,000 rpm at 4 °C. The volume was then filled with up to 10 mL of ACN. These concentrations and

benzoquinolizine (10 µg/mL) were injected (6 times) in HPLC. The formula shown below was used to calculate the actual percentage recovery [39,42]:

$$\text{Actual \% recovery} = \frac{\text{Actual concentration recovered}}{\text{Theoretical concentration}} \times 100$$

4.2.11.11. Precision

Precision was assessed using its repeatability and intermediate precision. To assure repeatability, six injections of the standard samples were made into the similar experimental setup on the exact same day. The intermediate accuracy was calculated by injecting standard samples six times under identical experimental condition but on separate days with different analysts (inter-analyst). % RSD was computed using the formula given below [39,44]:

$$\text{\% RSD} = \frac{\text{Standard deviation of peak area}}{\text{Average peak area}} \times 100$$

4.2.11.12. System suitability and estimation of LOD and LOQ

Tailing factor, theoretical plate, peak purity index, and HETP were used to assess system compatibility. LOD and LOQ were estimated using the slope and SD of response (sigma) of the calibration curve (S). The equations given were used to calculate the results [297] :

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

4.2.11.13. Stability study

The stability of spiked plasma samples with TBZ was investigated using short term for three hours at ambient conditions, three freeze-thaw cycles, and long-term at -20 °C for three weeks. For freeze thaw 3 mL plasma contained RIA vial was collected and vortex it for five minutes after 10 mg of TBZ (1000 µg/mL) was added to this vial. This sample was kept in a freezer at -20 °C. The frozen sample was withdrawn from the vial and thawed at 25 ± 2 °C. One millilitre of plasma was removed (Cycle 1) from the thawed samples, and deep freezer was used to kept remaining for further

study. From the extracted plasma (1 mL), the protein was precipitated, and the supernatant was centrifuged. Supernatant was collected after centrifugation and diluted to prepare standard solution. As in cycle 1, plasma sample was removed, and repeat the procedure. Each of these solutions received 10 µg/mL of IS addition. Inject the samples in HPLC and observe at 283 nm. For each concentration, the mean, SD, and RSD were computed. For long-term stability 1 mL of plasma was added to 3 RIA vials holding 1 mg of TBZ each. When the mixture had been vortexed for five minutes, all three vials were put at -20 °C. "The three vials were taken out of the freezer after one, two, and three weeks". The samples were taken from the plasma after each interval, prepared standard solution, and then benzoquinolizine (10 mg/mL) was added. For each concentration, the mean, SD, and percent RSD were examined. (SD ± 3)

4.2.12. Drug treatment schedule

There are 6 groups in the experimental protocol, with 6 rats per group.

G-I: Normal control group.

G-II: Disease control group received Haloperidol on the 1st day till 28th day (2mg/Kg intra-peritoneal) dissolved in 0.9% saline solution.

G-III: Haloperidol (2 mg/Kg IP) 1 hour before treatment + Naïve TBZ suspended in Carboxy Methyl Cellulose (CMC) (10mg/Kg p.o.) was given for 28 days from day 1st to 28th, respectively.

G-IV: Haloperidol (2 mg/Kg IP) 1 hour before treatment + CMC (1 mL/Kg) + 1 mL/Kg Placebo SNEDDS p.o. was administered for 28 days from day 1st to 28th day.

G-V: Haloperidol (2 mg/Kg IP) 1 hour before treatment + CMC (1 mL/Kg) + 1 mL/Kg TBZ loaded SNEDDS in low dose i.e., 5mg/Kg p.o. was administered for 28 days from day 1st to 28th day.

G-VI: Haloperidol (2 mg/Kg IP) 1 hour before treatment + CMC (1 mL/Kg) + 1 mL/Kg TBZ loaded SNEDDS in high dose i.e. 10 mg/Kg p.o. was given for 28 days from day 1st to 28th day.

Behavioural activity was performed on 0th, 7th, 14th, 21st and 28th day. Animals were sacrificed on 28th day for oxidative stress, permeability study (pharmacokinetic study) and histopathology study were estimated.

Table 10: Pharmacodynamics' study

Groups	Treatment	Dose (Route of administration)	No of animals (n=6)
I	Normal control (NC)	1mL 0.9% Nacl, i.p	6
II	Disease Control (Disease induced through Haloperidol)	2mg/kg haloperidol in 0.9% Nacl, i.p	6
III	Standard Control (Suspension of TBZ)	10mg/kg, p.o.	6
IV	SNEDDS Placebo	1mL of SNEDDS preconcentrate, p.o.	6
V	SNEDDS low dose	5mg/kg, p.o.	6
VI	SNEDDS high dose	10mg/kg, p.o.	6

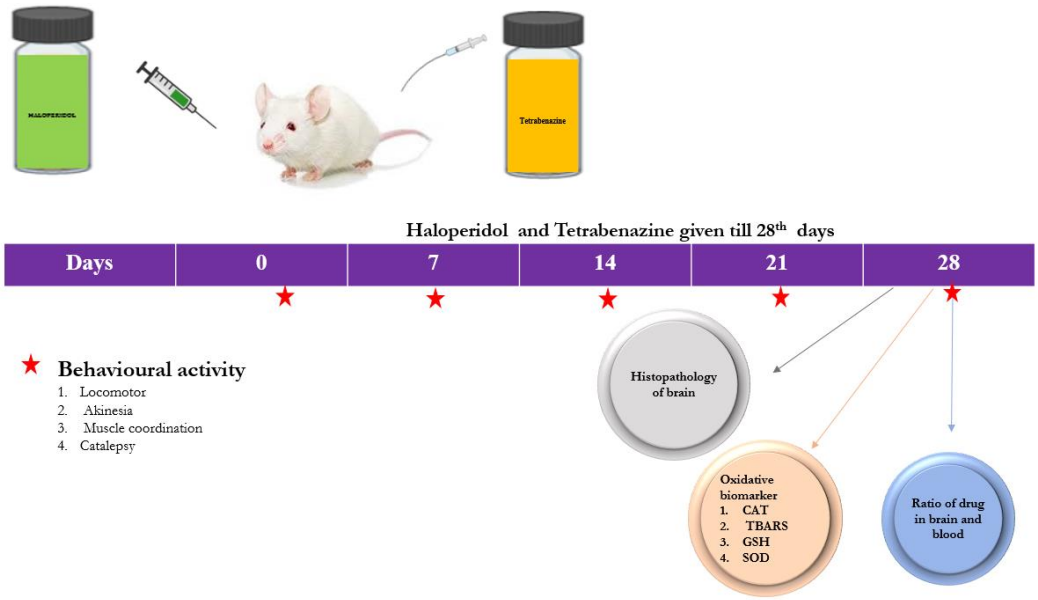


Figure 16: Protocol for animal study

4.2.13. Calibration curve of TBZ in CSF

Blank brain homogenate was taken in which 0.1 mL of drug solution of known concentration was spiked to get a linearity range from 50 to 250 ng/mL and 0.8 mL of

acetonitrile as precipitating agent was added to get total volume of 1 mL. This solution was vortexed 4–5 min and then centrifuged at 10,000 rpm for 10 min at 4 ℃. 0.2 mL of supernatant liquid was taken and diluted with methanol for up to 2 mL, this solution was injected to get response for linearity.

4.2.14. Pharmacokinetic study

4.2.14.1. Brain homogenate preparation

Animals were sacrificed using the decapitation method, and the brains were removed, collected, and washed in chilled isotonic saline. Ice-cold 0.1 mol/L phosphate buffer (pH 7.4) were used to homogenise whole brain tissue samples for 10 times. After homogenization the samples were centrifuged at 10,000 g for 15 min, and the clear supernatant were collected and utilised for oxidative stress and permeability [298].

4.2.14.2. Detection TBZ in plasma and brain

This study was carried out on the last day (i.e. 28^{th}) of study post-dosing on rats of group 3 and group 5. In order to collect the blood and plasma sample, five rats from both groups were taken individually.

The first rats from both groups were sacrificed at 0.5 h and blood as well as brain were collected. One ml blood was collected in EDTA containing RIA vial. Afterwards the blood sample (0.5 mL) was centrifuged at 3500 *g* for 15 min and plasma was collected. The drug was extracted from plasma using protein precipitation method using 1% v/v per chloric acid. The extracted sample was reconstituted with mobile phase and injected to HPLC for estimation of drug. This injection was carried out in triplicate and mean ± SD were recorded. In order to perform brain distribution study, the brain tissues were homogenised along with PBS at 10000 *g* for 30 min. The supernatant was isolated, filtered through 0.45 µm membrane filter and injected to HPLC. The injection was done in triplicate and mean ± SD were recorded. Similarly, the second rats, third rats, fourth rats and fifth rats from both groups were sacrificed at 1h, 2h, 4h and 24 h, respectively and the drug concentration in plasma and brain were recorded as per aforementioned protocol [298].

4.2.15. Behavioural

4.2.15.1. Locomotor

Actophotometer apparatus was used, which is square shaped metallic box (activity cage) with a transparent lid. This cage consist infrared rays which are across the axis of that box. The rats were placed one by one to check their activity. The number of rays crossed by rats were recorded by the meter present on instrument. The cut-off time for this apparatus is 10 minutes and used to measure voluntary movements [299,300].

4.2.15.2. Muscle coordination

Muscle coordination was checked using rota rod apparatus. It consist of horizontal rod which was present 20 cm above the base of the apparatus, which is rotating on its long axis and the speed of this rod was fixed at 25 rpm. Before performing this test rats were trained 2-3 times in a day. The time when rats were placed on rod to falling from rod were noted down, the maximum cut-off time is 120s and data was compared between the groups [301,302].

4.2.15.3. Catalepsy

The catalepsy was assessed using the bar test. Rats were positioned for this test with both front paws on a horizontal bar that was 9 cm above and parallel to the base in the half-rearing posture. With the use of a stopwatch, the passing of time was now documented, and the animals' paw removal from the bar was timed. Observation (maximum) cut off time was set at 180 seconds [51,52].

4.2.15.4. Akinesia test

Akinesia test was performed to measure rat's facing difficulty in initiating move. To conduct this test, the rats' latency to move all four limbs was timed in seconds. Prior to the trial, each rat was trained on a wooden elevated platform for five minutes. Stopwatch was used to measure the time used by animal for movement by all limbs. (SD ± 3) [149].

4.2.16. Oxidative biomarkers

4.2.16.1. Catalase (CAT) assay

To check the catalase activity add 1.95 mL (50 mM, pH 7.0) of phosphate buffer in 0.05 mL of the supernatant. After that, using a UV-spectrophotometer, absorbance was measured at 240 nm for 30 seconds at 15-second intervals after adding 1 mL of H_2O_2 (30 mM). Then calculation was done using formula given below [243].

$$CAT = \{[(2.3 * \log OD\ initial/\ OD\ final) / \Delta t * 100]/0.693\}/mg\ of\ protein$$

OD stands for optical density at 240 nm. Δt is the 15-second time period during which the absorbance was measured.

4.2.16.2. Thiobarbituric acid reactive substances (TBARS) assay

Malondialdehyde (MDA) levels in the TBARS assay are used to determine the degree of lipid peroxidation. Supernatant was mixed with Tris HCl (pH 7.4) in an amount of 0.2 mL, and the mixture was then incubated at 37 °C for two hours. TCA (1 mL chilled) was added after incubation, and the mixture was centrifuged for 10 minutes at 1000 g. After that, 1 mL of both TBA (0.67%) and supernatant were combined, kept in water bath for 10 minutes, samples were set aside to cool, and add distilled water (1 mL), and UV was used measured as 532. The resulting concentration was expressed as nanomole of MDA per mg of protein after further computation using the extinction coefficient of MDA, which is 0.156 M^{-1} [245].

By detecting the concentration of malondialdehyde (MDA), the TBARS assay gives information on the degree of lipid peroxidation. Supernatant and Tris HCl (pH 7.4) were combined in 0.2 mL, and the mixture was then incubated at 37°C for 2 hours following by added 1 mL TCA (10%), and the mixture was centrifuged at 1000 g for 10 min. After 10 minutes, the samples were removed and allowed to cool before being mixed with 1 ml of distilled water, and the absorbance was measured at 532 nm. Next, 1 mL of supernatant was obtained and mixed with 1 mL of 0.67% TBA. The resulting concentration was “expressed as nanomole of MDA per mg of protein”. MDA extinction coefficient, is 0.156 μM^{-1} [245].

4.2.16.3. Reduced glutathione (GSH) assay

The technique outlined by Beutler was used to measure the level of GSH. After centrifuging at 1000 g for 10 min, add 1 mL of supernatant and TCA (10% w/v in water). Following centrifugation, 0.5 mL of the supernatant was collected, and 2 mL of disodium hydrogen phosphate (0.3 M) and 0.25 mL of freshly produced DTNB (0.001 M in 1% w/v sodium citrate) were added. The concentration of GSH was calculated using a UV spectrophotometer at 412 nm from the standard plot (10-100 M) of the GSH.

4.2.16.4. Superoxide dismutase (SOD) assay

By using the hydroxylamine method, the activity of SOD was measured. 0.1 mL of the homogenate's supernatant was added to a 2 mL solution (EDTA 0.1 mM, NBT 96 m, and sodium carbonate 50 mm, pH 10.8) and 0.1 mL of hydroxylamine hydrochloride was added. (20 mM, pH 6). Then, with the use of the following equation, activity was determined by comparing the change in absorbance in the presence of enzyme to the absence of enzyme over the course of two minutes at 60-second intervals [303].

$$\text{SOD} = \frac{(\Delta\text{OD of control} - \Delta\text{OD of the sample}) * 100/50/\text{volume of homogenate/mg of protein}}{\Delta\text{OD of control}}$$

Here, Δ OD stands absorbance change of sample and control at 560 nm.

*Data were expressed as mean ± SD and analysed using Graph Pad Prism 5.01 software. Data of behavioural test were analysed by two way analysis of variance (ANOVA), followed by Turkey multiple comparison test and biochemical tests were analysed by one way analysis of variance (ANOVA), followed by Turkey multiple comparison test. The p value < 0.05 was considered significant.

4.2.17. Histopathology

Investigations on the neuronal damaged induced by haloperidol were made using histopathology. For this reason, all of the rat's group brain section were kept in hematoxylin and eosin staining. All group's brains were promptly fixed in 10% formalin and embedded in paraffin after being removed. A brain piece that was five

micro-metres thick was cut, individually treated, and deparaffinized using xylene and ethanol. This sections were then examined at a 100x magnification under the microscope [304].

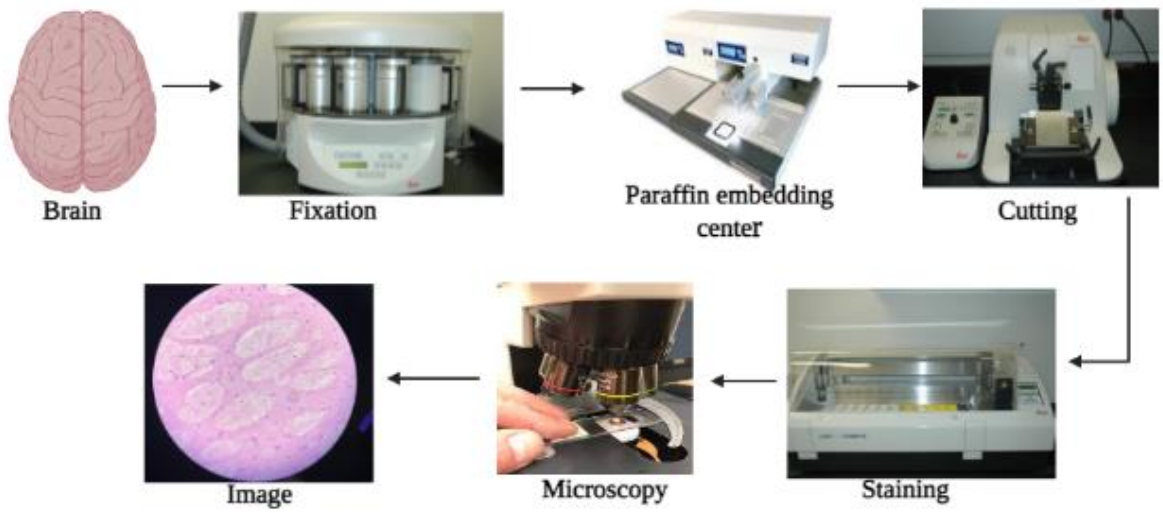


Figure 17: Steps for histopathology study

CHAPTER 5

RESULT AND DISCUSSION

5. RESULT AND DISCUSSION

Characterization of active pharmaceutical ingredient (API)

5.1. Spectrum analysis

TBZ has maximum absorption at 284 nm [305], from the UV results maximum absorption was found at 283 nm as depicted in Figure 17. Hence this absorbance was used for further evaluations.

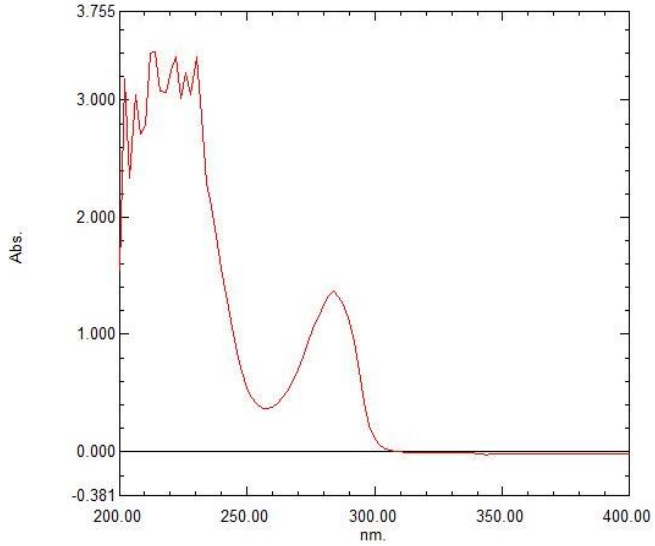


Figure 18: Absorbance of Tetrabenazine

5.2. Analytical method development and validation

5.2.1. Selection of mobile phase for TBZ estimation

To select a suitable mobile phase, number of trials were made by varying the composition, ratio, and pH of the mobile phase. ACN: 5 mM ammonium acetate [306], mobile phase provided peak with splitting and noise (Figure 20A). Shouldering was observed when ACN: 0.1% glacial acetic acid was used (Figure 20B). There was no sharp peak upon using ACN: 0.1% ortho-phosphoric acid as a mobile phase instead, two peaks with shouldering were observed (Figure 20C). The reported methods had retention time (RT) between 6.5-10 min, but the developed method with 0.1% formic (pH 3.2) acid and ACN in the ratio of 90:10 have RT 4.34±0.03 (Figure 20D), with better resolution, sharp peaks. Hence, this mobile phase combination was

selected for validation. When a blank of 0.1% formic acid and ACN was injected, there was no peak which interfered with the TBZ RT (Figure 19).

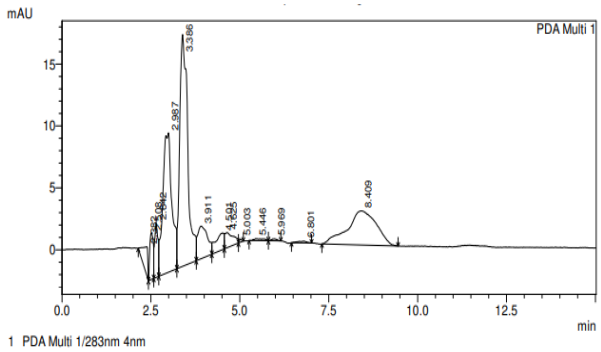


Figure 19: Chromatogram of blank of ACN and formic acid

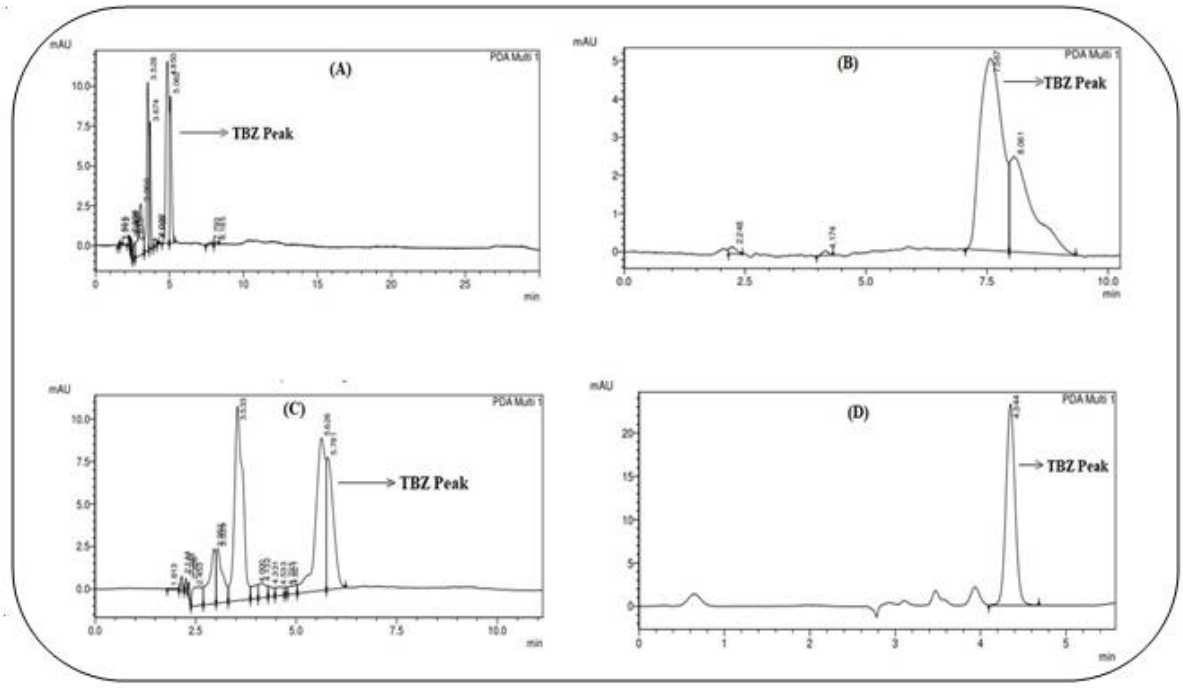


Figure 20 : Chromatogram of TBZ in ACN and 5mM ammonium acetate (A), Chromatogram of TBZ in ACN and 0.1% glacial acetic acid (B), Chromatogram of TBZ in ACN and 0.1% ortho-phosphoric acid (C), Optimized chromatogram of TBZ in ACN and 0.1% formic acid (90:10) (D)

5.2.2. Method validation

5.2.2.1. System suitability

TBZ dilution of 10 µg/mL was injected for system suitability. Tailing factor was found 1.10 ± 0.002 which is less than 2 ensure peak regularity. Theoretical plate

was found 6848±43, which is more than 2000 ensure excellent peak efficiency (Table 11).

Table 11: System suitability results for TBZ

Parameters	Value	Limits
HETP	22.01±0.37	Depends upon theoretical plate
Theoretical plate	6848±43	>2000
Theoretical plate/meter	45652±287	>20000
Tailing factor	1.10±0.002	<2
Peak purity index	0.999	>0.5

5.2.2.2. Linearity

The potential of an analytical process to bring out results that are "directly proportional" to the concentration (quantity) of analyte in the sample is known as linearity [307]. The calibration curve in Figure 21 showed that TBZ followed good linearity with the concentration 2-10 µg/mL. Graph was plotted between concentration (x-axis) and peak area (y-axis) with satisfactory r^2 value was found 0.9992.

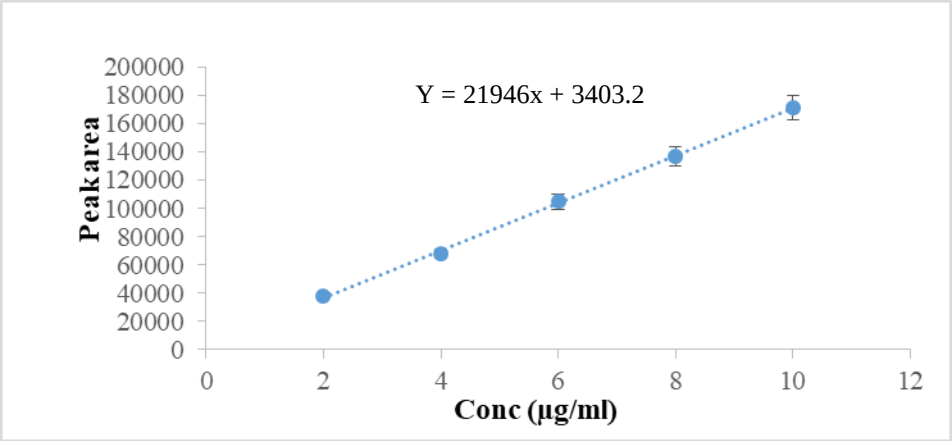


Figure 21: Calibration curve of TBZ

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

5.2.2.3. Accuracy

Accuracy of standard solution was execute by percentage recovery of standard solutions. Percentage recovery was found in between 85.83% - 91.38%. The accuracy of a test relates to how closely the results match the true value. The obtained results are depicted in Table 12.

