UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF PARENTERAL NANOEMULSION SYSTEMS LOADED WITH CARBAMAZEPINE FOR EFFICIENT BLOOD-BRAIN BARRIER CROSSING IN EPILEPSY TREATMENT

TAN SIM LING

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By

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Thesis Submitted to the School Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

DEVELOPMENT OF PARENTERAL NANOEMULSION SYSTEMS LOADED WITH CARBAMAZEPINE FOR EFFICIENT BLOOD-BRAIN BARRIER CROSSING IN EPILEPSY TREATMENT

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August 2016

Chairman : Hamidon Bin Basri, PhD
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Epilepsy is a neurological disorder characterised by epileptic seizures. Antiepileptic drug is commonly used to reduce the frequency and severity of this disease. Carbamazepine is an effective antiepileptic drug, however, it is limited by the side effects. In addition to the aforementioned problem, carbamazepine delivery to the brain is also, impeded by a biological barrier, the blood-brain barrier. The unique transport-barrier property of the blood-brain barrier further reduces the bioavailability of carbamazepine to the brain. Last but not least, carbamazepine is only available in oral form. To date, parenteral formulation of carbamazepine in not available. In the present study, carbamazepine-loaded, brain targeting parenteral nanoemulsions were developed to overcome these shortcomings. Polyunsaturated fatty acids-rich plant oils such as safflower seed oil, pine nut oil and oleic acid were used in nanoemulsion formulations. Optimisation, characterisation and stability evaluation were carried out to ensure these nanoemulsions meet the requirements of parenteral formulations. The particle size of these nanoemulsions were 119.7 ± 0.90 and 113.97 ± 0.72 nm respectively, the zeta potential were -60.50 ± 1.7 and -58.33 ± 0.58 mV respectively and the polydispersity index were 0.20 ± 0.01 and 0.21 ± 0.01 respectively. Carbamazepine-loaded safflower seed oil nanoemulsion and carbamazepine-loaded pine nut oil nanoemulsion were developed in this experiment and they were subjected to *in vitro* and *in vivo* studies. *In vitro* blood-brain barrier model was developed to determine the penetration efficiency of formulated nanoemulsions. Immortalised cerebral brain endothelial cell lines, hCMEC/D3 and astrocytes (CC-2565) were used to develop co-cultivation *in vitro* BBB model. Optimisation and characterisation were carried out to ensure the validity of this model. The *in vitro* study showed that these formulated nanoemulsions possessed higher apparent permeability (0.03 ± 0.01 and 0.05 ± 0.01 cm/h) when compared to carbamazepine solution (0.02 ± 0.001 cm/h). These formulations were also intraperitoneally injected in rats. *In vivo* pharmacokinetic profiles were generated in this experiment. According to the *in vivo* study, these formulated nanoemulsions successfully enhanced the plasma (11.20 ± 0.10 and 13.20 ± 0.30 vs. 10.20 ± 0.10 µg/mL) and brain concentrations of carbamazepine (4.10 ± 0.20 and 7.30 ± 0.30 vs.

2.50 ± 0.30 µg/g) when compared to carbamazepine solution. According to the results obtained, drug-loaded nanoemulsions could be an effective carrier for drug transport into the brain. In summary, carbamazepine-loaded, parenteral nanoemulsions have successfully developed and they can markedly increase the level of carbamazepine after intraperitoneal administration in both plasma and brain.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN SISTEM EMULSI PARENTERON TERMUAT KARBAMAZEPINA UNTUK CEKAP PENEMBUSAN HALANGAN OTAK DARAH UNTUK RAWATAN EPILEPSI

Oleh

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Epilepsi merupakan gangguan neurologi dicirikan oleh serangan epilepsi yang tiba-tiba. Ubat anti-epileptik biasanya digunakan untuk mengurangkan kekerapan dan keseriusan penyakit ini. Karbamazepina merupakan ubat anti-epileptik yang berkesan, bagaimanapun penggunaannya agak terhad kerana kesan sampingan dialami apabila menggunakannya. Selain masalah yang dinyatakan di atas, penghantaran karbamazepina ke otak juga terhalang oleh halangan biologi iaitu halangan darah otak. Ciri halangan pengangkutan unik bagi halangan darah otak menurunkan lagi bioketersediaan karbamazepina ke otak. Setakat ini karbamazepina boleh didapati di pasaran hanya dalam bentuk untuk dimakan. Formulasi parenteron karbamazepina di pasaran pula masih lagi belum wujud.Dalam kajian ini, nanoemulsi parenteron menyasarkan otak termuat karbamazepina dihasilkan untuk mengatasi kekurangan ini. Minyak tumbuhan kaya asid lemak politaktepu seperti minyak biji kesumba, minyak kacang pain dan asid oleik digunakan dalam penghasilan formulasi nanoemulsi. Pengoptimuman, pencirian dan penilaian kestabilan dijalankan bagi memastikan nanoemulsi ini mematuhi keperluan formulasi parenteron. Size nanoemulsi yang dihasilkan dalam eksperimen ini adalah 119.7 ± 0.90 and 113.97 ± 0.72 nm, potensi zeta adalah -60.50 ± 1.7 and -58.33 ± 0.58 mV dan indeks polydispersity adalah 0.20 ± 0.01 and 0.21 ± 0.01. Nanoemulsi minyak biji kesumba termuat karbamazepina dan nanoemulsi minyak kacang pain termuat karbamazepina berjaya dihasilkan dalam eksperimen ini dan kajian *in vitro* dan *in vivo* dijalankan bagi kedua-dua sampel tersebut. Model *in vitro* halangan otak darah dihasilkan bagi menentukan keberkesanan penembusan nanoemulsi yang diformulasi. Garis sel endotelial otak serebrum terabadi, hcMEC/D3 dan astrosit (CC-2565) digunakan bagi membangunkan penanaman bersama (co-cultivation) model BBB *in vitro*. Kajian *in vitro* menunjukkan yang kedua-dua nanoemulsi ini mempunyai kebolehtelapan yang jelas lebih tinggi (0.03 ± 0.01 and 0.05 ± 0.01 cm/h) bila dibandingkan dengan larutan karbamazepina (0.02 ± 0.001 cm/h). Formulasi ini juga disuntik secara intraperitoneum kepada tikus. Profil farmakokinetik *in vivo* dijana melalui eksperimen ini. Daripada kajian *in vivo* didapati, nanoemulsi terfomulasi ini berjaya meningkatkan kepekatan karbamazepina dalam plasma

(11.20 ± 0.10 and 13.20 ± 0.30 vs. 10.20 ± 0.10 µg/mL) dan otak (4.10 ± 0.20 and 7.30 ± 0.30 vs. 2.50 ± 0.30 µg/g) bila dibandingkan dengan larutan karbamazepina. Hasil keputusan ini menunjukkan nanoemulsi termuat ubat dapat dijadikan sebagai pembawa berkesan bagi pengangkutan ubat ke dalam otak.Sebagai ringkasan, nanoemulsi parenteron termuat karbamazepina berjaya dihasilkan dan ia dengan ketaranya dapat meningkatkan tahap karbamazepina selepas pemeriksaan intraperitoneum bagi plasma dan otak.

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I certify that a Thesis Examination Committee has met on (the date of viva voce) to conduct the final examination of Tan Sim Ling on her thesis entitled "Development of Parenteral Nanoemulsion Systems Loaded with Carbamazepine for Efficient Blood-Brain Barrier Crossing in Epilepsy Treatment" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABC	ATP binding cassette
AED(s)	Antiepileptic drug(s)
ADME	Absorption, distribution, metabolism, elimination
AGM	Astrocyte growth medium
AME	Adsorptive-mediated endocytosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANG1	angiopoetin 1
AUC	Area under curve
AUMC	Area under the moment concentration-time curve
BBB	Blood-brain barrier
BCRP(s)	Breast cancer resistance protein(s)
bFGF	Basic fibroblast growth factor
BM	Basement membrane
cAMP	Cyclic adenosine mono-phosphate
CBECs	Cerebral brain endothelial cells
CBZ	Carbamazepine
CBZ-NE(s)	Carbamazepine-loaded nanoemulsion(s)
CL	Clearance
C_{max}	Maximum concentration
CNS	Central nervous system
CPPs	Cell-penetrating peptides
CPT-cAMP	8-(4-Chlorophenylthio)-adenosine-3',5'-cyclic monophosphates
CYP3A4	Cytochrome P450 3A4
DEX	Dexamethasone
DHA	Docosahexaenoic acid
DLS	Dynamic light scattering
EBM-2	Endothelial basal medium-2
ECM	Extracellular matrix
ECs	Endothelial cells
EEG	Electroencephalogram
EGM-2	Endothelial growth medium-2
E-PUFAs	Esterified polyunsaturated fatty acids