Table 12: Result of accuracy studies

Levels	**Concentration of standard solution (µg/mL)**	**Concentration of drug added (µg/mL)**	**Recovered concentration (µg/mL)**	**Recovery (%)**	**Mean Recovery (%)**
LQC	4.8	6.0	4.3	89.58	
MQC	6.0	6.0	5.15	85.83	88.93
HQC	7.2	6.0	6.58	91.38	

5.2.2.4. Precision

Precision studies have been performed to check whether the method is repeatable. The obtained results are presented in Table 13. The observed % RSD for intraday (0.64-1.96%), interday (0.60-1.880%), and interanalyst (0.60-1.91%) which were less than 2% for all the samples, which prove this method was satisfactorily, repeatable and precised.

Table 13: Outcome of TBZ precision experiments

Parameters	**Level**	**Concentration**	**Analytical responses (area), injections**						**Mean**	**SD**	**%RSD**
		(µg/mL)	**1**	**2**	**3**	**4**	**5**	**6**	**(*N=6)**		
Repeatability (intraday precision)											
	LQC	4.8	99103	98742	98195	99752	98042	98570	98734.00	627.51	0.64
	MQC	6.0	104088	103671	106085	101326	103843	101238	103375.17	1839.80	1.78
	HQC	7.2	148847	147940	145179	146371	153138	145941	147902.70	2895.31	1.96
Intermediate precision (interday)											
Day 1	LQC	4.8	100800	99300	97149	101644	97810	98708	99235.17	1727.22	1.74
	MQC	6.0	113618	114959	114233	110419	110570	111176	112495.83	2005.40	1.78
	HQC	7.2	160419	160818	159205	158758	159698	157338	159372.67	1251.83	0.79
Day 2	LQC	4.8	102340	103721	102991	103161	102146	102269	102771.33	621.51	0.60
	MQC	6.0	107957	106914	106610	106254	105701	106287	106620.50	769.87	0.72
	HQC	7.2	155035	152976	153634	151066	151668	155023	153233.67	1662.72	1.09
Day 3	LQC	4.8	106031	104058	105570	105581	106324	105939	105583.83	800.29	0.76
	MQC	6.0	137157	136692	137042	137505	138961	139253	137768.33	1072.92	0.78
	HQC	7.2	189687	190240	197962	196294	197082	196424	194614.83	3655.29	1.88
Intermediate precision (inter analyst)											
Analyst 1	LQC	4.8	97511	99155	98424	99086	98382	98581	98523.16	595.58	0.60
	MQC	6.0	111095	112099	116379	114497	111248	114692	113335.00	2161.41	1.91
	HQC	7.2	152384	156990	150695	153388	156093	153763	153885.50	2332.69	1.52
Analyst 2	LQC	4.8	140015	145172	141923	141502	142676	142410	142283.00	1697.07	1.19
	MQC	6.0	120018	117776	117572	113830	118311	116454	117326.83	2071.83	1.76

	HQC	7.2	160327	157810	162809	160533	161919	158078	160246.00	2004.55	1.25
Analyst 3	LQC	4.8	120202	126452	122477	124958	124815	123884	123798.00	2195.78	1.77
	MQC	6.0	158504	154018	156134	150555	152525	154409	154357.50	2767.10	1.79
	HQC	7.2	196724	199904	200742	197805	194714	200525	198402.30	2408.82	1.21

5.2.2.5. Robustness

Robustness study was done by changing the "pH of the mobile phase (3.0, 3.2 and 3.4), flow rate (0.8, 1.0 and 1.2 mL/min) and the ratio of mobile phase ACN: 0.1% formic acid (88:12, 90:10 and 92:08)", respectively. The result of percentage RSD was in between 1.24-1.67% which were less than 2% (Table 14), which showed this method was unaffected by these changes, and satisfactory robust.

Table 14: Results of tests for TBZ's various parameters' robustness

Various Parameters	**Value**	**Concentration (µg/mL)**	**Peak (mean±SD) (*N=6)**	**Mean of peak area of three value (*N=3)**	**Retention time (in minutes) (mean±SD) (*N=6)**	**Mean of retention times of three values (*N=3)**	**% Recovery (mean±SD) (*N=3)**	**Mean of % recoveries of three values (*N=3)**
pH	3.0	6.0	112306.70±7105.37	118090.73	4.93±0.01	4.99	105.09±0.97	104.78
	3.2	6.0	114787.50±3189.42	SD=2152.71	5.05±0.77	SD=0.06	103.35±1.02	SD=1.30
	3.4	6.0	119041.00±3597.69	%RSD=1.82	5.01±0.58	%RSD=1.19	105.90±1.23	%RSD=1.24
Flow rate	0.8	6.0	137009.70±3287.00	136192.00	4.42±0.02	4.50	100.00±1.57	100.33
	1.0	6.0	137768.30±2299.50	SD=2107.68	4.50±0.01	SD=0.09	102.00±1.22	SD=1.52
	1.2	6.0	133798.00±2437.00	%RSD=1.54	4.60±0.03	%RSD=2.00	99.00±1.58	%RSD=1.52
Mobile phase ratio (A:B)	88:12	6.0	126103.00±3958.50	124069.30	4.82±0.06	4.94	93.00±1.16	91.33
	90:10	6.0	124168.20±1437.50	SD=2084.75	5.03±0.04	SD=0.06	91.00±1.05	SD=1.52
	92:08	6.0	121937.00±1571.00	%RSD=1.68	4.44±0.09	%RSD=1.12	90.00±1.13	%RSD=1.67

5.2.2.6. *Estimation of LOD and LOQ*

As per ICH guidelines, LOD and LOQ were calculated by the SD of response and slope. Low LOD and LOQ values i.e. 0.31 µg/mL and 0.96 µg/mL, respectively, indicating that the presented method for TBZ estimation has high sensitivity [308].

5.3. Appearance

Table 15: Physical properties of the drug

Sr. No	Property	Observation	Literature value	Reference
1	Colour	White to off white	White to off white	https://www.webmd.com/drugs/2/drug-151522/tetrabenazine-oral/details
2	Purity (as per label)	98 ± 0.5 %		

5.4. Melting Point Determination

5.4.1. Capillary fusion method

Results obtained from capillary method was found in the acceptable range of reported value, as the melting point of TBZ was found 125-128°C and the reported melting point was 126-127 °C (https://www.trc-canada.com/product-detail/?T284002) (Access date 05/06/2021).

5.4.2. DSC

DSC was performed to determine the melting point and state of TBZ in the temperature range of 10° C to 160° C according to the method described and thermogram was represented in Figure 22. TBZ showed a sharp endothermic peak at 128.14°C with enthalpy of 118.32 J/g indicating crystalline nature of TBZ. Since, there is no additional peak observed, that confirmed the purity of the drug and also absence of polymorphic form.

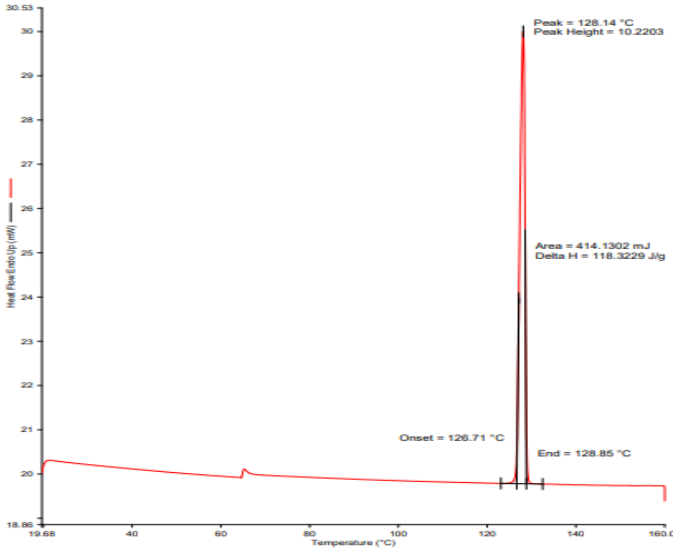


Figure 22: DSC thermogram of TBZ

5.5. FTIR

FTIR spectrum of TBZ was taken by using FTIR spectrophotometer (Perkin Elmer) and compared with reported FTIR spectra of TBZ (Table 16). The FTIR spectrum was presented in Figure 23.

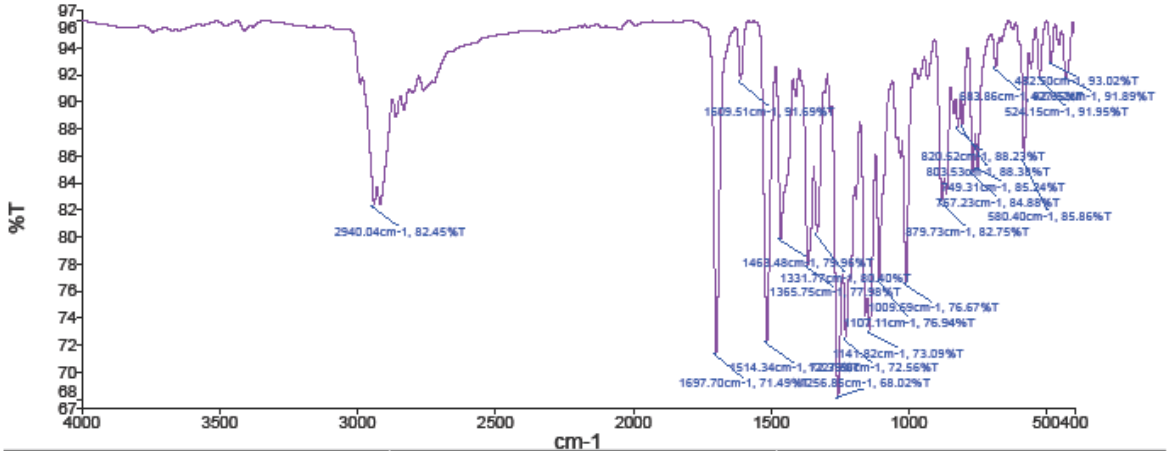


Figure 23: FTIR spectrum of TBZ

Table 16: Comparison of TBZ spectra with reported spectrum

Wavenumber (cm^{-1}) (Reported)	**Wavelength (cm^{-1}) (Obtained)**	**Vibration assignment**
2912.52	2940	C-H (stretching)

1697, 1257	1697.90, 1256.85	C=O (stretching)
1604, 1514	1609.51, 1514.34	C=C (stretching)
1226	1227	C-N (stretching)
1107	1107.11	C-O-C (stretching)
1007.91	1009.69	C-O (stretching)
641.40	643.86	NH (rocking)

The FTIR spectrum of TBZ showed characteristics peak at 2940, 1697.90, 1256.85, 1609.51, 1514.34, 1227, 1107.11, 1009.69, 643.86 which was for the stretching and rocking vibration of –CH, -C=O, -C=C, -C-N, -C-O-C, -C-C, and –NH. The functional group showed almost same wavelength inferred authentication of TBZ drug.

5.6. PXRD

PXRD was used to verify the crystallinity of TBZ. The PXRD of TBZ exhibit sharp diffraction peaks at 2θ angles of 6.2º, 22.1º, and 23.2º showing crystalline nature of the drug which is approximately same as in reported diffractogram. Figure 24 represent the diffractogram of TBZ.

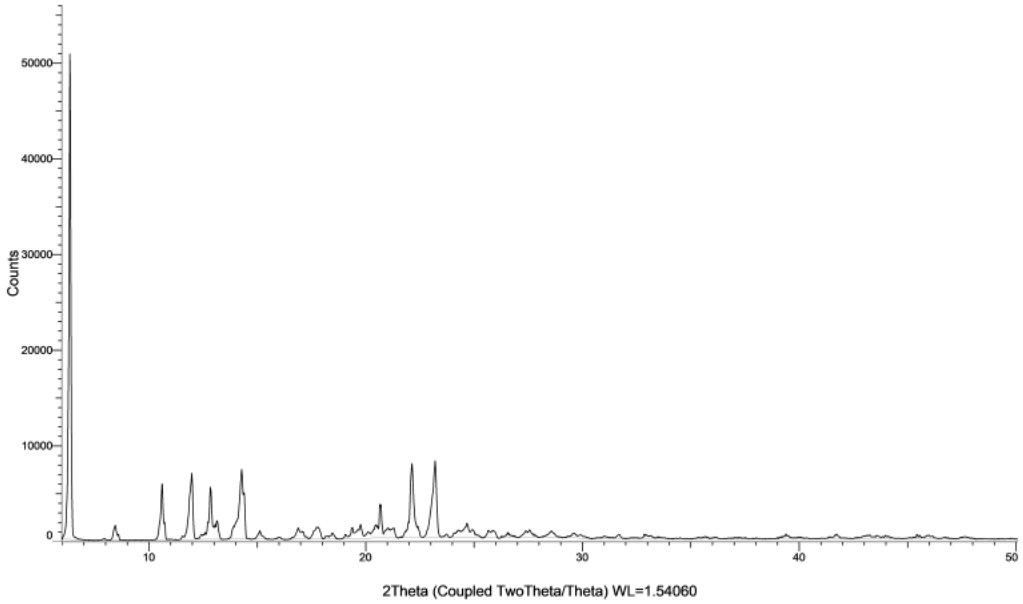


Figure 24: PXRD pattern of TBZ

Table 17: Comparison of TBZ PXRD spectrum with reported spectrum

S. No	Observation (at 2θ diffraction angle)	Reported (at 2θ diffraction angle)
1	6.2º	6.4 º
2	22.1º	22.3º
3	23.2º	23.1 º

5.7. Formulation development of SNEDDS

5.7.1. Solubility of TBZ in various oil, surfactant and co-surfactant

The results of solubility of tetrabenazine in oils, surfactants and co-surfactant are given in Figures 25, 26, and 27.

Tetrabenazine solubility followed the order given below.

Solubility in oils

TBZ has maximum solubility in capryol PGMC followed by peanut oil and capryol 90 which was 8 mg/mL, 6.83 mg/mL, and 6.42 mg/mL respectively. So on the basis of solubility capryol PGMC (HLB value 5) was selected as oil phase.

Capryol PGMC> Peanut oil> Capryol 90> Labrafil M 1944 CS> Capmul MCM> Olive oil> Paraffin oil> Eucalyptus Oil> Isopropyl myristate

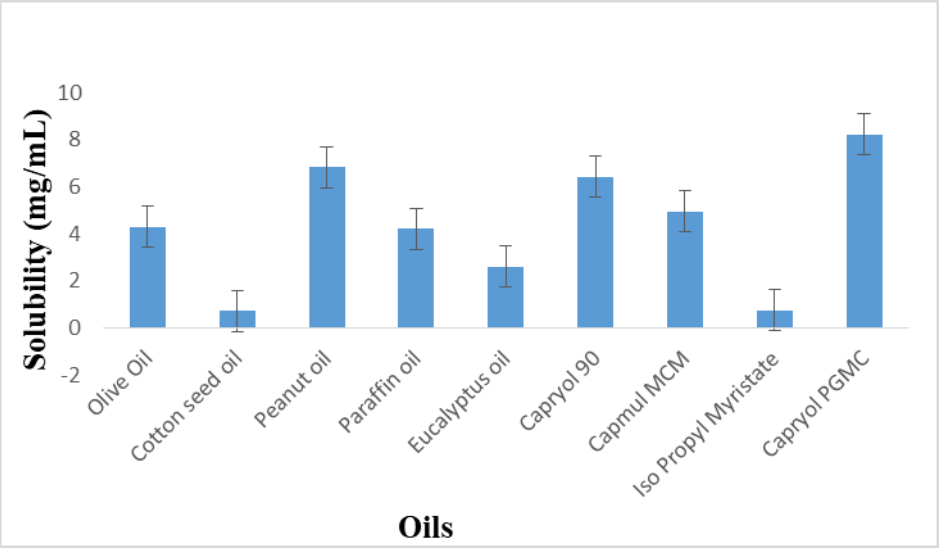


Figure 25**:** Solubility of tetrabenazine in various oil

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

Solubility in surfactant

TBZ had maximum solubility in Labrafac WL 1349 i.e. 9.92 mg/mL with HLB value 1 followed by Tween 20 (HLB 17) and Labrasol (HLB 14) with 6.25 mg/mL and 5.03 mg/mL respectively. Miscibility studies were used as the basis for choosing the surfactant.

Labrafac WL 1349> Tween 20> Labrasol> Tween 60> Peceol> Span 80> Tween 80

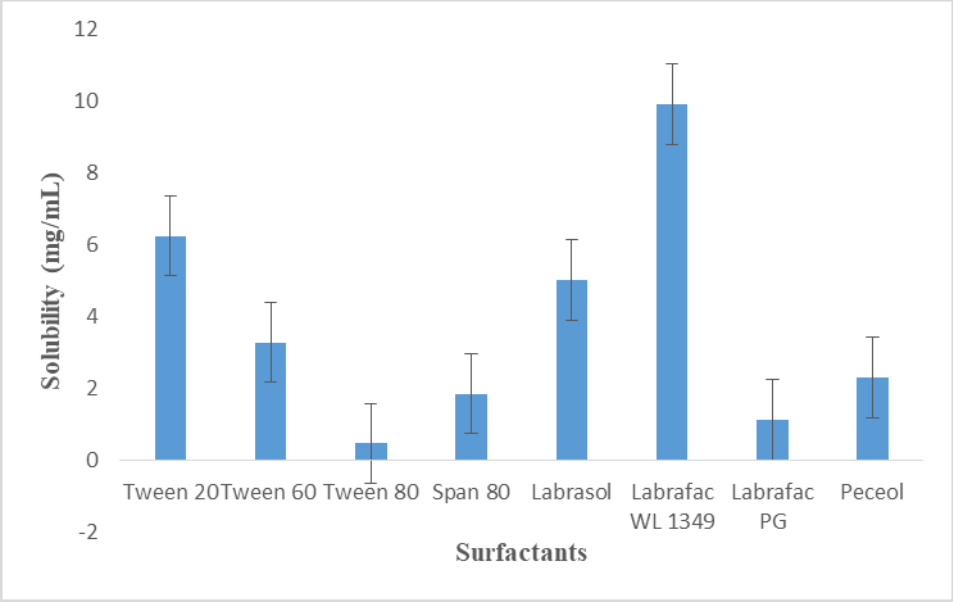


Figure 26: Solubility of tetrabenazine in various surfactant

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

Solubility in Co-surfactant

Maximum solubility of TBZ was found in Transcutol© P (HLB 4.2) and Laurogycol 90 (HLB 5) i.e. 9.06 mg/mL and 7.73 mg/mL respectively followed by other co-surfactant. Miscibility studies were used as the basis for choosing the co-surfactant.

Transcutol© P> Lauroglycol 90> Transcutol© HP> Lauroglycol FCC> PEG 400> Plurol Oleique> Maisin CC

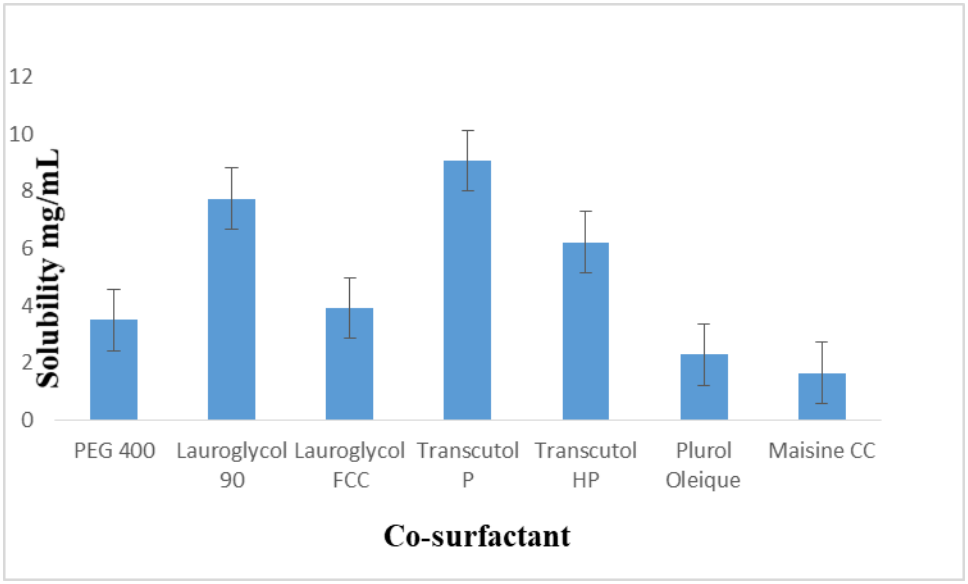


Figure 27: Solubility of tetrabenazine in various co-surfactant

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

5.8. Miscibility Study

5.8.1. Screening of surfactant

Labrafac WL 1349 being a lipophilic surfactant undergoes phase separation after 2 hours. The transmittance produced by Tween 20 (HLB 17) which produces higher transmittance with less number of flask inversions as shown in table 18. So, Tween 20 was selected as the surfactant.

Table 18: Miscibility study for the screening of surfactant

Oil	Surfactant	No. of flask inversion	% Transmittance	Observation
Capryol PGMC	Labrafac WL 1349	13	69.13±0.318	Turbid
Capryol PGMC	Tween 20	5	95.53±0.534	Clear

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

5.8.2. Screening of Co-surfactant

Transcutol© P (HLB 9.06) and Lauroglycol 90 (HLB 7.73) was selected on the basis of solubility. Transcutol© P has less number of flask inversions and the high purity nature. So it is a suitable co-surfactant for formulations as shown in Table 19.

Table 19: Miscibility study for the screening of co-surfactant

Oil	**Surfactant**	**Co-surfactant**	**No. of flask inversion**	**% Transmittance**	**Observation**
Capryol PGMC	Tween 20	Lauroglycol 90	9	74.76±0.489	Turbid
		Transcutol© P	6	97.19±0.506	Clear

5.9. Construction of pseudo ternary phase diagram

From Table 20 it was evident that, increasing the oil concentration lead to decrease in the transparency of the emulsion. This can be attributed due to increase in the droplet size of the oil phase and the effects of reduced surfactants and co-surfactants which were insufficient to make a transparent emulsion.

Table 20: Appearance of formulation of SNEDDS using oil Capryol PGMC and S_{miz} (Tween 20 and Transcutol© P)

Formulation Code	**Capryol PGMC (µL)**	**Tween 20 (µL)**	**Transcutol© P (µL)**	**Appearance (µL)**
$F1_{(1:1)}$	100	450	450	SMEDDS
$F2_{(1:1)}$	200	400	400	SMEDDS
$F3_{(1:1)}$	300	350	350	SNEDDS
$F4_{(1:1)}$	400	300	300	SMEDDS

$F5_{(1:1)}$	500	250	250	SMEDDS
$F6_{(1:1)}$	600	200	200	Coarse emulsion
$F7_{(1:1)}$	700	150	150	SMEDDS
$F8_{(1:1)}$	800	100	100	SMEDDS
$F9_{(1:1)}$	900	50	50	Coarse emulsion
$F1_{(2:1)}$	100	600	300	SNEDDS
$F2_{(2:1)}$	200	530	270	SMEDDS
$F3_{(2:1)}$	300	470	230	SNEDDS
$F4_{(2:1)}$	400	400	200	SMEDDS
$F5_{(2:1)}$	500	330	170	SMEDDS
$F6_{(2:1)}$	600	270	130	SMEDDS
$F7_{(2:1)}$	700	200	100	Coarse emulsion
$F8_{(2:1)}$	800	130	70	Coarse emulsion
$F9_{(2:1)}$	900	70	30	Coarse emulsion
$F1_{(1:2)}$	100	300	600	SMEDDS
$F2_{(1:2)}$	200	270	530	SNEDDS
$F3_{(1:2)}$	300	230	470	SMEDDS
$F4_{(1:2)}$	400	200	400	SNEDDS
$F5_{(1:2)}$	500	170	330	SMEDDS
$F6_{(1:2)}$	600	130	270	SMEDDS
$F7_{(1:2)}$	700	100	200	SMEDDS
$F8_{(1:2)}$	800	70	130	Coarse emulsion

$F9_{(1:2)}$	900	30	70	Coarse emulsion

A ternary phase diagram was constructed according to the extent of transparency after dilution with water. Out of 27 formulations in the prototype 5 formulations were found in the SNEDDS region which showed transparency upon dilution. The rest of the formulations showing translucent (Slightly cloudy) or opaque (Very Cloudy) were termed SMEDDS or coarse emulsions. The SNEDDS region is indicated by a red star in the pseudo ternary phase diagram as shown in Figure 28 which forms o/w emulsion upon slight agitation. As we can infer from the figure the low concentration of oil and a higher ratio of Smix gives transparent emulsions.

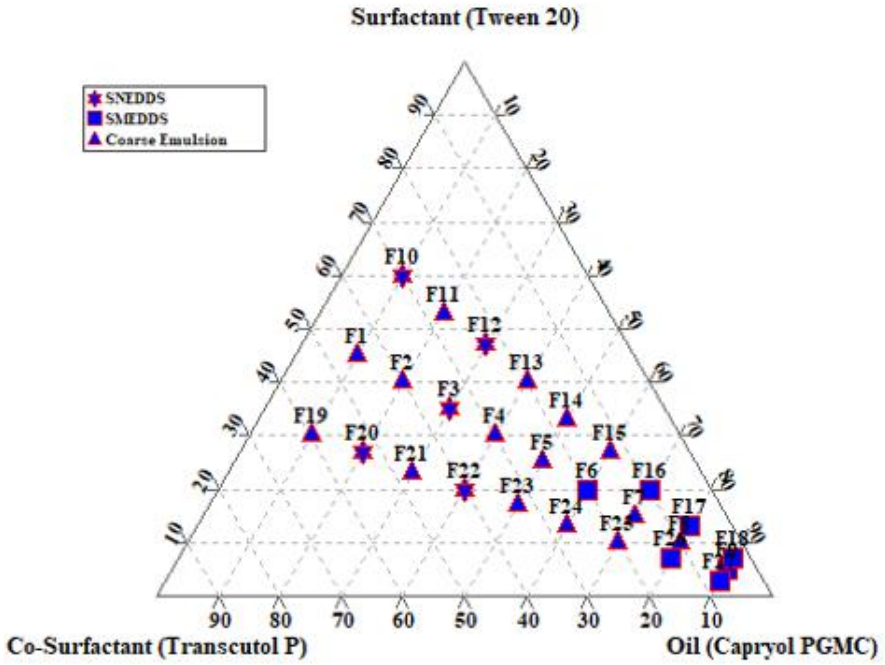


Figure 28: Pseudo ternary phase diagram

5.10. Stability evaluation of selected formulations

Selected 5 formulations were considered to perform thermodynamic stability studies and observation was that all formulations were stable which indicated no drug precipitation, phase separation and turbidity. Also cloud point temperature of different formulations were determined which was found in the range of 78-85°C as shown in

Table 21. Which infers good stability of all formulations because cloud point should be more than 37 ± 0.5℃.

Table 21: Results of thermodynamic stability studies

SNEDDS Formulations code	**Heating-cooling cycle**	**Centrifugation studies**	**Freeze thaw cycle**	**Cloud point (in ℃)**
F1	Transparent	Transparent	Transparent	80 ℃
F2	Transparent	Transparent	Transparent	82 ℃
F3	Transparent	Transparent	Transparent	78 ℃
F4	Transparent	Transparent	Transparent	85 ℃
F5	Transparent	Transparent	Transparent	80 ℃

5.11. Effect of pH on dilutions

After administer of L-SNEDDS, it undergoes limitless dilution in the GIT and passage through an acidic to basic pH condition. It is important that formulation should remain stable over dilutions and in different pH. Similar observations were noted when the optimized TBZ L-SNEDDS formulation was subjected to change in dilution and pH. Upon diluting the SNEDDS with 10, 100 and 500 mL of different buffers the size of optimized SNEDDS did not change significantly ($p>0.05$). Precipitation of drug and phase separation was not observed when diluted in different pH and volume (Table 22).

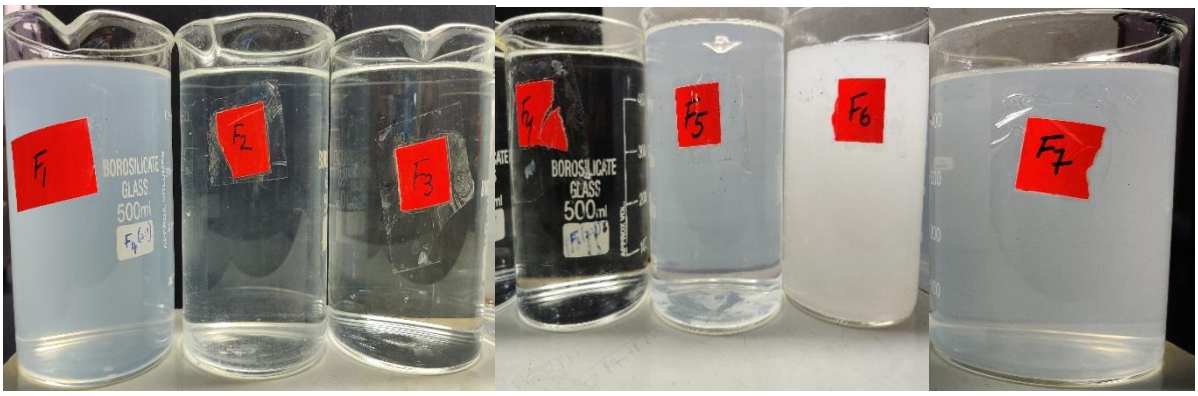
Table 22: Results of effect of pH on dilution

SNEDDS formulation code	**pH**		
	1.2 HCl buffer buffer	**6.8 phosphate buffer**	**7.4 phosphate**
F1	Transparent and stable	Transparent and stable	Transparent and stable
F2	Transparent and stable	Transparent and stable	Transparent and stable
F3	Transparent and stable	Transparent and stable	Transparent and stable
F4	Transparent and stable	Transparent and stable	Transparent and stable
F5	Transparent and stable	Transparent and stable	Transparent and stable

5.12. Formulation optimization using DoE

Table 23: Box-Behnken study design was used to examine the effect of variable A-C on responses Y_1-Y_5

Run	Oil conc. (µl)	Surfactant conc. (µl)	Co-surf conc. (µl)	Globule size (nm)	PDI	Zeta potential (mV)	Self-emulsification time (s)	Drug loading (%)
1	300	230	380	256.5	0.165	-19.5	15.41	73.96
2	200	230	230	100.7	0.214	-18.6	11.4	69.89
3	100	600	380	78.58	0.215	-19.1	9.62	62.89
4	100	230	380	70.79	0.204	-19.7	16.7	66.01
5	200	415	380	104.1	0.205	-19.3	16.1	73.4
6	200	230	530	195.6	0.155	-18.4	18.4	68.92
7	300	600	380	104.9	0.201	-20.3	-9.3	72.47
8	300	415	230	206	0.188	-19.7	-13.18	76.66
9	200	415	380	91.87	0.209	-19.1	-14.21	67.97
10	200	600	230	141.9	0.131	-19.8	-10.5	66.3
11	200	415	380	91.96	0.210	-19.3	13.1	68.91
12	200	600	530	134.4	0.188	-18.2	8.3	61.11
13	200	415	380	104.8	0.213	-19.8	15.13	65.42
14	300	415	530	184.5	0.144	-19.5	13.11	75.36
15	100	415	230	68.29	0.202	-19.3	18.7	65.66
16	100	415	530	63.5	0.185	-18.5	11.37	64.04
17	200	415	380	88.3	0.210	-19.4	14.88	68.85



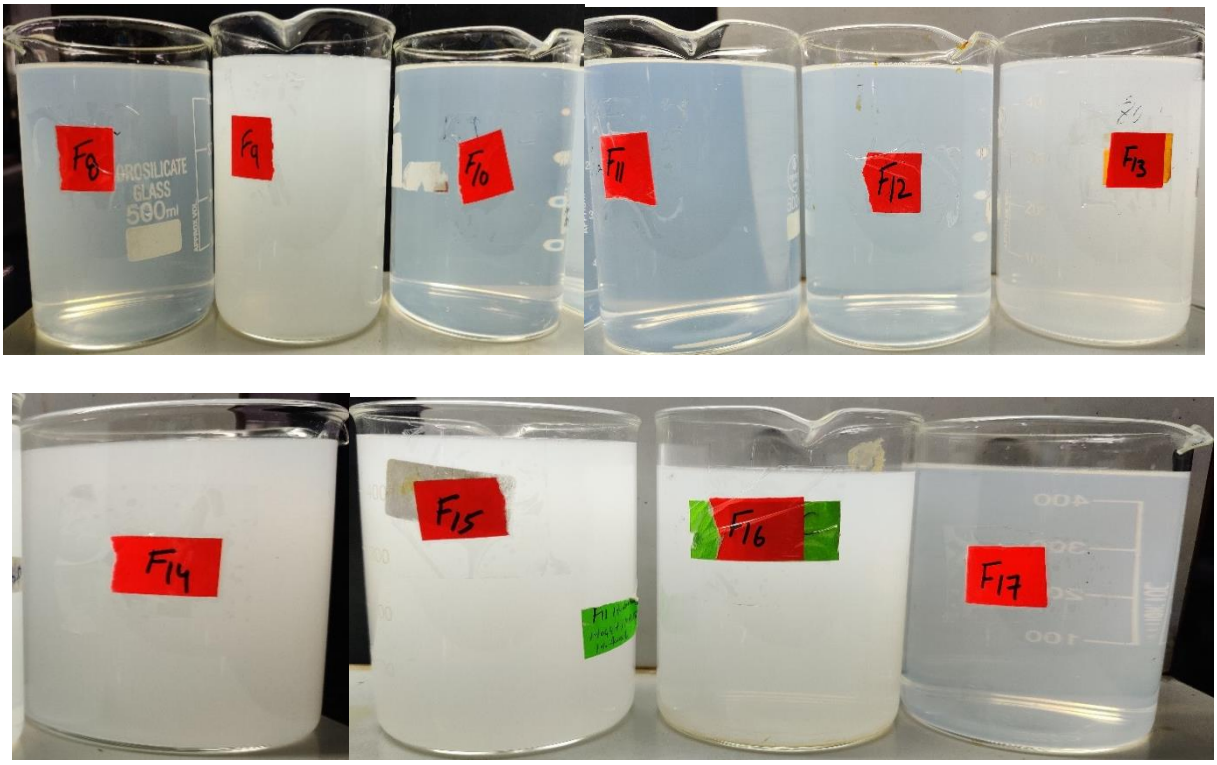


Figure 29: Various Nano-emulsions according to DOE

Table 24: Results of an ANOVA for a Box-Behnken Design

ANOVA Parameters	Y_1 globule size	Y_2 PDI	Y_3 Zeta potential	Y_4 SEF	Y_5 drug loading
Adequate precision	10.71	6.87	11.59	8.03	11.70
F-value	8.87	4.45	8.11	4.38	13.14
Std. deviation	24.02	0.0153	0.2530	2.07	2.43
R^2 value	0.9194	0.8512	0.9125	0.7245	0.7521
Adjusted R^2 value	08158	0.6598	0.8000	0.5592	11.701
Predicted R^2 value	-0.2214	-1.3380	0.3556	-0.1036	0.5852
Suggested model	Quadratic	Quadratic	Quadratic	Linear	Linear

5.12.1. Response analysis for optimization:

5.12.1.1. Influence of variables on globule size

In SNEDDS formulations GS played important role because it determines absorption extent and dissolution rate of the drug. It is always required that GS should be less. A quadratic polynomial model illustrating the interaction of multiple variables was

found to best reflect the experimental data after fitting them to various models and ANOVA results. The equation was described below:

$$Y_1 = +96.21+58.848A-20.48B+7.64C-39.85AB-4.18AC-25.60BC+9.45A^2+22.03B^2+24.91C^2$$

In Figure 30 showing the effect of surfactant and oil on GS as the concentration of oil increasing GS also increasing this might happen as a result of an increase in the frequency of oil droplet collisions, which therefore raises the likelihood of a coalescence event. When we increased the concentration of surfactant GS decreased. This might have been because there were high concentration of surfactant present interface for adsorption at the oil-water interface, which reduced the interfacial tension between the water and oil and, as a result, decreased the Laplace pressure, resulting in stronger stabilization. When the surfactant concentration was kept low, the molecules of the surfactant did not completely cover the oil droplets, resulting in insufficient surface covering. These occurrences caused the oil droplets to condense, which increased in GS [245].

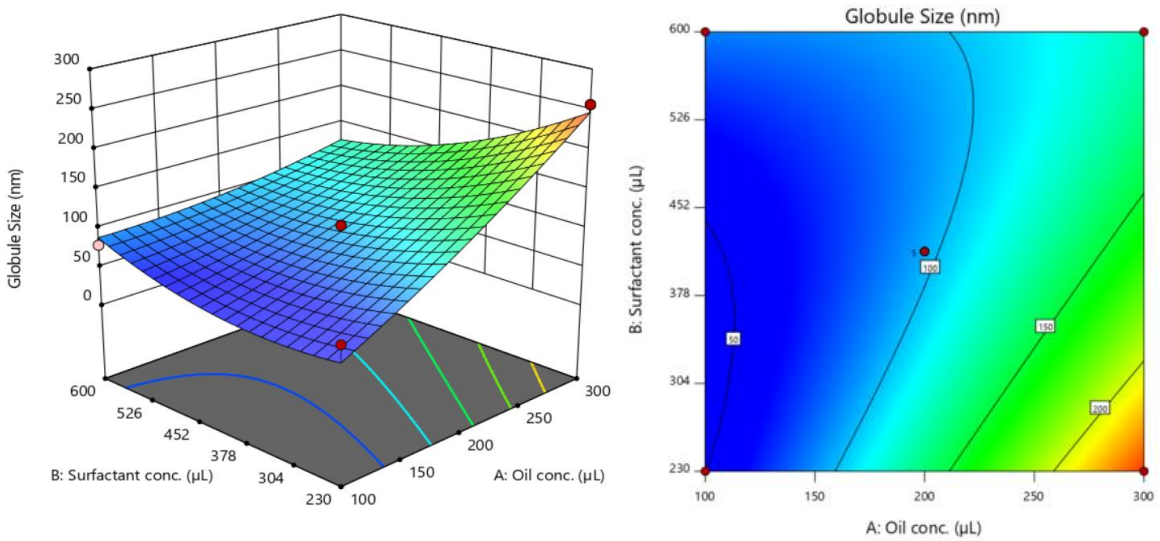


Figure 30: Effect of variables on globule size

5.12.1.2. Influence of variables on poly-dispersity Index

Poly-dispersity index is playing important role to evaluate the uniformity in GS of the SNEDDS. If the formulation has less PDI indicate good size distribution of droplets. '0' indicate monodispersed system and '1' indicate highly dispersed system. As we increased the concentration of oil and surfactant PDI decreasing as showing in Figure

31. A quadratic polynomial model illustrating the interaction of multiple variables was found to best reflect the experimental data after fitting them to various models and ANOVA results. The equation was described below:

$$Y_2 = +0.2094-0.0135A-0.0004B-0.0079C+0.0063AB-0.0068AC+0.290BC-0.0027A^2-0.0104B^2-0.0270C^2$$

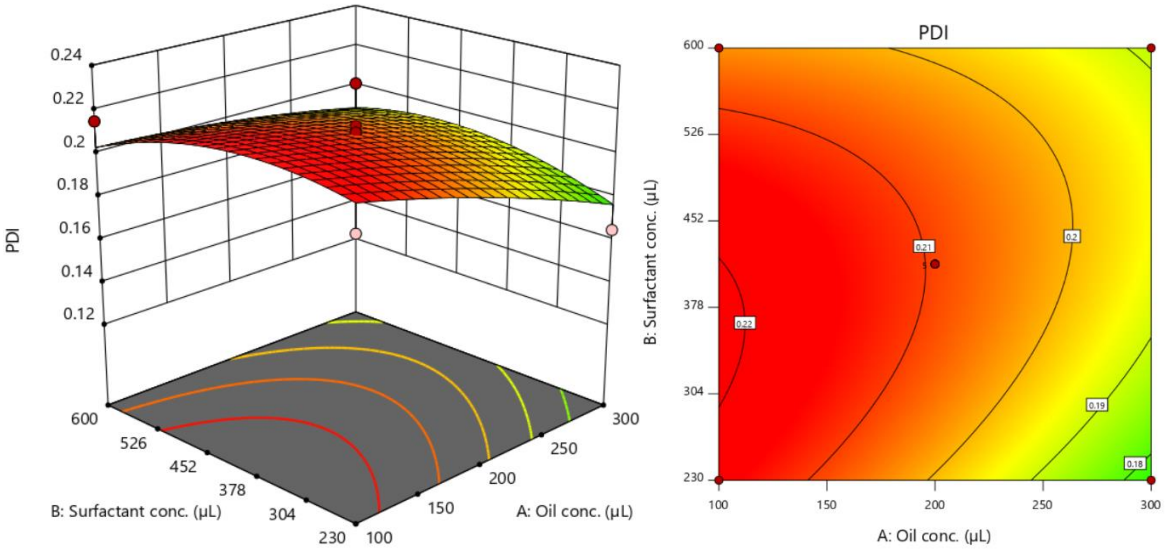


Figure 31: Effect of variables on PDI

5.12.1.3. Influence of variables on zeta potential

Zeta potential is used to evaluate the charge which is important for the stability of bilayer preparation. It indicate the strength of electrostatic repulsion between nearby charges that are similar in dispersion. According to Figure 32 as the concentration of oil and surfactant increased the ZP was decreased. A quadratic polynomial model illustrating the interaction of multiple variables was found to best reflect the experimental data after fitting them to various models and ANOVA results. The equation was described below:

$$Y_3 = -19.38-0.3600A-0.1500B+0.3500C-0.3500AB-0.1500AC+0.3500BC-0.3850A^2+0.1150B^2+0.5150C^2$$

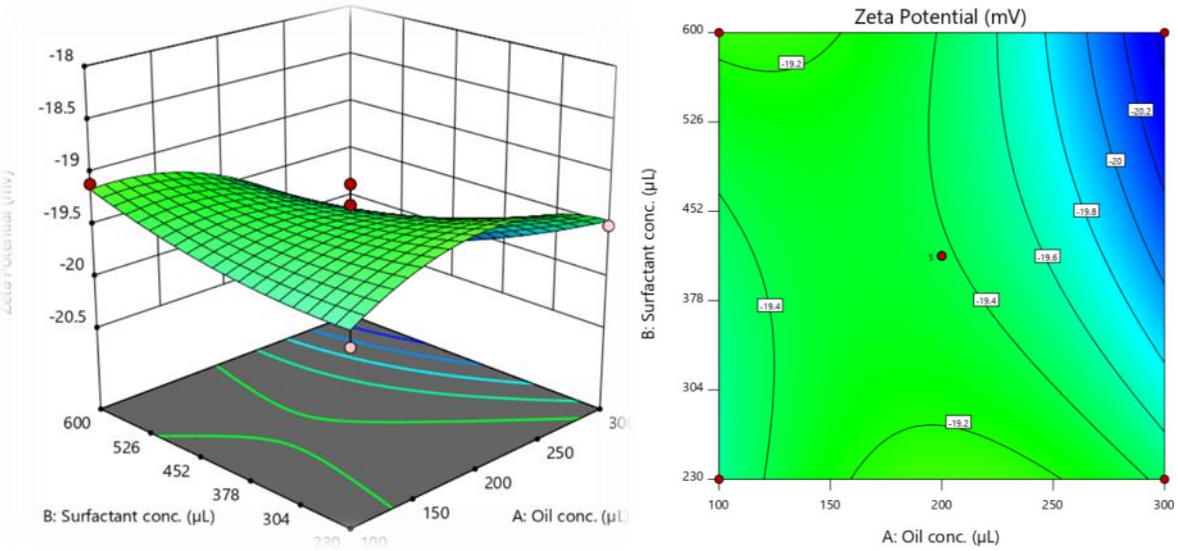


Figure 32: Effect of variables on zeta potential

5.12.1.4. Influence of variables on self-emulsification time

The SEF determines how well nano-emulsion develop inside the GIT. As oil and surfactant concentration was less the SEF is more as shown in Figure 33 so the intermediate value of oil and surfactant were appropriate. A "linear polynomial model" illustrating the interaction of multiple variables was found to best reflect the experimental data after fitting them to various models and ANOVA results. The equation was described below:

$$Y_4 = +13.49-0.6737A-3.02B-0.3250C+0.2425AB+1.82AC-2.30BC$$

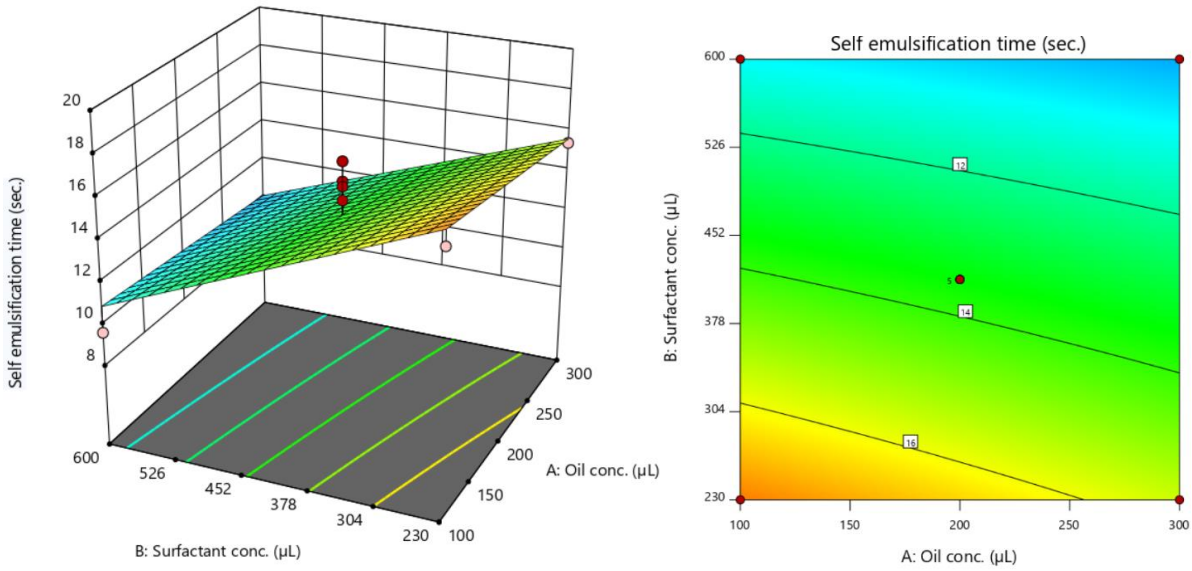


Figure 33: Effect of variable on self-emulsification time

5.12.1.5. Influence of variables on drug loading

The concentration of oil was directly affecting drug loading of formulation and whereas surfactant and concentration inversely affect the drug loading as shown in Figure 34. A linear polynomial model illustrating the interaction of multiple variables was found to best reflect the experimental data after fitting them to various models and ANOVA results. The equation was described below:

$$Y_5 = +68.66+4.91A-2.00B-1.06C$$

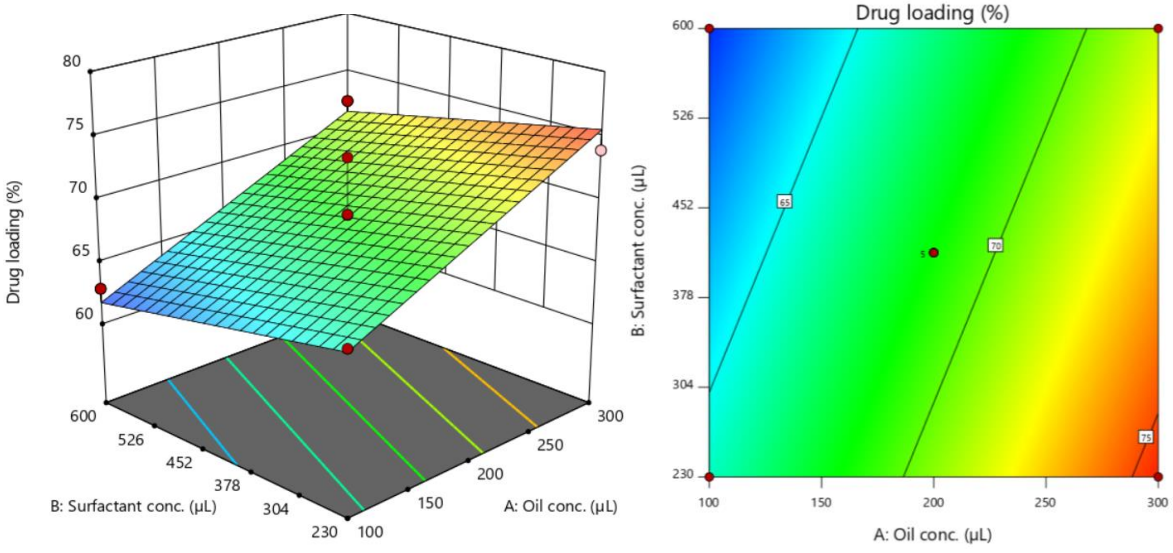


Figure 34: Effect of variables on drug loading

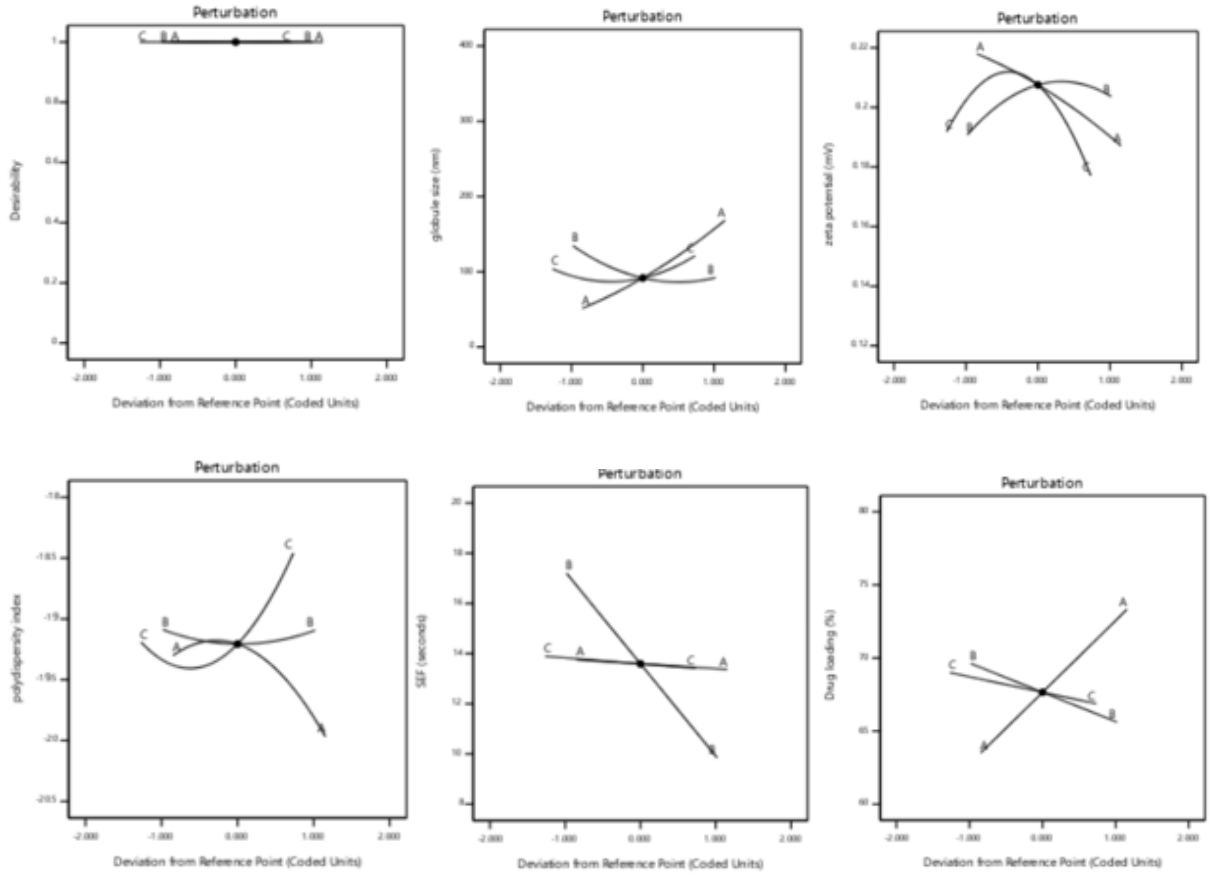
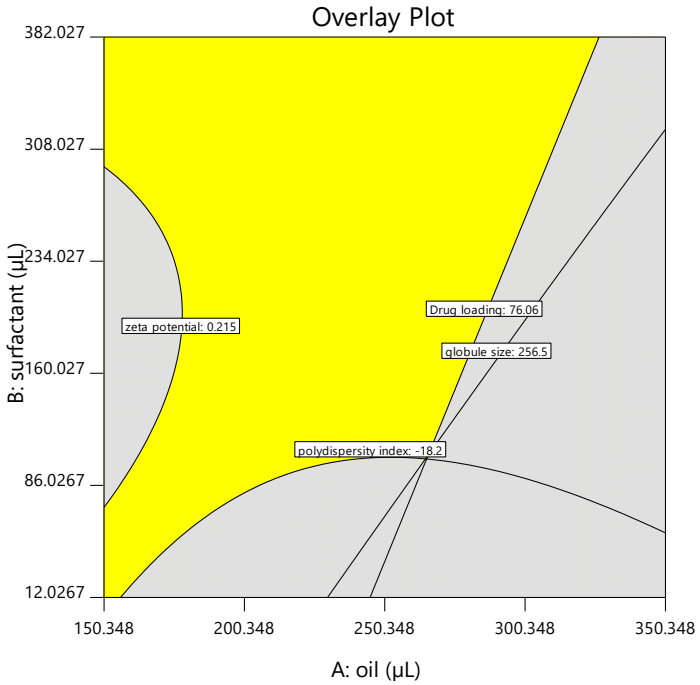
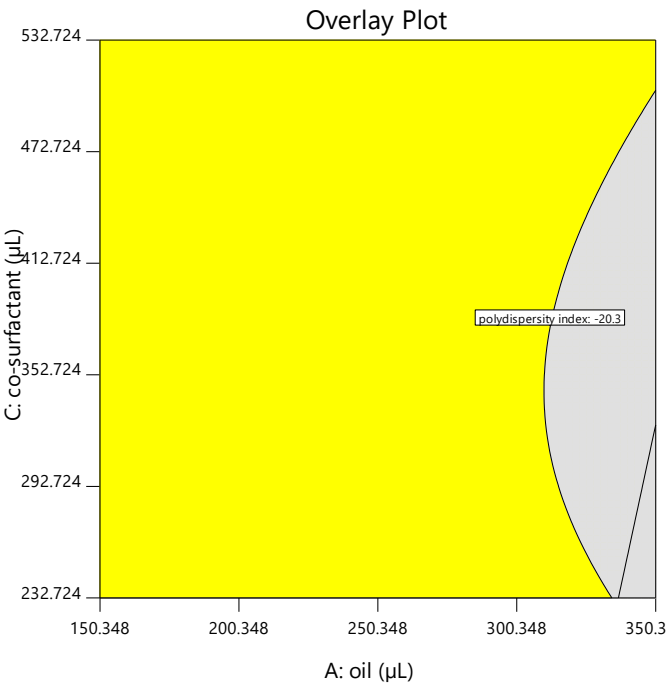


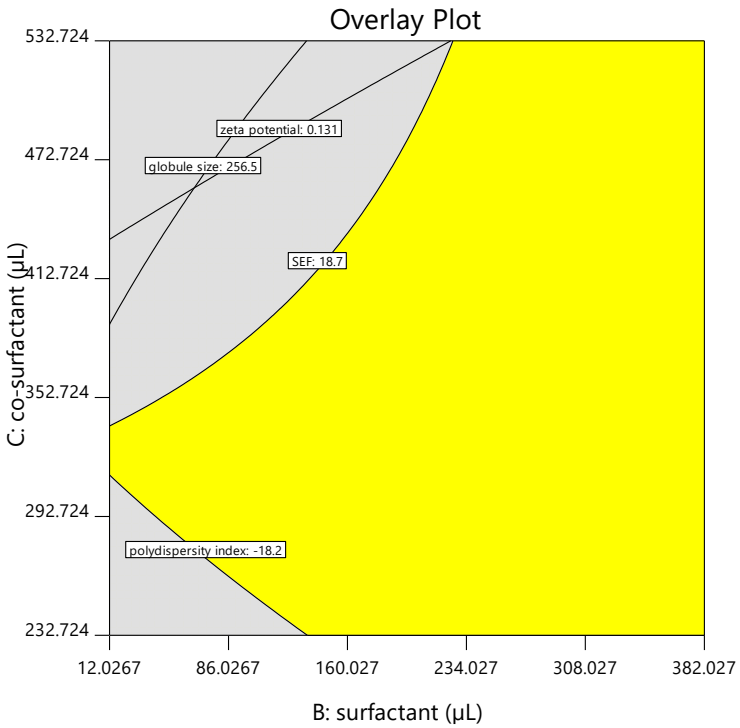
Figure 35: Perturbation plot of individual factors

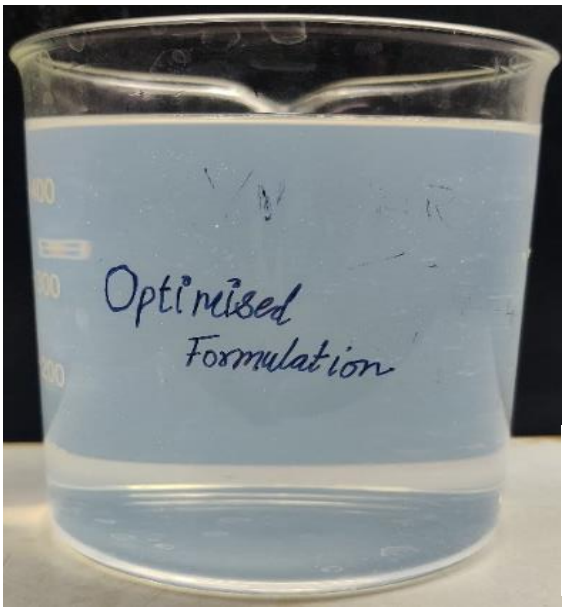
5.13. Optimization of L-SNEDDS

Using a graphical optimisation method, the levels of components A to C were optimised from design expert 11. The overlay plot has two region in which yellow colour indicated design space and grey colour indicate the value where results didn't meet the quality criteria. It has three variables i.e., oil, surfactant and co-surfactant has volume 240.68 µL, 506.85 µL and 252.47 µL respectively. The predicted value of GS, PDI, ZP, SEF, and drug loading was 167.04 nm, 0.148, -20.29 mV, 11.17s, and 70.81% respectively. The observed results of prepared OF with given compositions were shown in Figure 36.


Overlay Plot
382.027
308.027
234.027
160.027
86.0267
12.0267
B: surfactant (µL)
zeta potential: 0.215
Drug loading: 76.06
globule size: 256.5
polydispersity index: -18.2
150.348
200.348
250.348
300.348
350.348
A: oil (µL)


Overlay Plot
532.724
472.724
412.724
352.724
292.724
232.724
C: co-surfactant (µL)
polydispersity index: -20.3
150.348
200.348
250.348
300.348
350.3
A: oil (µL)


Overlay Plot
532.724
472.724
412.724
352.724
292.724
232.724
C: co-surfactant (µL)
zeta potential: 0.131
globule size: 256.5
SEF: 18.7
polydispersity index: -18.2
12.0267
86.0267
160.027
234.027
308.027
382.027
B: surfactant (µL)



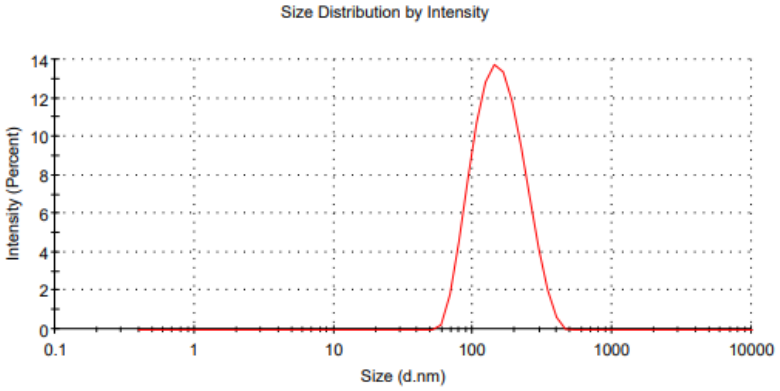
Parameters	Predicted	Observed
Globule size(nm) ± SD	167.04	142.6±0.98
Poly dispersity index ± SD	0.148	0.136±0.01
Zeta potential(mV) ± SD	-20.29	-19.6±0.23
Self-emulsification time (s)± SD	11.17	10.46 ± 0.67
Drug loading (%)± SD	70.81	72.42±0.53

Figure 36: Overlay plot of optimized formulation for TBZ loaded SNEDDS, prepared optimized formulation, predicted and observed result of optimized formulation

5.14. Characterization of optimized TBZ loaded SNEDDS

5.14.1. Zeta potential, globule size, and poly dispersity index

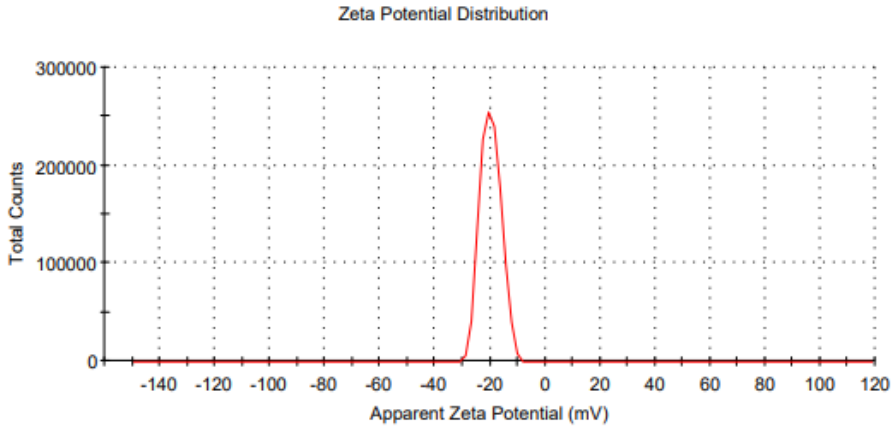
The average GS and PDI of OF were found to be 142.6 ± 0.98 nm and 0.136 ± 0.01 respectively which designate the uniformity of the GS distribution as shown in figure 37. The ZP -19.6±0.23 was indicative of the stability of the nano-emulsion (Figure 38). Surface has greater electrical charge (ZP > ±30 mV) of nano-droplets stops accumulation because of the powerful repulsion in between the particles.



Results

		Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 141.9	**Peak 1:**	163.3	100.0	65.54
PdI: 0.131	**Peak 2:**	0.000	0.0	0.000
Intercept: 0.950	**Peak 3:**	0.000	0.0	0.000
Result quality Good				

Figure 37: Globule size and PDI of optimized formulation



Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -19.7	**Peak 1:**	-19.7	100.0	3.64
Zeta Deviation (mV): 3.64	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm): 6.86e-4	**Peak 3:**	0.00	0.0	0.00
Result quality Good				

Figure 38: Zeta potential of optimized formulation

5.14.2. *Self-emulsification time*

SEF time of the OF was found 10.46 ± 0.53 s means the emulsification of SNEDDS was very quick. Short emulsification time was due to the appearance of less

concentration of oil and more concentration of co-surfactant and surfactant which results lower viscosity of the formulation.

5.14.3. *Drug loading*

The drug loading of the OF was found 72.42 ± 0.53% which was in the range of predicted value.

5.15. Transmission Electron Microscopy (TEM)

Images of TEM shown in Figure 39, revealed globe shaped with different sizes. Size of the particle perceptible from the size of the data obtained from Malvern zetasizer measurements of less than 200nm. This revealed the immediate formation of nanoparticles of nano emulsifying system. TEM is high resolution image technique and can capture any change in structure.

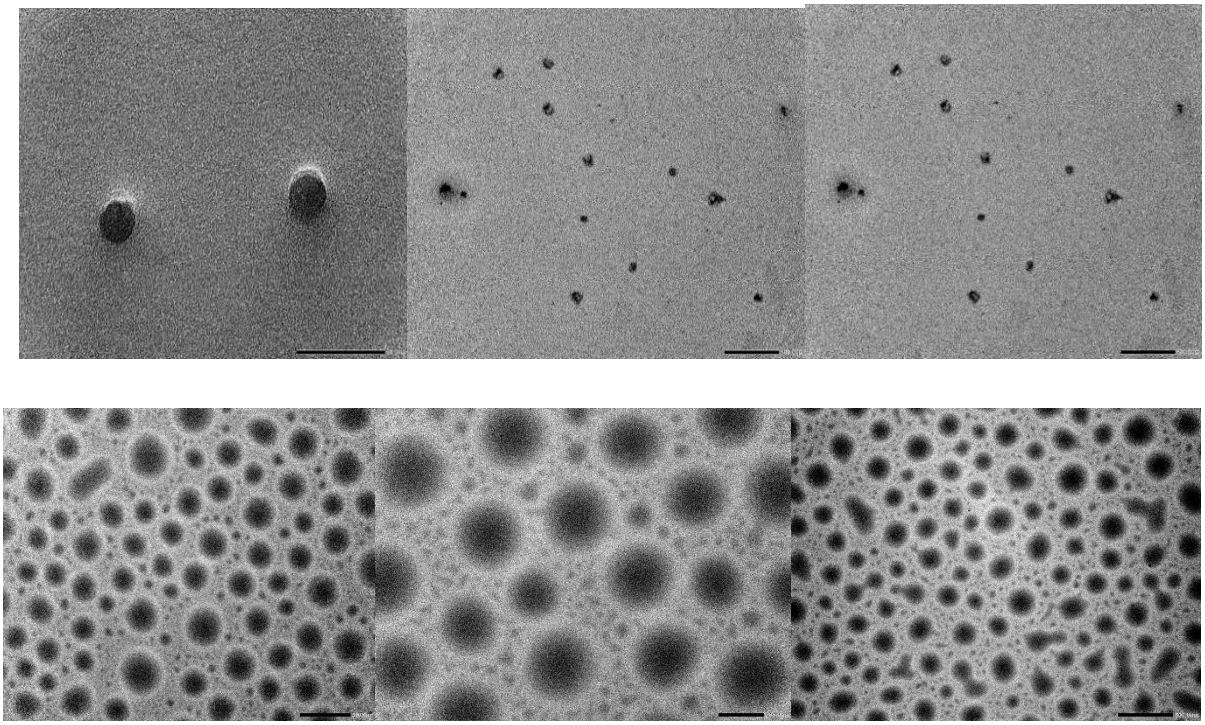


Figure 39: TEM micrographs of TBZ-loaded SNEDDS. (A–F) Images at different magnifications of optimized

5.16. Physicochemical Characterstics

Physicochemical compatibility between Naïve TBZ, oil, surfactant, co-surfactant and L-SNEDDS were studied by using FTIR spectroscopy. Figure 40 demonstrated no apparent physicochemical interaction. All significant peaks caused by the drug's functional group were seen to be present in the physical mixture. Wave number (cm^{-1}) of the medication did not significantly change, although a broadening impact was seen.

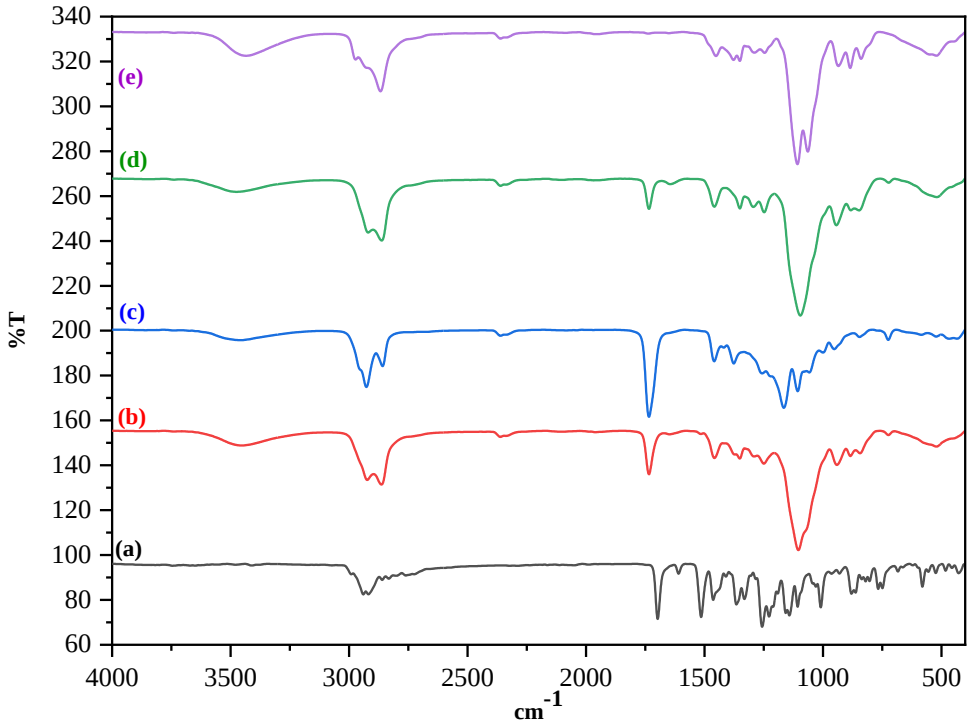


Figure 40: FTIR Spectra of (a) TBZ, (b) surfactant (c) oil, (d) formulation (e) co-surfactant

5.17. In vitro release study

Release study was observed for pure TBZ, marketed formulation (tablet), and TBZ-loaded SNEDDS. A significant increase was observed that more than 90% drug got release in pH 6.8 phosphate buffer dissolution media. In vitro drug release studies showed that naïve TBZ, marketed TBZ and optimized L-SNEDDS formulations evince drug release up to 0.62%, 10.34% and 91.19% respectively within 30 minutes. SNEDDS form of TBZ released more than 8.51 folds more as compare to marketed tablet in pH 6.8 phosphate buffer as compared to its marketed form. This

demonstrated that the TBZ dissolution rate has successfully been improved with the use of SNEDDS formulation.

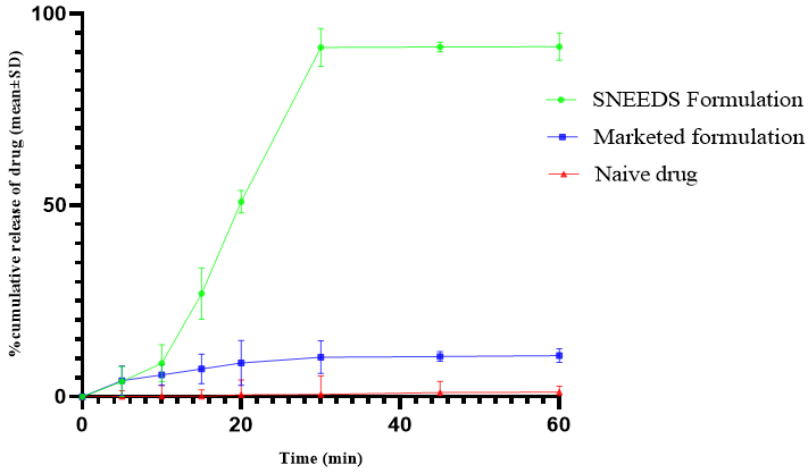


Figure 41: Release profile in phosphate buffer (pH 6.8) of naïve TBZ, marketed TBZ and L-SNEDDS

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

5.17.1. Kinetic modelling

The release kinetics of naïve, marketed and OF was evaluated using different release kinetic models such as First order, Korsemeyer Peppas, Zero order, Hixon Crowell, and Higuchi. As the R^2 value for naïve drug was maximum in zero order kinetics i.e. 0.9662 so it is concentration based release. Marketed formulation follow Korsemeyer Peppas model because of the higher R^2 value i.e. 0.9662 and the n value is 0.5827, so following non-fickian diffusion. The prepared OF of SNEDDS followed Korsemeyer Peppas model as the R^2 value was highest i.e. 0.9497 and other kinetic models had R^2 value less than 0.9. As the value of n for SNEDDS was 1.2454, so it followed super case II transport which means drug was released by fickian diffusion and relaxation of the drug delivery.