GABA	G-aminobutyric acid
GAT1	G-aminobutyric acid transporter type 1
GDNF	Glial-derived neurotrophic factor
GPI	Glycerophosphoinositol(L-a-Phosphatidyl-D-myo-inositol-3-monophosphate)
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee
IM	Intramuscular
IP	Intraperitoneal
IV	Intravenous
JAM	Junctional-adhesion molecule A
K_{el}	Elimination rate constant
K_p	brain-to-plasma partition coefficient
LCTs	Long chain triglycerides (LCTs)
LDL	Low density lipoprotein
LOQ	Limit of quantitation
MCTs	Medium chain triglycerides
MRPs	Multidrug resistance proteins
MRP-1	Multidrug resistance protein-1
MPS	Mononuclear phagocytic system
MRT	Mean residence time
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide
NE(s)	Nanoemulsion(s)
NE-PUFAs	Non-esterified polyunsaturated fatty acids
NMDA	*N*-methyl-D-aspartate
O/W	Oil-in-water
P_{app}	Apparent permeability
P-gp	P-glycoprotein
PDA	Photodiode array
PDI	Polydispersity index
PECAM-1	Platelet endothelial cell adhesion molecule
PET	Polyester
PHS	Plasma-derived setum
PIT	Phase inversion temperature

PNE	Carbamazepine-loaded pine nut oil nanoemulsion
pNP	p-nitrophenol
pNPP	p-nitrophenyl phosphate
R^2	Correlation coefficient
RES	Reticulo-endothelial system
RIPA	Radioimmunoprecipitation
RME	Receptor-mediated endocytosis
RO	Phosphodiesterase inhibitor
SD	Standard deviation
SLCs	Solute-carrier transporters
SNE	Carbamazepine-loaded safflower seed oil nanoemulsion
SV2A	Synaptic vesicle glycoprotein 2A
$T_{1/2}$	Half-life
TA	Therapeutic availability
TEER	Trans-endothelial electrical resistance
Tf	Transferrin
TGFβ	Transforming growth factor-β
T_{max}	Time to achieve maximum concentration
T-SV40	SV40 large T antigen
VE-cadherin	Vascular endothelial-cadherin
V_d	Volume of distribution
W/O	Water-in-oil
ZO-1	Zona occludens-1

CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Epilepsy is one of the most common, serious and chronic brain disorders that affects the entire age range, from neonates to elderly people. It has varied causes and manifestations, with many distinct seizure types, several identifiable symptoms, but also much that is poorly classified. Approximately, 50 million people worldwide have epilepsy and 80% of the people with epilepsy live in low- and middle-income countries (WHO, 2015). Less wealthy people show a higher incidence of epilepsy because of poor sanitation, inadequate health delivery systems and a higher risk of brain infections and infestations (WHO, 2015; Forsgren *et al*., 2005; Sander, 2003). In many parts of the world, people with epilepsy and their family suffer from stigma and discrimination.

1.2 Problem Statements

Carbamazepine (CBZ) is a commonly used anti-epileptic drug (Genaro, 2002). It inhibits the spread of seizure activity by preventing the high repetitive firing of neurons (Perucca, 2005). It is a lipophilic drug, it's solubility in an aqueous environment is less than 200 µg/mL, and has slow and irregular gastrointestinal absorption (Sethia and Squillante, 2004). CBZ is only available in the market in the forms of oral capsule or tablet (immediate release, extended release and chewable) and oral suspension (FDA, 2015). A parenteral formulation of CBZ is not available in the market. Parenteral formulation is an alternative route for the administration of lipophilic drugs with poor oral bioavailability (Robie *et al*., 1999). Additionally, parenteral formulation can be used in emergency situations as it provides an immediate onset of action. For certain circumstances, such as patients in a coma or having swallowing problems, parenteral administration is the best route. Some pharmaceutical strategies have been used to develop parenteral delivery of CBZ. Researchers have solubilised CBZ using co-solvents such as propylene glycol, ethanol and polyethylene glycol 400 (Strickley, 2004). However, the severe soreness at injection site and the occurrence of haemolysis can limit their application. This pharmaceutical strategy is also, less appealing to the patients in many cases (Akers, 2002).