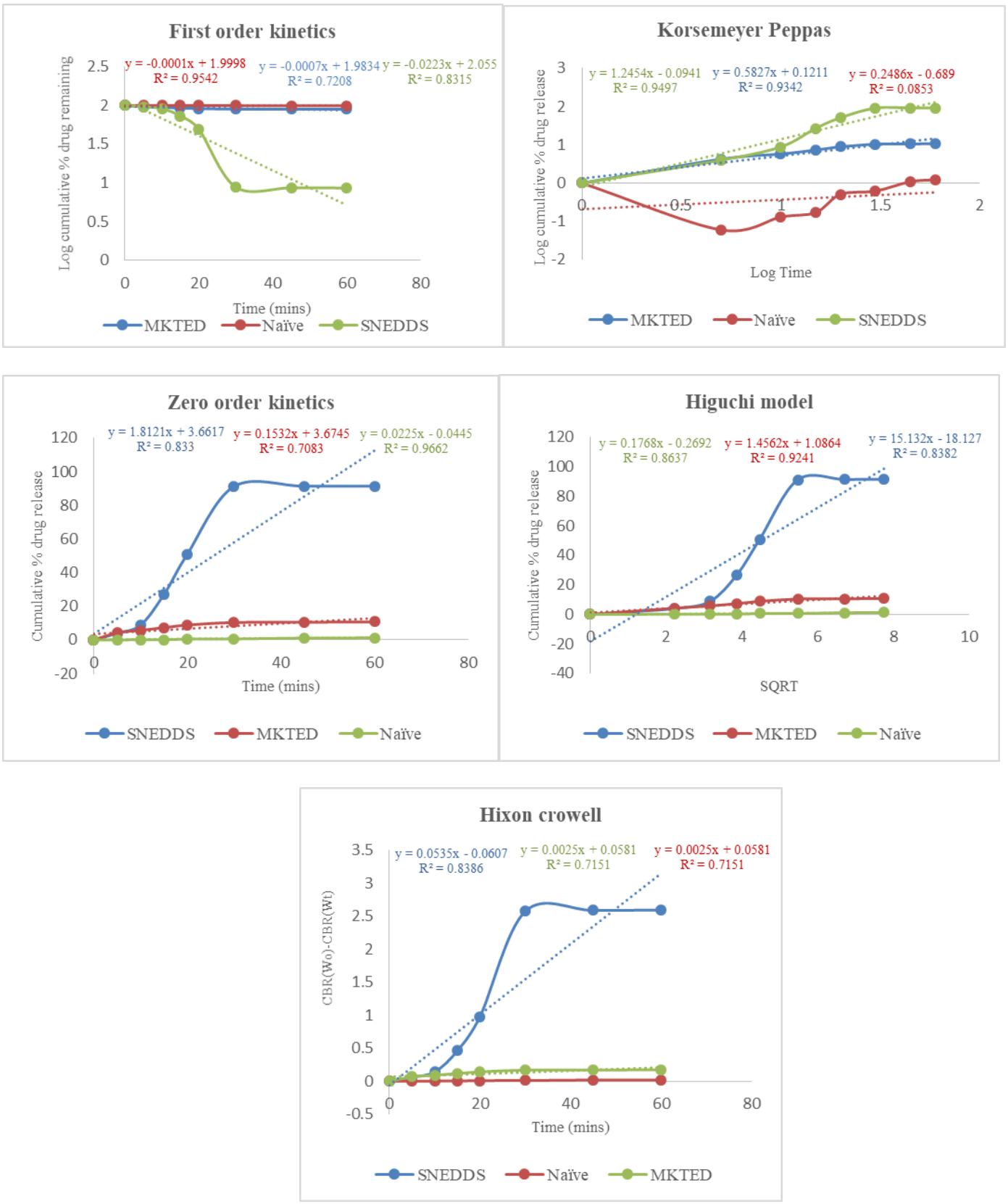


Figure 42: Release kinetics of naïve drug, marketed and optimized formulation.

Table 25: Release kinetic of naïve, marketed and optimized formulation

Kinetic model	Naïve drug R^2	Marketed formulation R^2	Optimized formulation R^2	N value of optimized formulation
Zero order	**0.9662**	0.7083	0.833	-
First order	0.9542	0.7208	0.8315	-
Korsmeyer peppas model	0.0853	**0.9342**	**0.9497**	**1.2454**
Higuchi model	0.8637	0.9241	0.8382	-
Hixon crowell	0.8386	0.7151	0.8386	-

5.18. Cell line study

Neuroblastoma SHSY-5Y cell lines were used to check the viability of the cells. This is a cell model for neurological disorders. Control used was PBS (pH 7.4) shown 100% viability of the SH-SY5Y cells. The blank had 18% cytotoxicity. The cytotoxicity was greater for the groups Y-12.5 and Y-6.25. Whereas, the toxicity is reduced upon serial dilutions. An optimum of 65%–80% cell viability was observed for the formulations upon dilution. So, SNEDDS loaded with tetrabenazine when coming in contact with gastric fluid get diluted and form nano-emulsion confirming that the carrier is non-toxic.

Cell viability% = Test sample Mean Absorbance/Negative Control Mean Absorbance x 100

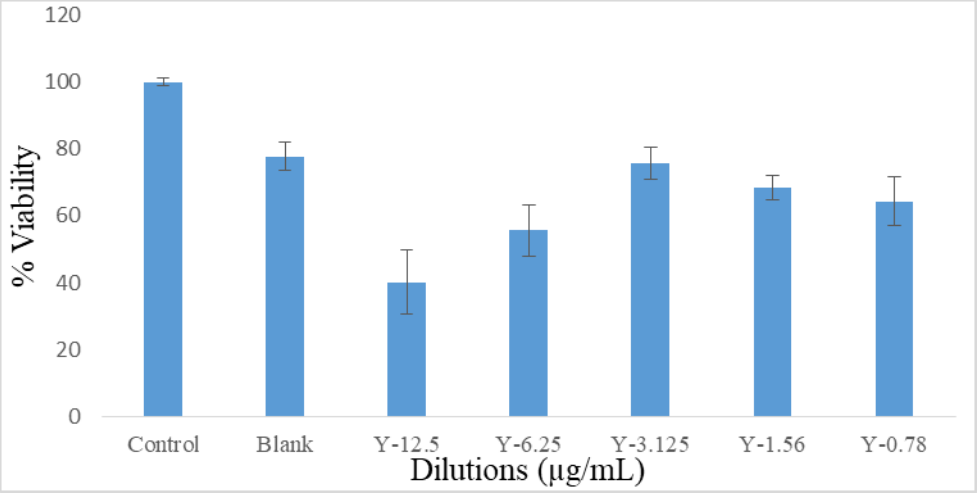


Figure 43: Cell line cytotoxicity of blank and TBZ-SNEDDS

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

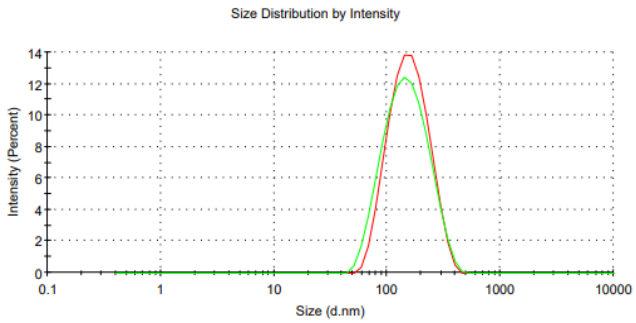
5.19. Accelerated stability study

Globule size, PDI, ZP, SEF, DL, and in-vitro drug release of aged formulation were performed and juxtapose with fresh formulation (zero month) at different interval (1, 2**,** 3, and 6 months). There was no observable difference found in any parameters which were considered for stability. No significant difference was found in the result of in-vitro release of TBZ loaded SNEDDS.

Table 26: Details of storage stability study

Time (month)	**Parameters**				
	Globule size (nm)	**PDI**	**Zeta Potential (mV)**	**SEF (s)**	**Drug loading (%)**
0 (Fresh TBZ SNEDDS)	142.6±0.98	0.136±0.01	-19.6±0.23	10.46±0.67	72.42±0.63
1 (Aged TBZ SNEDDS)	145.3±0.25	0.144±0.01	-19.5±0.13	11.12±0.72	72.13±0.14
2 (Aged TBZ SNEDDS)	144.5±0.54	0.140±0.02	-20.3±0.11	10.30±0.32	73.56±0.87
3 (Aged TBZ SNEDDS)	150.7±0.75	0.154±0.03	-19.7±0.21	11.15±0.57	73.21±0.41
6 (Aged TBZ SNEDDS)	141.9 ± 0.51	0.133 ± 0.06	-21.5 ± 0.34	11.89 ± 0.78	81.34 ± 0.38

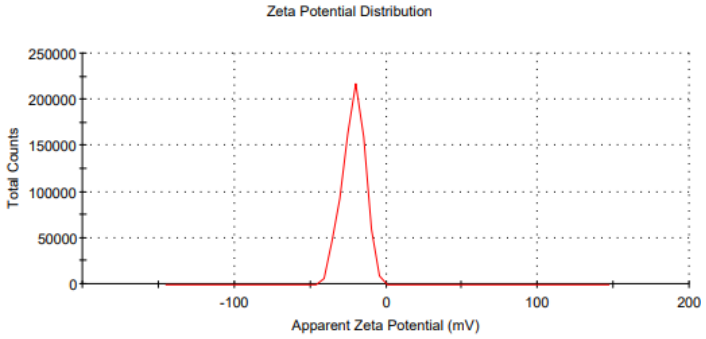
* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.



Results

		Size (d.n...	% Intensity:	St Dev (d.n...
Z-Average (d.nm): 141.9	Peak 1:	164.9	100.0	64.34
PdI: 0.133	Peak 2:	0.000	0.0	0.000
Intercept: 0.949	Peak 3:	0.000	0.0	0.000
Result quality Good				

Figure 44: Globule size and PDI of 6 months stored optimized formulation



Results

		Mean (mV)	Area (%)	St Dev (mV)
Zeta Potential (mV): -21.5	Peak 1:	-21.5	100.0	7.33
Zeta Deviation (mV): 7.33	Peak 2:	0.00	0.0	0.00
Conductivity (mS/cm): 0.0314	Peak 3:	0.00	0.0	0.00
Result quality Good				

Figure 45: ZP of 6 months stored optimized formulation

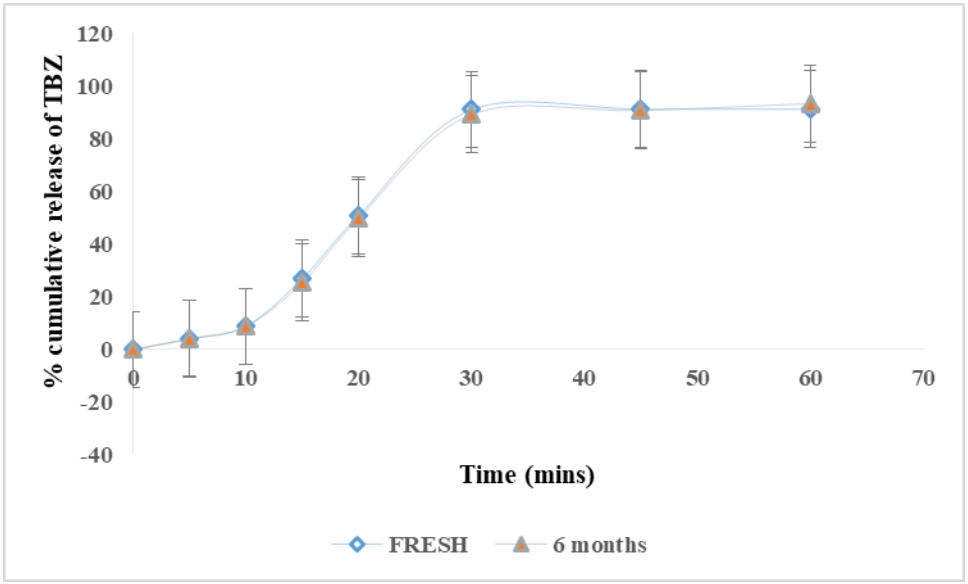


Figure 46: In vitro release of fresh formulation and 6 month stored formulation

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

5.20. In vivo study

5.21. Bioanalytical method development

Bioanalytical method RP-HPLC was developed using C_{18} column as stationary phase ACN and 0.1% formic acid in the ratio of 90:10 at 283 wavelength.

5.21.1. Specificity, linearity and range

Specificity of the devised approach was discovered specific for particular drug molecule as blank plasma sample chromatogram, there was no peak present at same retention time and wavelength. On the basis of concentration range (50-250 ng/mL) of plasma based sample the calibration curve was linear with 0.9964 coefficient of regression. The calibration curve and chromatogram of blank plasma and TBZ in rat plasma were shown in Figure 47 and 48 respectively.

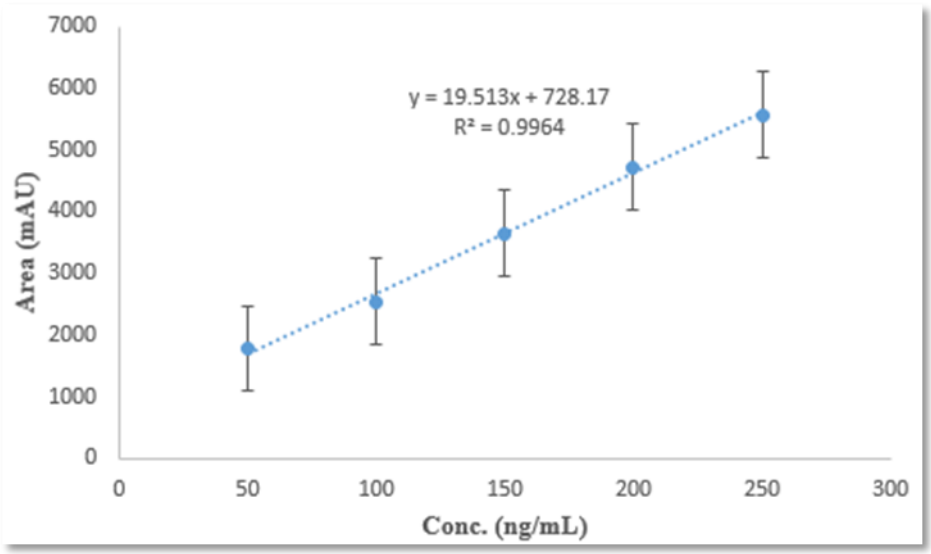


Figure 47: Calibration curve of TBZ in Plasma

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in hexaplicate.

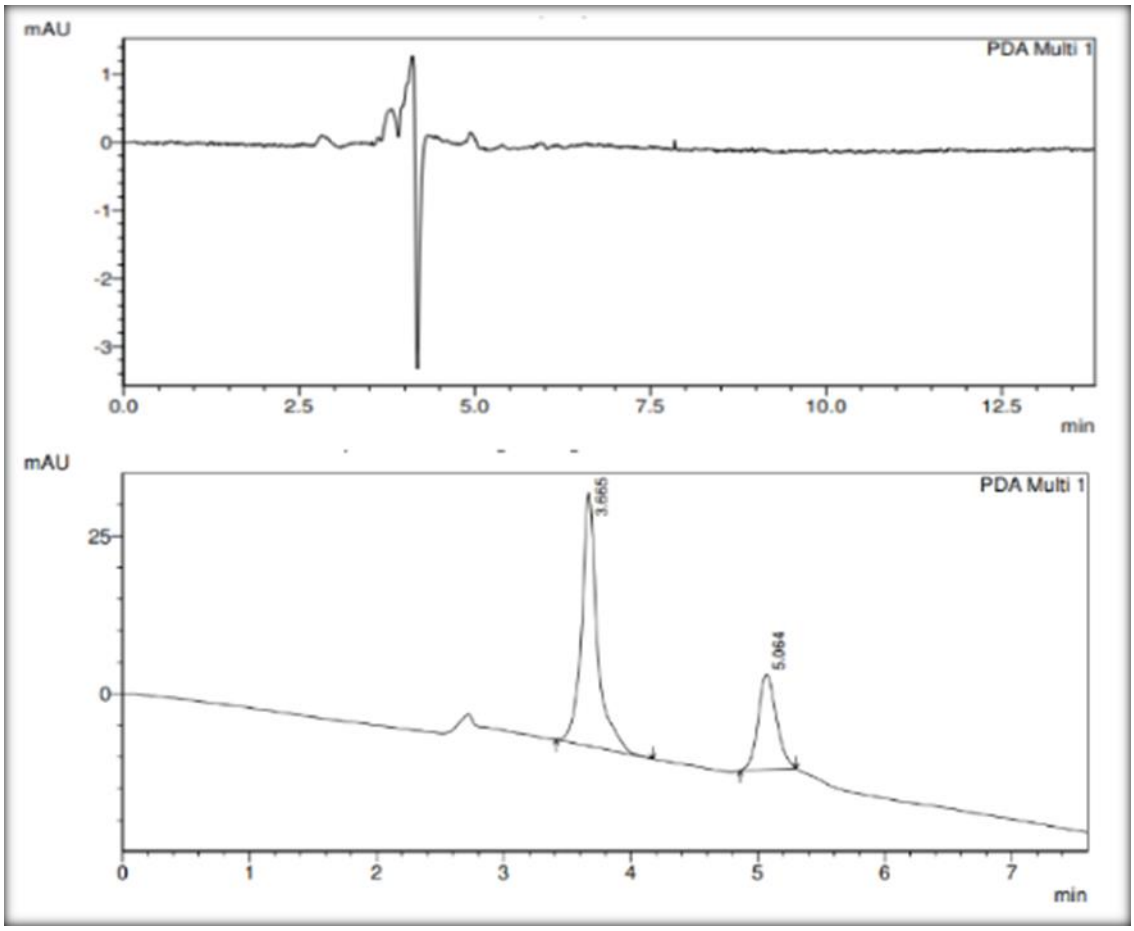


Figure 48: (A) Blank plasma, (B) Benzoquinolizine (RT. 3.665) and TBZ (RT. 5.064) in rat plasma chromatogram

5.21.2. Accuracy

As per the results, for all levels the value of mean percentage recovery were within 95-105% which is within the standard range. As shown in Table 27 method was accurate under experimental conditions.

Table 27: Accuracy study's results

Level	**Concentration of sample solution (ng/mL)**	**Total concentration of solution, actual (ng/mL)**	**Concentration of drug recovered (ng/mL), (n=5)**	**% Recovery**	**Mean % recovery**
LQC	50	120	112.62 ± 1.39	93.85 ± 0.86	
MQC	50	150	150.54 ± 0.87	100.36 ± 0.59	99.89 ± 0.64

HQC	50	180	189.87 ± 0.49	105.48 ± 0.48	

5.21.3. Precision

Developed method précised by determining the %RSD of the prepared standard solutions were injected in six replicates at intraday, interday, and interanalyst precision under same experimental conditions. The % RSD of standard dilutions in intraday, interday study were ranging from 0.49-0.92 % and 0.49-1.28 % respectively. In interanalyst analysis %RSD was changing between 0.25-1.60 % and results were mentioned in Table 28.

Table 28: Precision study's result

Parameters	**Level**	**Conc.**	**Analytical responses**						**Mean**	**SD**	**%RSD**
		(ng/mL)	**1**	**2**	**3**	**4**	**5**	**6**	**(N=6)**		
Repeatability (intraday precision)											
1 hour	LQC	120	2907	2956	2934	2987	2911	2946	2940.16	27.28	0.92
	MQC	150	3683	3694	3675	3629	3687	3672	3673.33	21.12	0.57
	HQC	180	4446	4413	4436	4482	4465	4491	4455.50	26.86	0.60
2 hour	LQC	120	2981	2945	2971	2943	2974	2991	2967.50	17.77	0.59
	MQC	150	3757	3786	3711	3794	3784	3786	3766.67	27.73	0.73
	HQC	180	4501	4587	4468	4472	4485	4531	4507.33	41.31	0.91
3 hour	LQC	120	2878	2876	2883	2914	2897	2905	2892.17	14.20	0.49
	MQC	150	3810	3845	3788	3791	3812	3867	3818.83	28.44	0.74
	HQC	180	4554	4513	4562	4538	4573	4560	4550.00	19.58	0.43
Intermediate precision (interday)											
Day 1	LQC	120	2971	2987	2978	2964	2943	2983	2971.00	14.61	0.49
	MQC	150	3740	3786	3757	3764	3776	3701	3754.00	27.75	0.73
	HQC	180	4424	4491	4398	4437	4401	4389	4423.33	34.36	0.77
Day 2	LQC	120	2914	2962	2933	2951	2978	3001	2956.50	28.46	0.96
	MQC	150	3717	3729	3799	3685	3825	3738	3748.83	48.16	1.28
	HQC	180	4424	4393	4457	4436	4382	4445	4422.83	27.05	0.61
Day 3	LQC	120	2963	2945	2925	2887	2903	2888	2918.50	28.54	0.97
	MQC	150	3724	3699	3785	3755	3759	3801	3753.83	34.45	0.91
	HQC	180	4510	4526	4508	4534	4511	4491	4513.33	13.73	0.30
Intermediate precision (interanalyst)											

Analyst 1	LQC	120	2895	2861	2872	2873	2894	2904	2883.16	15.33	0.53
	MQC	150	3801	3843	3784	3915	3851	3876	3845.00	43.89	1.14
	HQC	180	4454	4487	4435	4398	4482	4435	4448.50	30.41	0.68
Analyst 2	LQC	120	2987	2945	2915	2845	2888	2934	2919.00	44.68	1.53
	MQC	150	3936	3958	3972	3961	3999	3971	3966.16	18.88	0.47
	HQC	180	4501	4523	4572	4531	4511	4584	4537.00	30.65	0.67
Analyst 3	LQC	120	2832	2943	2951	2876	2947	2866	2902.50	46.50	1.60
	MQC	150	3847	3858	3892	3867	3794	3765	3837.16	43.77	1.14
	HQC	180	4605	4598	4572	4592	4584	4575	4587.16	11.86	0.25

5.21.4. Stability study of plasma samples

The stability of the drugs in plasma at three different levels i.e., standard solution in terms of freeze-thaw, short-term and long-term stability was conducted and results were presented in Table 29, 30, and 31 respectively. The results obtained indicated recovery of the short term stability was 93.83-106.93 % and %RSD was in the range of 0.32-1.97. Freeze thaw stability gave 92.70-109.73 % drug recovery and 0.85-1.79 was range of %RSD. Long term stability provide 91.09-110.99 % drug recovery and %RSD changes from 0.71-1.93. This signified the long-term storage of drugs in plasma samples.