In addition to the aforementioned shortcomings, drug delivery to the brain is also impeded by a highly selective biological barrier, the blood-brain barrier (BBB). The BBB regulates brain homeostasis by safeguarding the brain from circulating toxic or infectious agents (Abbott *et al*., 2006). It is the tight junctional characteristic of brain cerebral endothelial cells (BCECs), which results in the impermeability of BBB. In addition, the presence of a high level of efflux transporters at BCECs greatly reduces the bioavailability of most of the neurological drugs in the brain. The BBB also has accessional enzyme aspects, which help to safeguard the brain. Compounds or drugs

which cross the cell membrane are subsequently exposed to large number of degrading enzymes such as peptide degrading enzymes, which are present inside the BCECs (Deeken and Löscher, 2007). Lastly, CBZ is a substrate for efflux transporters, P-glycoprotein (P-gp) and multidrug resistance protein-1 (MRP-1), which prevents CBZ transport across the BBB (Löscher and Potschka, 2015).

1.3 Significance of the Study

In order to increase the bioavailability of CBZ, drug delivery approaches must address methods to cross the BBB, meanwhile, overcoming the efflux transporters. Emulsion based lipid nano-carriers like nanoemulsion (NE) possess the advantage of a greater ability to bypass the reticulo-endothelial system (RES) with enhanced brain targeting (Shinde *et al*., 2011). A NE system consisting of edible oils has been developed to deliver the HIV protease inhibitor, saquinavir (Vyas *et al*., 2008). The NE obtained was able to increase the plasma and brain drug availability by 3- and 5-fold. Therefore, NE has emerged as a promising alternative for the administration of drugs, including CBZ. Furthermore, NE offers an appealing alternative for the delivery of lipophilic drugs due to the benefits such as the ability to solubilise high amounts of drug, the ability to avoid drug hydrolysis, its ease of manufacture scale-up and it is economically friendly if compared to other colloidal carriers for instance liposomes (Sarker, 2005; Floyd, 1999). In addition, its potential for enhanced bioavailability, anticipated patient compliance due to the development of NE with BBB-targeted moiety and reduced side effects are appealing to both researchers and patients (Chen and Liu, 2012; Patidar *et al*., 2010). It should also be noted that parenteral emulsion has been used for parenteral nutrition for several years and has a good safety profile (Melgardt *et al*., 2009).

1.4 Hypothesis

Parenteral nanoemulsion can be an effective drug delivery system, to deliver CBZ across the BBB into the brain.

1.5 Objectives of the study

1.5.1 Main Objective

The aim of this study is to formulate and characterise parenteral nanoemulsions (NEs) of CBZ which possess the ability to cross the BBB and improved bioavailability.

1.5.2 Specific Objectives

a) To formulate, characterise, optimise and to evaluate the stability of CBZ-loaded nanoemulsions (CBZ-NEs)
b) To develop, optimise and characterise an *in vitro* BBB model
c) To determine the delivery potential of CBZ-NEs using *in vitro* BBB model
d) To determine the *in vivo* pharmacokinetic profile of CBZ-NEs

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LIST OF PUBLICATIONS

Publications

Tan S.L., Stanslas J., Basri M., Karjiban R.A.A., Kirby B.P., Sani D., Basri H.B. (2015). Nanoemulsion-based parenteral drug delivery system of carbamazepine: preparation, characterization, stability evaluation and blood-brain pharmacokinetics. *Current Drug Delivery*, 12(6): 795-804.

Zaynah M., **Tan S.L.**, Tan S., Geoff P. (2013). Influences of different blood-brain barrier basement membrane ECM molecules on brain trans-endothelial electrical resistance. *Neuro-oncology*, 15: 1-6.

Posters

Tan S.L., Stanslas J., Basri M., Karjiban R.A.A., Kirby B.P., Basri H.B. (2014). The preparation and characterization of carbamazepineencapsulated nanoemulsion targeting the brain via parenteral administration. *25th Annual Scientific Meeting of Malaysian Society of Neurosciences, 20th – 22nd June, Kuala Lumpur, Malaysia.*

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