Table 29: Short term TBZ plasma sample stability

Actual concentration of drug (ng/mL)	**Area 1 (cm^2)**	**Area 2 (cm^2)**	**Area 3 (cm^2)**	**Mean (cm^2)**	**S.D.**	**%RSD**	**Amount of drug recovered in plasma (ng/mL)**	**Recovery (%)**
1 hour								
120LQC	2912	2933	2931	2925.33	11.59	0.39	112.59	93.83
150MQC	3640	3657	3634	3643.66	11.93	0.32	149.41	99.61
180HQC	4475	4462	4438	4454.50	17.13	0.38	191.16	106.20
2 hour								
120LQC	2873	2974	2932	2926.33	50.73	1.73	113.72	94.76
150MQC	3682	3725	3701	3702.67	21.54	0.58	151.97	101.31
180HQC	4412	4546	4483	4480.33	67.03	1.49	191.70	106.50
3hour								

120LQC	2988	2873	2921	2927.33	57.76	1.97	112.70	93.91
150MQC	3759	3683	3625	3722.33	67.20	1.82	151.97	101.15
180HQC	4472	4567	4413	4484.00	77.69	1.73	192.47	106.93

Table 30: TBZ plasma sample's Freeze thaw stability

Actual concentration of drug (ng/mL)	**Area 1**	**Area 2**	**Area 3**	**Mean**	**S.D.**	**%RSD**	**Amount of drug recovered in plasma (ng/mL)**	**Recovery (%)**
Cycle 1								
120LQC	2871	2959	2867	2899.00	52.00	1.79	111.25	92.70
150MQC	3697	3683	3745	3708.33	32.51	0.87	152.72	101.82
180HQC	4512	4599	4636	4562.33	63.65	1.38	197.51	109.73
Cycle 2								
120LQC	2967	2894	2943	2934.67	37.20	1.26	113.07	94.23
150MQC	3622	3746	3685	3684.33	62.00	1.68	151.49	100.99
180HQC	4559	4467	4585	4537.00	62.00	1.36	195.19	108.44
Cycle 3								
120LQC	3011	3057	2966	3018.00	36.01	1.19	117.34	97.79
150MQC	3758	3699	3705	3720.66	32.47	0.87	153.35	102.23
180HQC	4512	4568	4587	4555.67	38.99	0.85	196.15	108.97

Table 31: Long term TBZ plasma samples stability

Actual concentration of drug (ng/mL)	**Area 1**	**Area 2**	**Area 3**	**Mean**	**S.D.**	**%RSD**	**Amount of drug recovered in plasma (ng/mL)**	**Recovery (%)**
1st week								
LQC	2876	2948	2971	2931.67	49.59	1.69	112.57	93.81
MQC	3652	3741	3678	3690.33	45.76	1.24	151.33	100.89
HQC	4564	4644	4538	4582.00	55.24	1.21	196.89	109.38
2nd week								
LQC	2864	2947	2972	2927.67	56.53	1.93	112.37	93.64
MQC	3757	3786	3691	3744.67	48.69	1.30	154.11	102.74

HQC	4587	4614	4549	4583.33	32.65	0.71	196.96	109.42
3rd week								
LQC	2836	2891	2876	2867.67	28.43	0.99	109.30	91.09
MQC	3754	3717	3675	3715.33	39.52	1.06	152.61	101.74
HQC	4587	4637	4696	4638.67	52.51	1.13	199.79	110.99

Table 32: System suitability parameters

Parameters	**Value**	**Limits**
HETP	25.86±0.2	Depends on theoretical plate
Tailing Factor	1.17±0.05	<2
Theoretical Plate	7337.33±34.83	>2000

5.21.5. LOD and LOQ

Rat plasma contained the following LOD and LOQ values: 18.19 ng/mL and 55.14 ng/mL, respectively. The outcome indicated that the technique was sensitive for drug identification at lower doses [296].

5.21.6. System suitability

By using system suitability test chromatographic conditions were examined for further use. For TBZ and Benzoquinolizine tailing factor and theoretical plates is <2 and >2000 respectively which was under acceptable range and showing good efficiency of column throughout the method shown in Table 32.

5.22. Calibration curve of TBZ in cerebrospinal fluid (CSF)

On the basis of concentration range (50-250 ng/mL) of CSF based sample the calibration curve was linear with 0.999 coefficient of regression. The calibration curve of TBZ in rat CSF were shown in Figure 49.

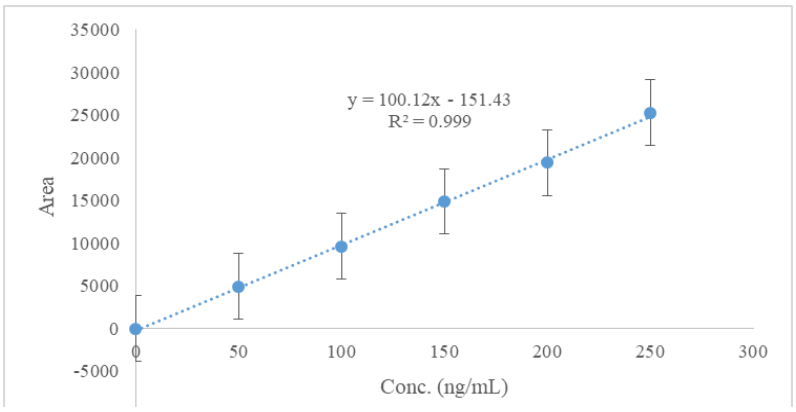


Figure 49: Calibration curve of TBZ in CSF

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

5.23. Pharmacokinetic studies

The overlay plot of TBZ in brain as well as in plasma at 0, 30, 60, 120, 240, and 1440 min as shown in figure. Naïve TBZ has low concentration in blood when compared with the OF of TBZ loaded SNEDDS same was present in brain. Maximum concentration of TBZ loaded SNEDDS in plasma as well as in brain at 1 h showed 156.68 ± 6.03 ng/mL and 61.08 ± 4.03 ng/mL respectively. But naïve drug showed maximum concentration at 4 h which was 29.22 ± 6.29 ng/mL and 3.27 ± 0.28 ng/mL in plsma and brain respectively. So it was understandable concentration of TBZ increased when administered through SNEDDS form (figure 50).

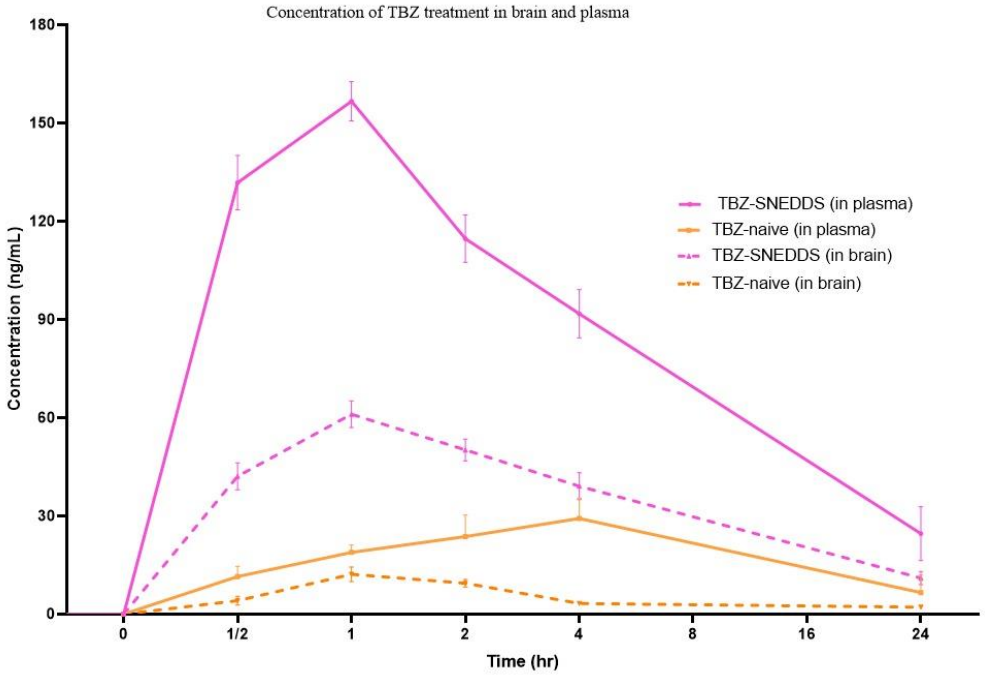


Figure 50: Concentration of TBZ in brain and plasma of Naïve and TBZ loaded SNEDDS

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

5.24. Body weight

In the present study the animals were subjected with haloperidol to induce dystonia. As per the experimental design, body weight of rats of all groups were recorded on 0, 7, 14, 21, and 28th days. The body weight of rats of normal control (G-I) was found as 181.11 ± 8.22 g, 181.27 ± 9.05 g, 182.22 ± 8.14 g, 181.12 ± 8.13 g, and 184.19 ± 9.01 g on day 0, 7, 14, 21 and 28th respectively. As the day progresses the significant reduction in the body weight was observed from 180.11 ± 12.6 g to 100.23 ± 16.11 g on 0th to 28th day in group of rats received haloperidol only (Gp-II). Similar kind of reduction in body weight was also observed in G-IV as rats of this group received excipients of SNEDDS and haloperidol i.e., SNEDDS Placebo which was 180.1 ±11.03 g, 142.19 ± 12.17 g, 129.18 ± 10.06 g, 113.11 ± 10.11 g, and 100.13 ± 9.22 g on 0, 7, 14, 21, and 28th days of study. The body weight of rats of G-III changes from 182.19 ± 10.03 g, 153.17 ± 11.12 g, 137.22 ± 9.23 g, 142.03 ± 9.12 g, and 149.13 ± 9.19 g on 0, 7, 14, 21, and 28th day. The body weight of group of rats received low doses of TBZ-SNEDDS (G-V) was 180.16 ± 10.04 g, 158.08 ± 9.23 g, 161.12 ± 9.17

g, 168.09 ± 9.11 g, and 171.23 g on day 0, 7, 14, 21 and 28th. On the other hand the body weight of group of rats received high doses of TBZ-SNEDDS (G-VI) was 181.17 ± 2.13 g, 163.08 ± 10.22 g, 169.04 ± 10.12 g, 174.23 ± 10.09 g and 179.02 ± 10.05 g on day 0, 7, 14, 21 and 28$^{th.}$ (Figure 51).

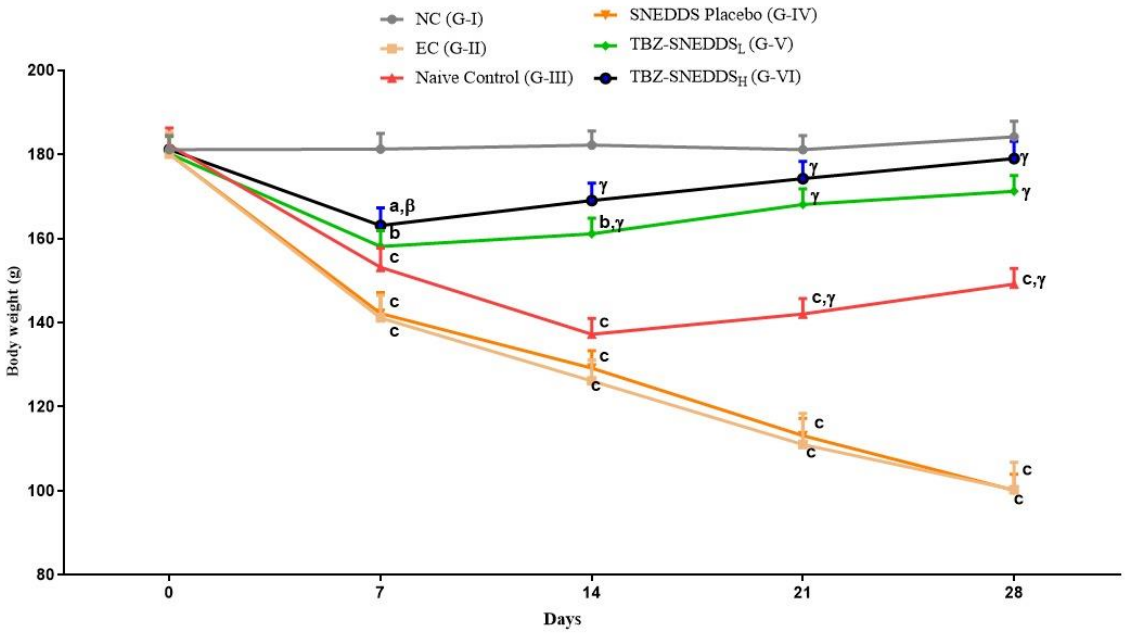


Figure 51: Effect on body weight of different treatment (Data are represented as mean ± SD (n=6) where α = p < 0.05, β = p < 0.01, γ = p < 0.001 compared to the Experimental Control Group (EC). A = p < 0.05, b = p < 0.01, c = p < 0.001 compared to the Normal Control Group)

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

Decrease in the weight of dystonic rats was due to difficulty in having feed than normal rats [309]. It was very interesting to note that the improvement in body weight was observed in G-III, from day 28th while in G-V and VI such effect was observed from day 14th only. Hence it indicates that the development of SNEDDS of TBZ not only treat the disease state but also brings amelioration in dystonia in early days of experimental period.

5.24. Behavioural

Behavioural study was execute to check the effect of TBZ on dystonic rats induced by haloperidol. This inductive agent cause movement disorder such as dystonia and tardive dyskinesia.

5.24.1 Locomotor

Utilized an Actophotometer to check one of the behavioural parameter of the rats. Locomotor activity was consider good if the counts of beam crossed by rats was more in number. As per the experimental design, locomotor activity of rats of all groups were performed on 0, 7, 14, 21, and 28th days. The beam crossed by normal control (G-I) was found as 463.04 ± 14.22 n, 462.07 ± 12.22 n , 463.16 ± 13.19 n, 463.12 ± 12.13 n, and 462.19 ± 12.06 n on day 0, 7, 14, 21 and 28th respectively. G-II reported least locomotor activity in which only haloperidol administered to rats were reduced from 463.11 ± 14.22 n to 108.03 ± 14.26 n on 0th to 28th day. Similar observation can be seen in rats of G-IV in which SNEDDS placebo and haloperidol was administered which was 463.03 ±10.22 n, 387.17 ± 12.2 n, 286.22 ± 11.21 n, 184.18 ± 11.25 n, and 104.23 ± 11.28 n on 0, 7, 14, 21 and 28th days of study (Figure 52).

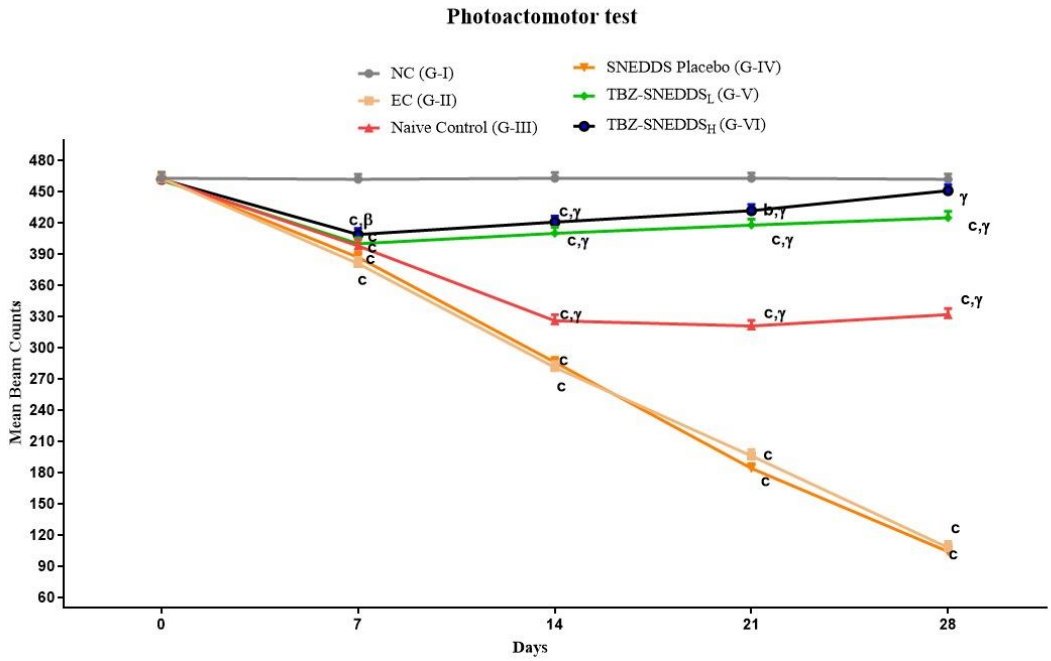


Figure 52: Effect on locomotor of different treatment (Data are represented as mean ± SD (n=6) where α = p < 0.05, β = p < 0.01, γ = p < 0.001 compared to the Experimental Control Group (EC). A = p < 0.05, b = p < 0.01, c = p < 0.001 compared to the Normal Control Group)

* Results were reported as values of Mean ± standard error mean (SEM) which were obtained from experiments carried out in triplicate.

On the other hand G-III which received pure form of drug changes in counts of beam crossing 462.19 ± 14.04 n to 332.09 ± 14.1 n on 0^{th} and 28^{th} day. Count of beam in low dose of TBZ loaded SNEDDS (G-V) was observed 461.19 ± 14.24 n, 400.11 ± 14.03 n, 410.11 ± 14.22 n, 418.06 ± 14.14 n, and 425.11 n on day 0, 7, 14, 21, and 28^{th}. Count of beam in high dose of TBZ loaded SNEDDS (G-VI) was observed 462.18 ± 14.4 n, 409.11 ± 14.11 n, 421.05 ± 14.2 n, 432.02 ± 14.19 n, and 451.22 ± 14.12 n on day 0, 7, 14, 21, and 28^{th}.

According to this study G-III had same effect throughout the study because pure form of the drug has low BA and couldn't cross BBB but G-V and G-VI which received SNEDDS form of the drug improved the locomotor activity significantly because of the enhancement in aqueous solubility and BA helped to cross BBB.

5.24.2. Muscle coordination

Rotarod apparatus was used to check muscle coordination of rats. Haloperidol significantly induced dystonia. Difficulty in muscle coordination was considered with fall time of the rats, as rapid time to fall rota rod indicated low muscle coordination and stay till cut-off time indicated in the improvement from dystonia occurred by haloperidol.

Time of fall of normal control (G-I) was found 152.04 ± 3.22 s, 151.07 ± 3.22 s, 151.16 ± 3.19 s, 153.12 ± 2.65 s, and 152.19 ± 2.26 s on 0, 7, 14, 21, and 28^{th} days respectively. Coordination of muscle was found least in G-II from the beginning of the study in which only inducer was administered. 153.11 ± 2.66 s, and 78.08 ± 3.16 s was recorded on 0^{th} and 28^{th} day respectively in G-II. SNEDDS placebo (G-IV) exhibited similar result as G-II in which haloperidol and blank SNEDDS was administered and showed 152.8 ± 2.29 s, 117.17 ± 2.19 s, 100.12 ± 2.19 s, 86.06 ± 2.44 s, and 75.14 ± 3.03 s fall time on 0, 7, 14, 21, and 28^{th} respectively (Figure 53).

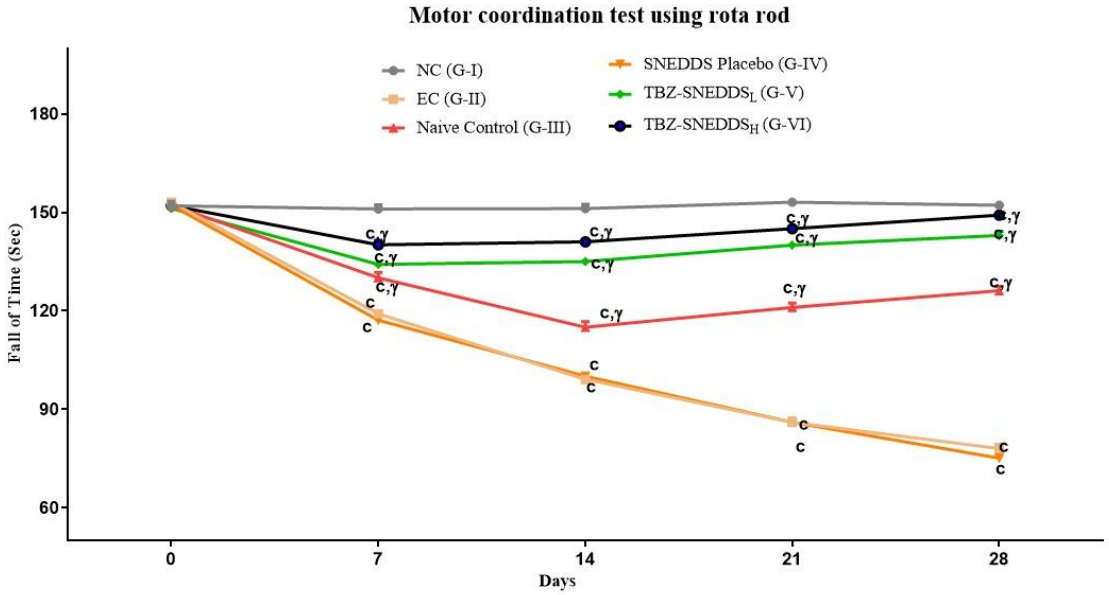


Figure 53: Effect on motor coordination of different treatment (Data are represented as mean ± SD (n=6) where α = p < 0.05, β = p < 0.01, γ = p < 0.001 compared to the Experimental Control Group (EC). A = p < 0.05, b = p < 0.01, c = p < 0.001 compared to the Normal Control Group)

*Results were reported as values of Mean ± standard error mean (SEM)

G-III (naïve control) which received pure form of the drug changes in fall time i.e., 152.19 ± 4.11 s, 130.11 ± 4.04 s, 115.02 ± 4.22 s, 121.06 ± 3.2 s, and 126.13 ± 3.17 s on 0, 7, 14, 21 and 28th day respectively.

Fall time in low dose of TBZ loaded SNEDDS (G-V) was observed 151.22 ± 2.11 s, 134.17 ± 3.01 s, 135.02 ± 2.88 s, 140.02 ± 3.09 s and 143 ± 3.12 s on 0, 7, 14, 21, and 28th day. But it was more significant in high dose of TBZ loaded SNEDDS (G-VI) and fall time observed as 152.08 ± 3.4 s, 140.17 ± 3.22 s, 141.05 ± 2.19 s, 145.02 ± 2.04 s, and 149.18 ± 2.1 s on 0, 7, 14, 21, and 28th day respectively.

As fall of time indicates coordination of muscles which was lack in disease induced rats but the coordination was found good in treatment received rats because of the improvement in pharmaceutical challenges of drug i.e., low solubility and permeability.

5.24.3. Catalepsy

Bar test was used to check catalepsy in rats. Haloperidol was used to induce dystonia. Time taken to remove the paw from bar was noted down to check the catalepsy if removal of paw was fast than it indicated amelioration in treatment and if it was more indicated diseased condition.

Time to remove paw of normal control (G-I) was found 7.03 ± 0.01 s , 6.94 ± 0.03 s, 7.01 ± 0.01 s, 6.99 ± 0.01 s, and 6.94 ± 0.02 s on 0, 7, 14, 21, and 28th days respectively. Difficulty in removing paw was found more in G-II from the starting of the study in which only haloperidol was administered. 6.89 ± 0.02 s, and 86.11 ± 4.28 s was recorded on 0th and 28th day respectively in G-II. Same response was observed in SNEDDS placebo (G-IV) as G-II in which inducer and blank SNEDDS was administered and observed 6.93 ± 0.03 s, 47.03 ± 4.21 s, 60.04 ± 4.23 s, 79.03 ± 4.32 s, and 86 ± 4.28 s fall time on 0, 7, 14, 21, and 28th respectively (Figure 54).

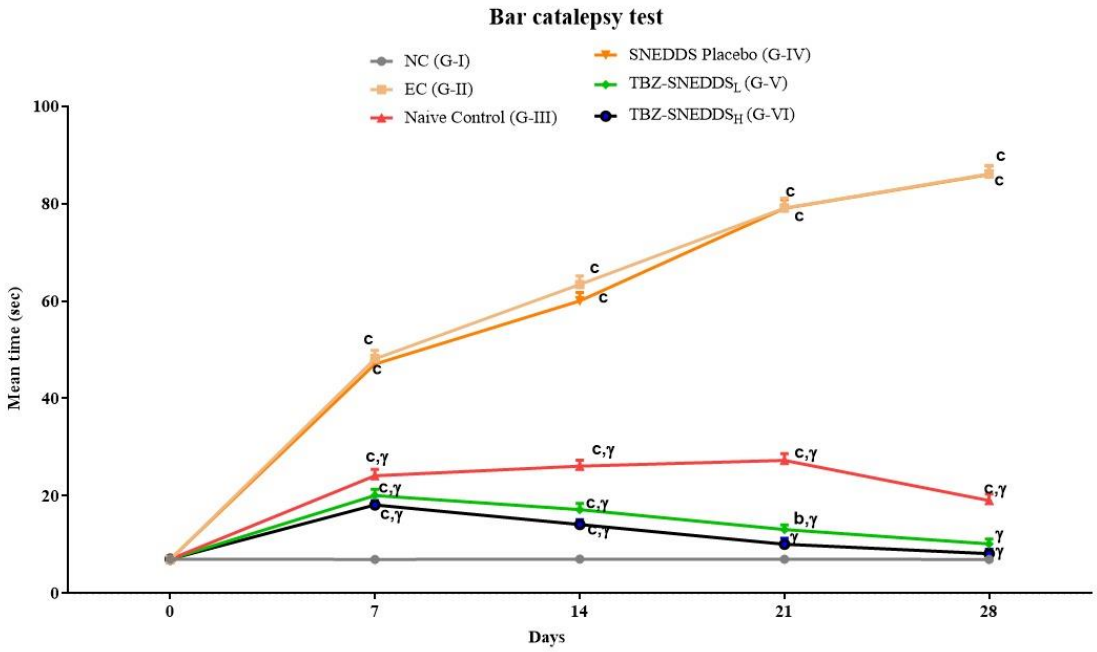


Figure 54: Effect on bar catalepsy of different treatment (Data are represented as mean ± SD (n=6) where α = p < 0.05, β = p < 0.01, γ = p < 0.001 compared to the Experimental Control Group (EC). A = p < 0.05, b = p < 0.01, c = p < 0.001 compared to the Normal Control Group)

*Results were reported as values of Mean ± standard error mean (SEM)

Naïve control (G III) which received raw form of the drug has some improvement in time to remove the paw from the day 28th and the observation were 6.96 ± 0.02 s, 24.13 ± 3.23 s, 26.09 ± 3.04 s, 27.29 ± 3.29 s, and 19.04 ± 3.21 s on 0, 7, 14, 21 and 28th day respectively.

In low dose of TBZ loaded SNEDDS (G-V) was observed 7.01 ± 0.03 s, 20.06 ± 3.09 s, 17.17 ± 3.11 s, 13.06 ± 2.37 s, and 10.14 ± 2.34 s on 0, 7, 14, 21, and 28th day. But it was less in high dose of TBZ loaded SNEDDS (G-VI) and fall time observed as 7 ± 0.01 s, 18.12 ± 2.23 s, 14.11 ± 2.39 s, 10.06 ± 3.03 s, 8 and 8.11 ± 2.23 s on 0, 7, 14, 21, and 28th day respectively.

5.24.4. Akinesia

Akinesia was performed to check the difficulty sense by the rats to start motion. Less time taken to initiate any step or movement by rat of any group was considered improvement in treatment. If more time consumed to start movement than worsening of the disease condition was considered.

Time to start movement of normal control (G-I) was found 5.11 ± 0.01 s, 5.08 ± 0.02 s, 5.1 ± 0.01 s, 5.06 ± 0.01 s, and 5.09 ± 0.03 s on 0, 7, 14, 21, and 28th days respectively. G-II (EC) in which only haloperidol was administered struggled more for any movement throughout the study. 5.08 ± 0.02 s, and 232.11 ± 5.21 s was recorded on 0th and 28th day respectively in G-II. SNEDDS placebo (G-IV) gave similar kind of response as G-II in which inducer and blank SNEDDS was administered and observed 6.93 ± 0.03 s, 96.12 ± 5.21 s, 131.22 ± 5.18 s, 186.11 ± 5.1 s, and 230.21 ± 5.13 s fall time on 0, 7, 14, 21, and 28th respectively (Figure 55).

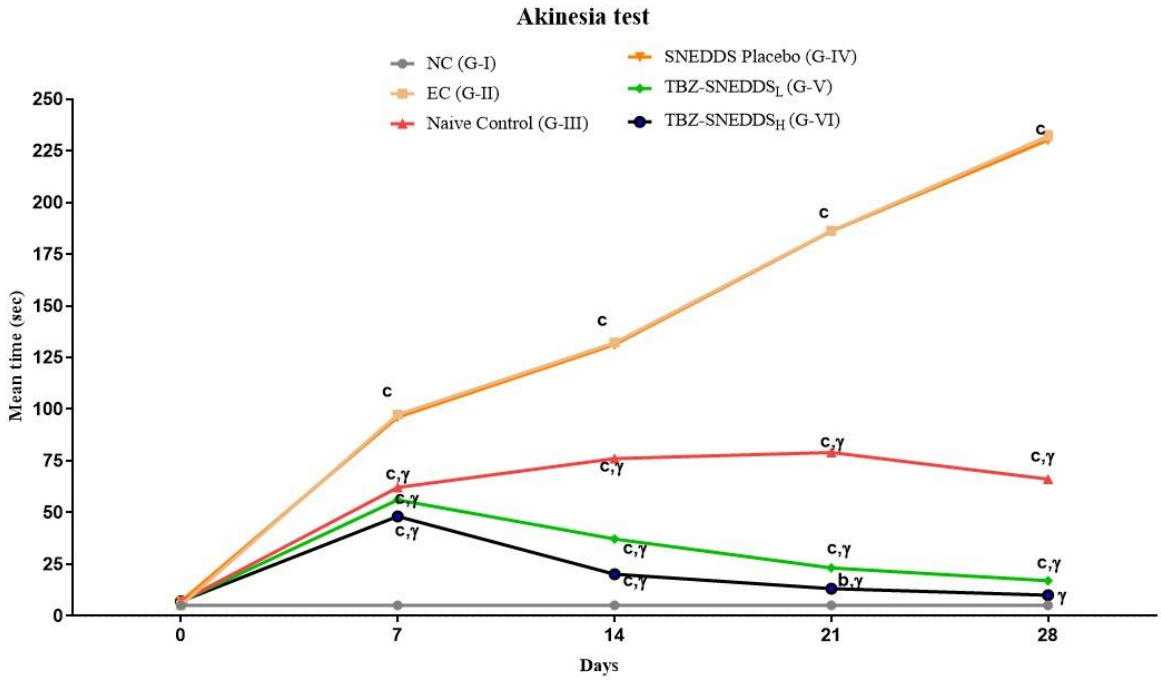


Figure 55: Effect on akinesia of different treatment (Data are represented as mean ± SD (n=6) where α = $p < 0.05$, β = $p < 0.01$, γ = $p < 0.001$ compared to the Experimental Control Group (EC). A = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$ compared to the Normal Control Group)

*Results were reported as values of Mean ± standard error mean (SEM)

Naïve control (G III) which received pure form of the drug showed some improvement in time to start any movement from the day 28th and the observation were 6.96 ± 0.02 s, 62.14 ± 2.32 s, 76.11 ± 2.08 s, 79.04 ± 2.21 s, and 66.11 ± 2.23 s on 0, 7, 14, 21 and 28th day respectively.

In low dose of TBZ loaded SNEDDS (G-V) was observed 7.01 ± 0.03 s, 56.06 ± 4.09 s, 37.17 ± 4.11 s, 23.16 ± 2.19 s and 17.03 ± 4.03 s on 0, 7, 14, 21, and 28th day. But it was rapid in high dose of TBZ loaded SNEDDS (G-VI) and time to start move observed as 7 ± 0.01 s, 48.12 ± 4.23 s, 20.09 ± 4.03 s, 13.16 ± 4.21 s, and 10.03 ± 4.18 s on 0, 7, 14, 21, and 28th day respectively.

From the behavioural study it was observed that naïve control group showed improvement from 28th day but TBZ loaded SNEDDS in low as well as high dose was showed form 14th day and it might be because of the enhancement in solubility and

better absorption of TBZ loaded in SNEDDS formulation. SNEDDS GS was in nano range which made increased level of drug in brain.

5.25. Oxidative biomarkers

When dystonia inducer i.e. haloperidol injected to the rat it produces excess of free radicals which reduces the level of GSH, SOD, catalase level and elevate the level of MDA. The attack of free radicals on –SH (sulfhydryl) is the main reason for the reduction of antioxidant enzymes. The interconnection between per-oxidative products and enzymes can obstruct enzyme activation. Biochemical parameters was performed to measure the level of GSH, SOD, CAT, and MDA in the brain tissue. Numerous cellular defence mechanisms exit to amend the ROS generation in brain. These mechanism mend destructed oxidative cells and reduced the level of free radicals. These all mechanisms are interrelated to each other (Figure 56).

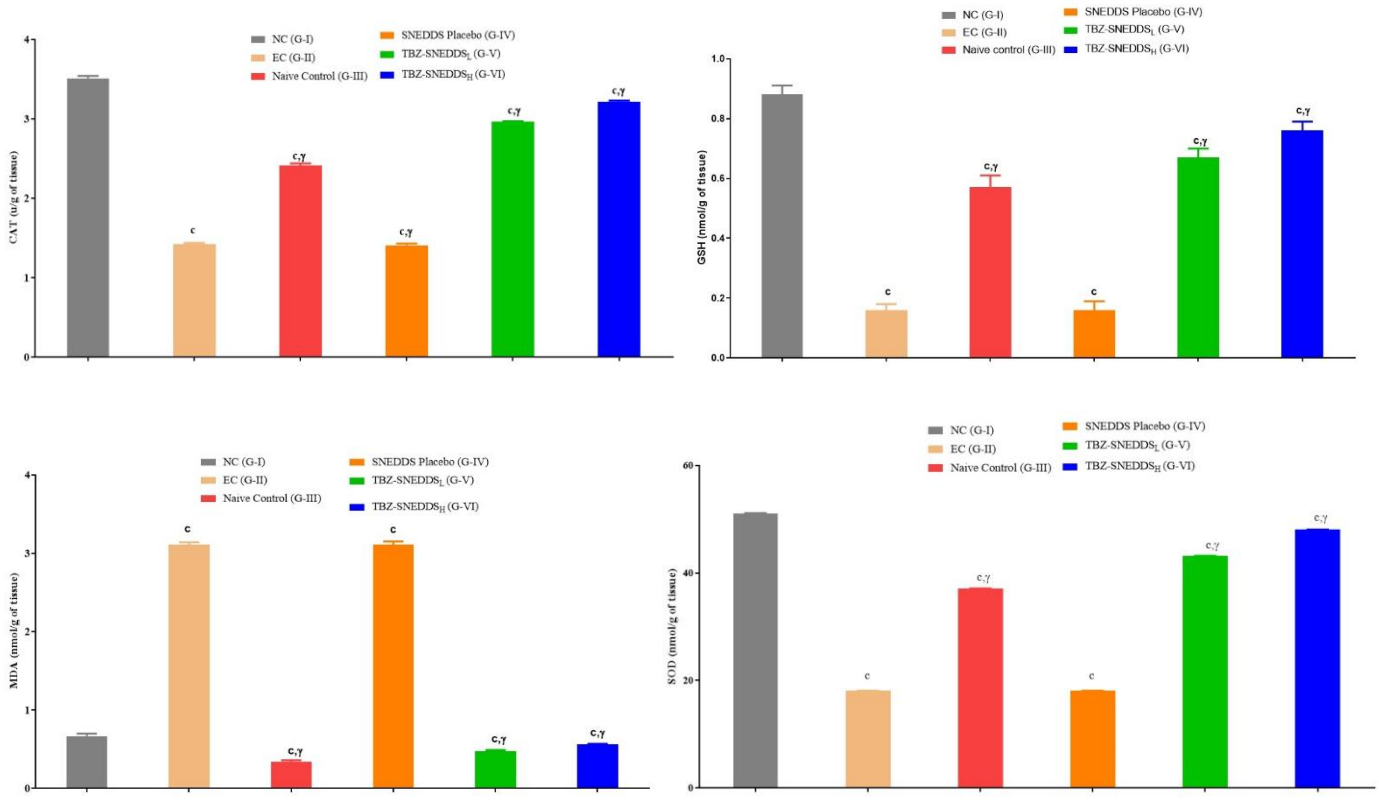


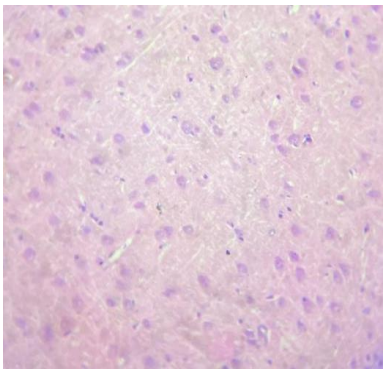
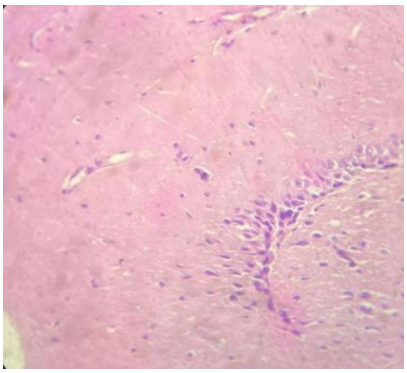
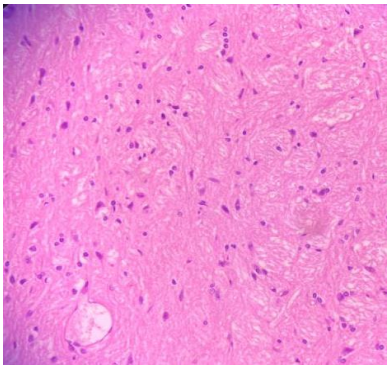
Figure 56: Variation in CAT, GSH, SOD, and MDA of rats in different group's in vivo studies. (Data are represented as mean ± SD (n=6) where α = $p < 0.05$, β = $p < 0.01$, γ = $p < 0.001$ compared to the Experimental Control Group (EC). A = $p < 0.05$, b = $p < 0.01$, c = $p < 0.001$ compared to the Normal Control Group)

*Results were reported as values of Mean ± standard error mean (SEM)

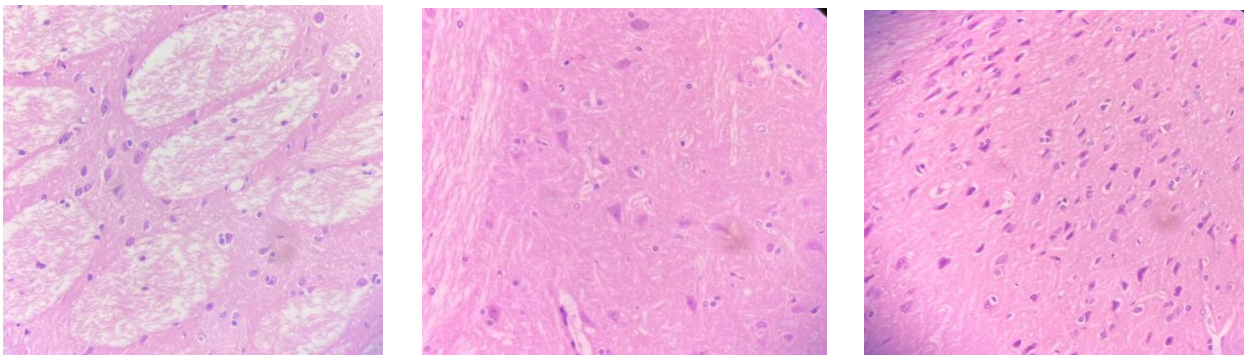
The effect of TBZ loaded SNEDDS on CAT, GSH, SOD, and MDA level was checked to indicate the effect on oxidative stress due to haloperidol. CAT, GSH, SOD, in EC (G-II), and SNEDDS placebo (G-IV) was very less when compared with NC (G-I) but level of MDA was high in both group because they didn't receive any treatment. The level of CAT, GSH, and SOD in naïve control (G-III), low dose of TBZ loaded SNEDDS (G-V), and high dose of TBZ loaded SNEDDS was found high but MDA level was found low. But it was very significant in the groups which were treated with TBZ loaded SNEDDS. Oxidative stress of rats of G-VI and V showed maximum protection i.e., in GSH 0.76 ± 0.03, 0.67 ± 0.03 n mol/g of tissue, in catalase 3.21 ± 0.02, 2.96 ± 0.01 µ/g, in MDA 0.56 ± 0.01, 0.48 ± 0.01 n mol/g of tissue and in SOD study 48.04 ± 0.03, 43.18 ± 0.03 n mol/g of tissue.

5.26. Histopathology study

Figure 57 showed photomicrographs (100x) of the brain slices of all groups. In normal control group rats' brains had characteristic normal architecture. In negative control group deteriorating tissue, and numerous pyknotic nuclei, fragmentation, and a large loss of neuronal cells. In naïve control group significantly reduction of neuronal loss, and fragmentation. It was discovered that the striatal tissue had pyknotic nuclei. Numerous pyknotic nuclei, fragmentation, and a considerable loss of neuronal cells in the deteriorating tissue were present in the placebo group. In SNEDDS with low dose less reduction in neuronal loss and fragmentation was observed. Pyknotic nuclei were seen in the striatal tissue. In SNEDDS with high dose prominent reduction of observed in the striatal tissue were neuronal loss, fragmentation, and the presence of pyknotic nuclei [310].

NC (G-I) **EC (G-II)** **Naïve control (GIII)**



SNEDDS-Placebo (G-IV) **TBZ-SNEDDS$_L$ (G-V)** **TBZ-SNEDDS$_H$ (G-VI)**

Figure 57: Photomicrographs of various groups of rats

Table 33: Inference of histopathology of brain

Groups	Treatment	Remarks
I	Normal control (NC)	Rats' brains had characteristic normal architecture
II	Disease Control (Disease induced through Haloperidol)	In the deteriorating tissue, there were numerous pyknotic nuclei, fragmentation, and a large loss of neuronal cells.
III	Naïve Control (Suspension of TBZ)	Significantly reduction of neuronal loss, and fragmentation. The presence of pyknotic nuclei in striatal tissue was observed
IV	SNEDDS Placebo	Many pyknotic nuclei, fragmentation, and significant loss of neuronal cells in the degenerating tissue
V	SNEDDS low dose	• Less reduction in neuronal loss and fragmentation was observed. • Presence of pyknotic nuclei in striatal tissue was observed. • The observation recorded here is lesser than the sample of Slide VI
VI	SNEDDS high dose	• Prominent reduction of the neuronal loss, fragmentation and presence of pyknotic nuclei in striatal tissue were observed. • The observation recorded here is lesser than the sample of Slide III

SUMMARY & CONCLUSION

6. SUMMARY AND CONCLUSION

Lipid-based formulations have received a lot of evidence based research with the focus on improvement in oral bioavailability as well as solubility of drugs belongs to BCS class II and IV category. As per the previous reports, a hydrophobic medication can be administered in the form of SNEDDS to make them sufficiently solubilized and can reduce the pharmaceutical road backs of the drugs. Lipid-based drug delivery system has potential to enhance solubility of hydrophobic drugs in the gastrointestinal tract. Different kinds of formulations can be prepared microemulsion, self-emulsifying drug delivery system (SEDDS), and emulsion. The presence of S_{mix} (surfactant and co-surfactant) and oil restrict the precipitation of drugs and bypass rapid first-pass metabolism. Drugs are absorbed through lymphatic circulation along with oil [311]. After this drug enters the systemic circulation and crosses the BBB because of the nano-sized particles. Additionally, SNEDDS promote supersaturation, surfactant-induced membrane fluidity, and an increase in permeability, which is frequently adequate for drug absorption, and protects the drugs against enzymatic degradation.

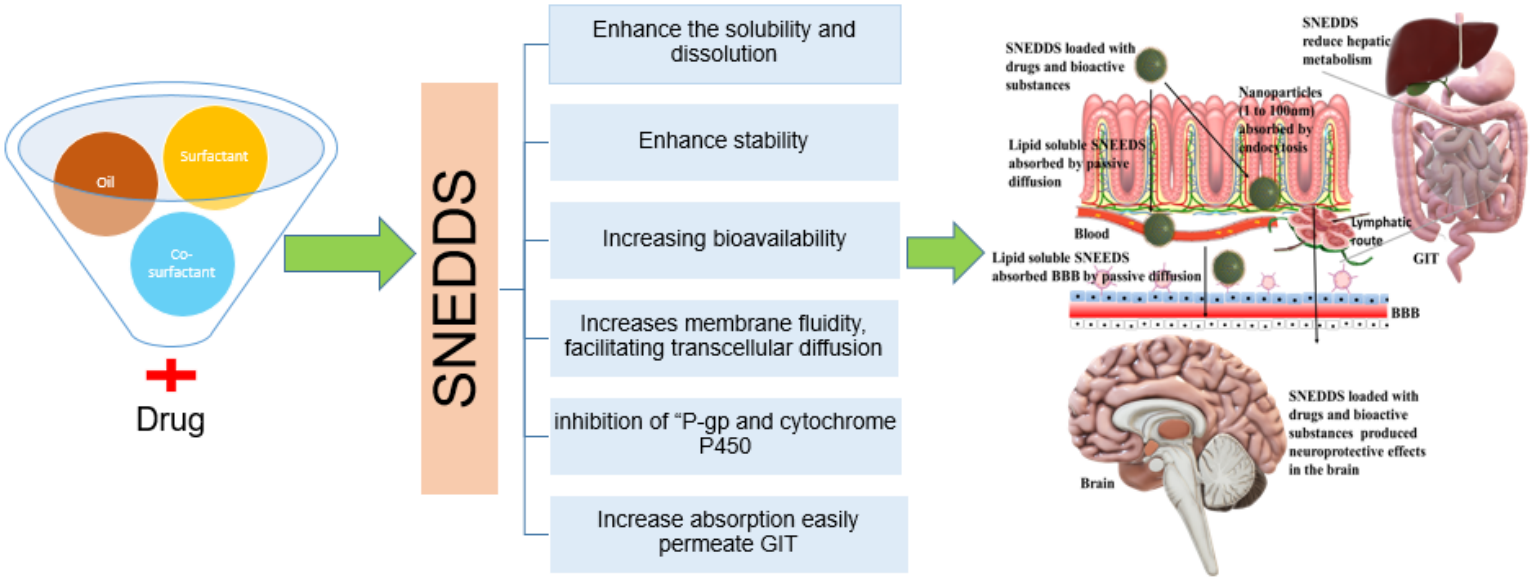


Figure 57: Flow chart of self-nano-emulsifying drug delivery system formulation through oral route to brain

The aim of the present study was to formulate and evaluate Tetrabenazine (TBZ) loaded SNEDDS for the treatment of Dystonia. Dystonia is a neurological disorder that affected 1% population worldwide. TBZ is the most effective drug for the treatment of hyperkinetic movement disorders. But it has so many pharmaceutical challenges such as low aqueous solubility, permeability, and bioavailability (BA) as it belongs to BCS class IV. It is degraded by cyto-P450 enzymes so the drug couldn't provide its effect. To overcome these problems TBZ loaded SNEDDS was designed because SNEDDS are giving promising effects for the enhancement of solubility and BA. After enhancing these parameters TBZ can cross the BBB and give maximum effect. From 1972-2004, three preclinical studies were reported in which 128 mg/day, 175 mg/day dose of TBZ was administered to 8, 8, and 41 patient but the outcomes was not satisfactory because 3 out of 8 (38%), 2 out of 8 (25%) and 20 out of 41 (49%) patients were treated [229–231]. The developed optimized formulation was administered to rats to check the effectiveness in haloperidol-induced dystonic rats.

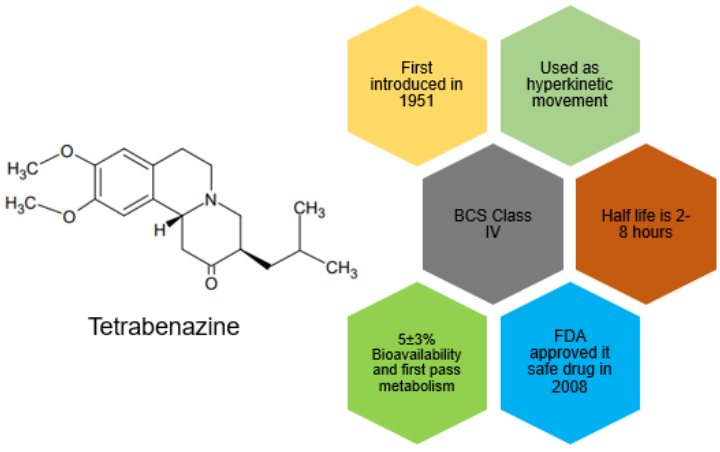


Figure 58: Pharmaceutical challenges of tetrabenazine

To achieve this aim, TBZ was characterized for purity such as FTIR, XRD, DSC, and melting point. An analytical RP-HPLC method was developed which was helpful in checking the solubility, drug loading, in-vitro release, and pharmacokinetic study. The method was validated as per ICHQ_2 (R_1) guidelines such as accuracy, linearity, precision, and robustness study. So this method was used in the TBZ-loaded SNEDDS.

For the development of TBZ loaded SNEDDS, solubility study was performed in various surfactants, co-surfactants, and oils and tween 20, transcutol© P, and capryol PGMC were selected respectively as the solubility of TBZ was maximum in these excipients. Twenty seven prototypes formulations were developed by combination of capryol PGMC and combination of Tween 20 and Transcutol© P as S_{mix} in the ratio of 1:9 to 9:1, whereas internal ratio of S_{mix} was varied from 1:1, 2:1, and 1:2 and inferred as SNEDDS, SMEDDS (self-micro-emulsifying drug delivery system), and coarse emulsion. Thermodynamic stability study, and effect of pH on dilution was performed of selected 5 formulations. Pseudo-ternary phase was plotted using triplot 4.1.2 software.

Thermodynamic stability study were performed in terms of permanent phase separation, cracking, coalescence, and creaming. The optical clarity of the formulation is determined by the percentage transmittance of the formulation, since clear dispersion will scatter incident light less than opalescent dispersion [259].

BBD was used to optimize the formulation using 3 independent variables and 5 dependent variables. Optimized formulation of TBZ loaded SNEDDS showed GS 142.6 ± 0.98 nm, PDI 0.136 ± 0.01, ZP -19.6 ± 0.23 mV, SEF 10.46 ± 0.67, and DL 72.42 ± 0.53%.

TEM exhibit spherical shape and smooth surface of SNEDDS. SHSY-5Y cell line study provide cell viability in the range of 65-80%. Accelerated stability study was performed at 40 °C ± 2 °C/ 75 % RH ± 5 % RH for 6 months showed significant stability. In-vitro release study showed increase in dissolution rate of from SNEDDS was about 8.51 folds in pH 6.8 phosphate buffer as compared to its marketed form. As the value of n for SNEDDS was 1.2454, so it followed super case II transport which means the drug was released by fickian diffusion and relaxation of the drug delivery.

Bio-analytical method was developed and validated as per ICH M10 guidelines, and benzoquinolizine was considered as an internal standard (IS).

Sprague Dawley rats were used to perform pharmacokinetic and pharmacodynamics studies. Dystonia was induced by haloperidol in the dose of (2 mg/Kg) (i.p.) for 28

days and 1 hour before giving the treatment. Behavioural activity such as locomotor, rotarod, akinesia, and catalepsy were performed on 0, 7, 14, 21, and 28th day of the study.

Pharmacokinetic study showed the amount of drug present in plasma as well as in the brain. Plasma as well as brain samples were taken at 30, 60, 120, 240, and 1440 min after treatment. Maximum concentration of TBZ-loaded SNEDDS in plasma as well as in the brain at 1 h showed 156.68 ± 6.03 and 61.08 ± 4.03 ng/ml respectively. But naïve drug showed maximum concentration at 4 h which was 29.22 ± 6.29 and 3.27 ± 0.28 ng/mL in plasma and brain respectively. Similar observation was observed in histopathological study were maximum protection was recorded in brain of G-V and G-VI. Oxidative stress were evaluated and here rats of G-VI and V showed maximum protection i.e., in GSH 0.76 ± 0.03, 0.67 ± 0.03 n mol/g of tissue, in catalase 3.21 ± 0.02, 2.96 ± 0.01 µ/g, in MDA 0.56 ± 0.01, 0.48 ± 0.01 n mol/g of tissue and in SOD study 48.04 ± 0.03, 43.18 ± 0.03 n mol/g of tissue.

Finally the efficacy of drug treatment follow the following order

$TBZ\text{-}SNEDDS_H$ > $TBZ\text{-}SNEDDS_L$ > Naïve TBZ

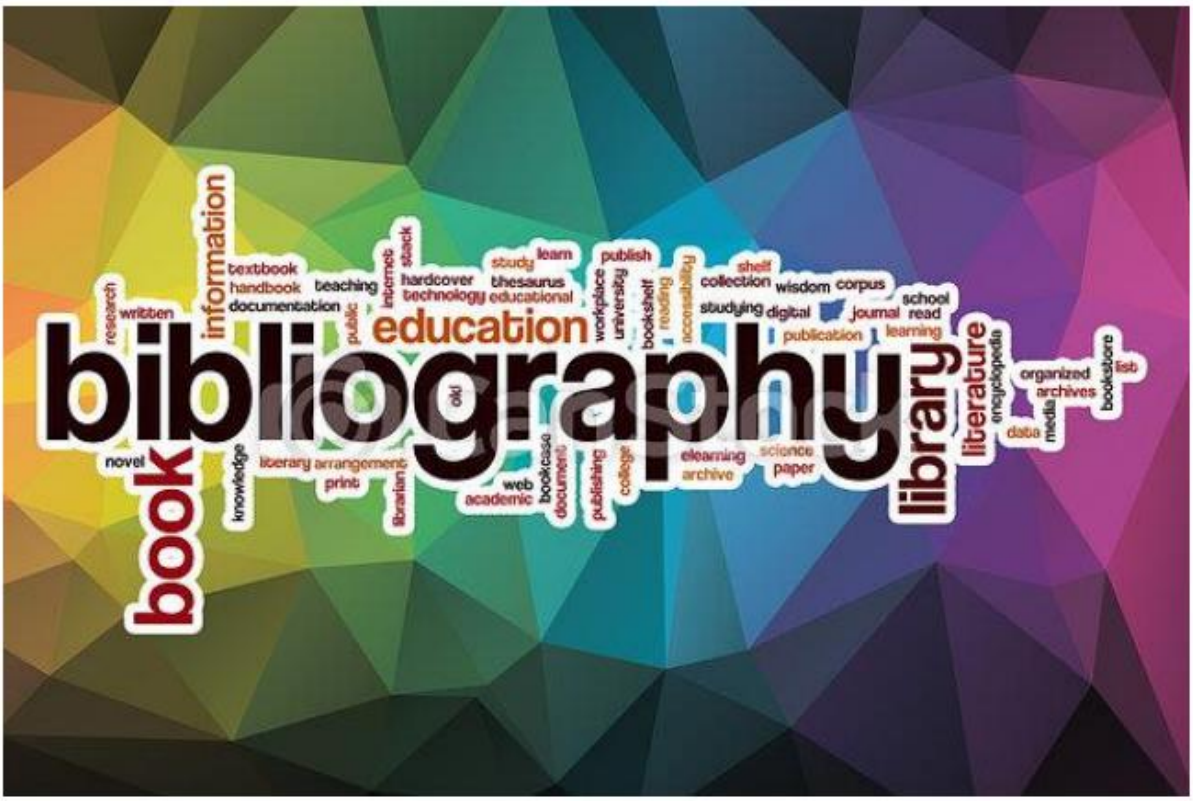
The efficacy in duration as the treatment was recorded in the same order as given above.

Consequently all the excipients used were easily available in safe concentrations. The surfactant that was used in formulation is non-ionic so it is less irritant and non-toxic in nature. SNEDDS can be easily prepared in large scale using blenders available in industries. The formulation of TBZ-loaded SNEDDS was successfully formulated, developed and characterized in vitro as well as in vivo for the treatment of dystonia.

FUTURE PROSPECTIVE

Overall, the study showed successful effective development of a SNEDDS formulation containing TBZ in the form of nanomedicine for the treatment of dystonia. The formulation was developed using readily available excipients in a safe concentration, ensuring long-term use without any significant negative effects. Additionally, the SNEDDS preconcentrate can be produced at industrial scale and on a wider scale using readily available blenders. “The research has also given a future direction to the scientists working in i.e., field of modification of synthetic drug development to explore the role of selected excipients for the formulation of novel drug delivery systems”. However, before the translation of a nano-formulation from laboratory to commercial scale, it is important to look into various aspects such as market demand, scalability and potential clinical toxicity related to the formulation. The demand is completely based on severity and prevalence of disease and safety of formulation during their long-term use. The prevalence rate of dystonia is increasing day by day and its one of the major traumatic situation in which person feels disable. Sincere efforts are required to avoid the prolong side effects of prepared formulations.

Here, the proposed composition provides a composition of TBZ with capryol PGMC, tween 20, and Transcutol© P as oil, surfactant, and co-surfactant. This is very interesting that TBZ is already available in market for the treatment of dystonia but it has got several pharmaceutical challenges which renders its utilization. These challenges are low aqueous solubility, permeability, enzymatic degradation. Based on the performed study TBZ have shown good results at its both doses i.e. 5 and 10 mg/kg in rats.



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Annexures

ANNEXURES

Annexure 1: Candidacy letter of Ph.D.

Annexure 2: List of publications, patents, awards, certificates

Annexure 3: Certificate of analysis of TBZ

LOVELY PROFESSIONAL UNIVERSITY

Centre for
Research Degree Programmes

LPU/CRDP/PHD/EC/20210322/000588

Dated: 01 Dec 2020

Shashi
Registration Number: 11919680
Programme Name: Doctor of Philosophy (Pharmaceutics)

Subject: Letter of Candidacy for Ph.D.

Dear Candidate,

We are very pleased to inform you that the Department Doctoral Board has approved your candidacy for the Ph.D. Programme on 01 Dec 2020 by accepting your research proposal entitled: "Formulation and evaluation of self-nano emulsifying drug delivery system loaded with Tetrabenazine for the treatment of Dystonia"

As a Ph.D. candidate you are required to abide by the conditions, rules and regulations laid down for Ph.D. Programme of the University, and amendments, if any, made from time to time.

We wish you the very best!!

In case you have any query related to your programme, please contact Centre of Research Degree Programmes.

Head

Centre for Research Degree Programmes

Section A-Research paper

Bioanalytical reverse phase high performance liquid chromatography method development and validation for the estimation of Tetrabenazine in rat plasma

Shashi[1], Yadav Sarvi Rajesh[1], Bala Vikash R[1], Kusman Sekhar Behra[1], Narendra Kumar Pandey[1], Bimlesh Kumar[1], Sachin Kumar Singh[1], Dileep Singh Baghel[1], Ritu Gupta[2], Saurabh Singh[1], Kalvatala Sudhakar[1]

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[2] Charles River Labs, Mattawan, Michigan, USA.

Corresponding author: Dr. Narendra Kumar Pandey, School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India-144411 herenarendra4u@gmail.com; Tel. +91-9888749238

Abstract

Tetrabenazine (TBZ) in rat plasma can be estimated using a reverse-phase high-performance liquid chromatography (RP-HPLC) approach employing benzoquinolizine as an internal reference. ICH M10 criteria were used to validate the created approach. Acetonitrile (ACN) and 0.1% formic acid were used in a ratio of 90:10 v/v for the separation, with a flow rate of 1 mL/min and a detection wavelength of 283 nm. Benzoquinolizine and TBZ had retention times (RT) of 3.665 and 5.064 minutes, respectively. The developed method's regression coefficient (r2) of 0.996 revealed that it is linear in the 50–250 ng/mL range. Indicating their accuracy and precision, the TBZ recovery (%) was found to be over 92% and their relative standard deviation (%) was determined to be less than 2%. The plasma concentration of TBZ had a limit of detection (LOD) and limit of quantitation (LOQ) of 18.19 ng/mL and 55.14 ng/mL, respectively. To ensure the stability of the medication in plasma,

The Epidemic of Monkeypox Virus- A Review

Bala Vikash[1], Shashi[1], Narendra Kumar Pandey[1]*, Bimlesh Kumar[1], Sachin Kumar Singh[1], Sarvi Yadav Rajesh[1], Nilopher Chhotaray[1]

[1]School of Pharmaceutical Sciences, Lovely Professional University, Punjab-144411, India

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Lovely Professional University, Punjab-144 411, India
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Abstract

A multi-country outbreak of monkeypox has been emerging as an epidemic in various parts of the globe. The source of the monkeypox virus outbreak, the pattern of viral attack, and the impact of the disease on humans remain unclear. The laboratory goal of the scientists is to enable accurate, precise, and cheap methods that enable confirmation of the infection, break the transmission chain, and stop the outbreak of the monkeypox virus attack. Economic burdens had been imposed on developing countries due to their fragile healthcare system. This review aims to provide an outline of the ongoing epidemic of monkeypox and show light on the same by the scientific knowledge gathered to date about the disease, its global prevalence, and the methods of preventing further transmission.

Keywords

Epidemic, monkeypox virus, global prevalence, vaccine.

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journal homepage: www.elsevier.com/locate/jddst

Formulation and evaluation of ocular self-nanoemulsifying drug delivery system of brimonidine tartrate

Bala Vikash [a], Shashi [a], Narendra Kumar Pandey [a,*], Bimlesh Kumar [a], Sheetu Wadhwa [a], Umesh Goutam [b], Aftab Alam [c], Faisal Al-Otaibi [d], Pramila Chaubey [e], Gulam Mustafa [e], Gaurav Gupta [f,g,h], Kamal Dua [i,j], Sachin Kumar Singh [a,j]

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[g] Department of Pharmacology, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India
[h] Uttaranchal Institute of Pharmaceutical Sciences, Uttaranchal University, Dehradun, India
[i] Discipline of Pharmacy, Graduate School of Health, University of Technology Sydney, Ultimo, NSW, 2007, Australia
[j] Faculty of Health, Australian Research Centre in Complementary and Integrative Medicine, University of Technology Sydney, Ultimo, NSW, 2007, Australia

ARTICLE INFO

Keywords:
Glaucoma
SNEDDS
Box-Behnken
Transcorneal permeability
Ex-vivo toxicity

ABSTRACT

The aim of the current study was to develop an ocular Self-nanoemulsifying drug delivery system (SNEDDS) loaded with Brimonidine tartrate (BRT) and evaluate its ex-vivo permeability. SNEDDS loaded with BRT were prepared by low energy method. Physicochemical properties, drug release, ex-vivo permeation, and toxicity studies were investigated. The BRT-loaded SNEDDS formulation was composed of Capmul MCM (Oil), Tween 80 (Surfactant), and Transcutol HP (Co-surfactant) and was optimized by Box-Behnken design. Optimized SNEDDS loaded with BRT had a droplet size of 118.3 nm, a PDI of 0.251, a pH of 6.34 ± 0.31, an osmolarity of 329 mosm/L after reconstitution with NaCl, and a viscosity was found to be 12.39 ± 2.31 cP. The optimized SNEDDS loaded with BRT had excellent stability in the shelving period. The apparent permeability coefficient of optimized SNEDDS was found to be ($7.078 \pm 0.218 \times 10^{-6}$ cm/s) when compared to the marketed formulation with ($3.127 \pm 0.173 \times 10^{-6}$ cm/s). These results suggest that Brimonidine tartrate-loaded SNEDDS can be used as a promising nanocarrier for ocular drug delivery.

Office of the Controller General of Patents, Designs & Trade Marks
Department of Industrial Policy & Promotion,
Ministry of Commerce & Industry,
Government of India

(http://ipindia.nic.in/index.htm)

(http://ipindia.nic.in/index.htm)

Application Details	
APPLICATION NUMBER	202211073089
APPLICATION TYPE	ORDINARY APPLICATION
DATE OF FILING	16/12/2022
APPLICANT NAME	**LOVELY PROFESSIONAL UNIVERSITY**
TITLE OF INVENTION	SNEDDS BASED TETRABENAZINE FORMULATION FOR THE TREATMENT OF DYSTONIA
FIELD OF INVENTION	CHEMICAL
E-MAIL (As Per Record)	director@bansalip.com
ADDITIONAL-EMAIL (As Per Record)	director@bansalip.com
E-MAIL (UPDATED Online)	
PRIORITY DATE	
REQUEST FOR EXAMINATION DATE	--
PUBLICATION DATE (U/S 11A)	06/01/2023

Application Status	
APPLICATION STATUS	**Awaiting Request for Examination**

Office of the Controller General of Patents, Designs & Trade Marks
Department of Industrial Policy & Promotion,
Ministry of Commerce & Industry,
Government of India

INTELLECTUAL PROPERTY INDIA
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GEOGRAPHICAL INDICATIONS

Application Details	
APPLICATION NUMBER	202211032676
APPLICATION TYPE	ORDINARY APPLICATION
DATE OF FILING	08/06/2022
APPLICANT NAME	**Lovely Professional University,**
TITLE OF INVENTION	A NOVEL OCULAR ANTI-GLAUCOMA FORMULATION AND PROCESS THEREOF
FIELD OF INVENTION	CHEMICAL
E-MAIL (As Per Record)	dip@lpu.co.in
ADDITIONAL-EMAIL (As Per Record)	dip@lpu.co.in
E-MAIL (UPDATED Online)	
PRIORITY DATE	
REQUEST FOR EXAMINATION DATE	--
PUBLICATION DATE (U/S 11A)	17/06/2022

Application Status	
APPLICATION STATUS	**Awaiting Request for Examination**

View Documents

3ICP2022LOC-28

Serial No. _____

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Certificate of Appreciation

This is to certify that Prof./Dr./Mr./Ms. SHASHI has participated as Member LOC/ ~~Student Volunteer~~ in the 3rd International Conference of Pharmacy (ICP-2022) on the **Theme of "Practice, Promotion & Publication of Innovation : A Way of Transforming Health"** held on 09th & 10th **November 2022** organized by School of Pharmaceutical Sciences in a collaboration with Indian Pharmaceutical Association (IPA) at Lovely Professional University, Punjab.

Mr. Suresh Khanna
National Hon. Gen. Secretary, IPA

Dr. Bimlesh Kumar
Organizing Secretary

Dr. T.V. Narayana
National President IPA

Dr. Monica Gulati
LOC Chairperson

Sri Herbasia Biotech

Tishk International University, Iraq

Serial No. 31CP20221043

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सत्यमेव जयते
IPC

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. SHASHI has successfully participated as Delegate & Presented Poster/ Oral Presentation on Application of Developed RP-HPLC method of Tetrabenzine for characterization of Self-nano emulsifying drug delivery system in the 3rd International Conference of Pharmacy (ICP-2022) on the Theme of "Practice, Promotion & Publication of Innovation : A Way of Transforming Health" held on 09th & 10th November 2022 organized by School of Pharmaceutical Sciences in a collaboration with Indian Pharmaceutical Association (IPA) at Lovely Professional University, Punjab.

Mr. Suresh Khanna	Dr. Bimlesh Kumar	Dr. T.V. Narayana	Dr. Monica Gulati
National Hon. Gen. Secretary, IPA	Organizing Secretary	National President IPA	LOC Chairperson

Sri Herbasia Biotech

Tishk International University, Iraq

ISFCON- 2021 Online International Conference on Recent Advances in Pharmaceutical Sciences: Vision 2030

July 01 - 03, 2021.

In Collaboration with

Punjab State Council for Science & Technology, Chandigarh

Organized by

ISF COLLEGE OF PHARMACY, MOGA

(An Autonomous College)

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms.

SHASHI

.....

has participated as **Delegate**

in

ISFCON- 2021 Online International Conference

Organizing Secretary	Organizing Secretary	Finance Secretary	Convener	Patron
Dr. Vineet K. Rai	Dr. Pooja Chawla	Prof. (Dr.) R. K. Narang	Prof. (Dr.) G. D. Gupta	Parveen Garg

Registration Number: ISFCON2021/097.....

HUMAN RESOURCE DEVELOPMENT CENTER

[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T. Road, Phagwara (Punjab)]

Certificate No. 241676

Certificate of Participation

This is to certify that **Ms. Shashi** D/o Sh. Surender Singh participated in **Training on The Care and Use of Laboratory Animals in Accordance with Ethical Guidelines** organized by Lovely Professional University w.e.f. **March 11, 2022** to **March 12, 2022** and obtained "O" Grade.

Deputy Dean
Human Resource Development Center

Professor and Dean
Human Resource Development Center

GITAM
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NATIONAL WEBINAR

CERTIFICATE

This is to certify that Shashi of Lovely professional University
has participated in National Webinar
on
TARGETING CANCER STEM CELLS FOR THERAPY
on 30 July 2021.

Prof. G Shiva Kumar	Dr. S. Sunitha	Prof. Ganapaty S.
Chairperson	Convener	Chairperson

GITAM School of Pharmacy, Hyderabad

ISFCON- 2021 Online International Conference on Recent Advances in Pharmaceutical Sciences: Vision 2030

July 01 - 03, 2021.

In Collaboration with

Punjab State Council for Science & Technology, Chandigarh

Organized by

ISF COLLEGE OF PHARMACY, MOGA

(An Autonomous College)

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms.

SHASHI

has participated as **Delegate** and presented an **e-poster**

in

ISFCON- 2021 Online International Conference

Organizing Secretary	Organizing Secretary	Finance Secretary	Convener	Patron
Dr. Vineet K. Rai	Dr. Pooja Chawla	Prof. (Dr.) R. K. Narang	Prof. (Dr.) G. D. Gupta	Parveen Garg

Registration Number: ISFCON2021/PHA008

FACULTY OF APPLIED MEDICAL SCIENCES

[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T. Road, Phagwara (Punjab)]

Certificate No. 235864

Certificate of Participation

This is to certify that Mr./Ms. **Shashi** S/O,D/O,W/O **Mr. Surender Singh**

student of **School of Pharmaceutical Sciences** Registration No. **11919680**

pursuing **PhD** participated in the **National Pharmacovigilance Week-2021**

on the theme of **"Pharamacovigilance: A step towards patient safety"** held from **17-09-2021** to **23-09-2021**

organized by **Adverse Drug Reaction Monitoring Centre (AMC) and Medical Device Adverse Event Monitoring Centre (MDMC)**

in association with School of Pharmaceutical Sciences at Lovely Professional University, Punjab.

Date of Issue : 12-11-2021
Place of Issue: Phagwara (India)

Prepared by
(Administrative Officer-Records)

Dr. Bimlesh Kumar
Organizing Secretary

Prof. (Dr.) Monica Gulati
Executive Dean
LFAMS

GLA UNIVERSITY MATHURA

Accredited with A Grade by NAAC

12-B Status from UGC

Institute of Pharmaceutical Research
GLA University, Mathura

ICBPS-2021

Certificate

This is to certify that **Ms. Shashi** from Lovely Professional University, Phagwara, Punjab, Presented Poster on **Method Development and Validation for Estimation of Tetrabenazine by Using Reverse Phase High Performance Liquid Chromatography, Secured 2nd Position** in 1st International Conference on **"Innovations in Chemical, Biological and Pharmaceutical Sciences (ICBPS-2021)"** held on November 26-27, 2021 through online mode.

Dr Ahsas Goyal	Dr Yogesh Murti	Dr Anuj Garg	Dr Prabhat K Upadhyay	Prof. Meenakshi Bajpai
(Co-Convener)	(Co-Convener)	(Convener)	(Convener)	(Chairperson)

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Certificate No.253341

Certificate of Presentation

This is to certify that **Dr./Mr./Ms. Shashi** of **School of Pharmaceutical Sciences, Lovely Professional University- Phagwara** has presented a paper on **Famotidine Loaded Microspheres Entrapped Into Floatable In-Situ Gel With Extracted Natural Gum** in the **"3rd International Conference on Functional Materials, Manufacturing and Performances (ICFMMP-2022)"** held on July29-30th, 2022, organized by **Division of Research and Development, Lovely Professional University, Punjab**.

Date of Issue: 30-08-2022
Place: Phagwara (Punjab), India

Prepared by (Administrative Officer-Records)	Dr. Hitesh Vasudev Convenor	Dr. Pranav Kumar Prabhakar Organizing Secretary	Dr. Chander Prakash Conference Secretory

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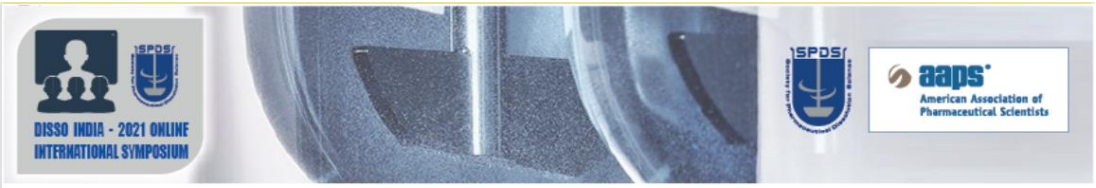

CERTIFICATE

OF PARTICIPATION

PRESENTED TO

Shashi

for attending **10th International Conference DISSO india 2021 - Online**
organised by **SPDS** in collaboration with **AAPS**
during 24th, 25th & 26th June 2021

Vijay Kshirsagar
President, SPDS India

Arvind K Bansal
Scientific Chair - SPDS India

Andrew M. Vick
President, AAPS

CERTIFICATE

Anton Paar

Shashi

Lovely Professional University, Punjab

attended the webinar on

Importance of Particle Characterization and Zeta Potential in Pharmaceutical

Date: July 22, 2021

Time: 11:30 to 12:30 hours

Contents

The training provided the essential knowledge for:

- Role of particle size in pharmaceutical development
- Discussing the impact of particle size in different diseases
- Brief about the basics of Dynamic Light Scattering
- Outlining the ideal Light Scattering Instrument

Date of issue: July 23, 2021

Dr. Rishi Gupta
Application Specialist - Characterization Division
Anton Paar India Pvt. Ltd.

CERTIFICATE

Anton Paar

Shashi
Lovely Professional University, Punjab

attended the webinar on

Basics of Rheology for Pharmaceutical Applications

Date: June 11, 2021
Time: 11:30 to 12:30 hours

Contents

The training provided the essential knowledge for:

- What is Rheology
- Rheometer and Measuring Geometries
- Rotation Tests
- Oscillation Tests
- Powder Rheology

Date of issue: June 15, 2021

Mr. Mayank Varshney
Application Specialist
Characterisation Division
Anton Paar India Pvt. Ltd.