UNIVERSITI PUTRA MALAYSIA

FABRICATION OF NANOEMULSION LOADED WITH CEFUROXIME FOR EFFICIENT TRANSLOCATION ACROSS THE BLOOD BRAIN BARRIER

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By

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

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January 2018

Chairman: Professor Hamidon Bin Basri, M.D.M.Med
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Meningitis is one of the commonest and most debilitating acute neurological conditions. Drugs that are effective against diseases in the central nervous system and reach the brain via the blood compartment must pass the blood brain barrier (BBB), the unique interface that formed protection against potentially harmful molecules. Antibiotics in high doses had been used to treat this illness however, with significant increase in side effects. Nanoemulsion was an effective drug nanocarrier due to their biocompatibility, relative stability, high drug loading capacity, preserved cytotoxicity and ability to protect drugs from hydrolysis and enzymatic degradation in physiologic conditions.

In this research, a nanosystem for blood-brain barrier translocation utilizing nanoemulsions loaded with cefuroxime were developed. This new form of drug delivery will be able to reduce the peripheral side effects of the cefuroxime and at the same time increase the penetration across the BBB. Optimization, characterization and stability evaluation were perfomed to ensure the formulated nanoemulsion fulfilled the requirement for parenteral drug delivery. The characterization revealed particle size of 100.32 ± 0.75 nm, polydispersity index of 0.18 ± 0.01, zeta potential of −46.9 ± 1.39 mV, viscosity of 1.24 ± 0.34 cps and osmolality of 285.33 ± 0.58 mOsm/kg, indicating the nanoemulsion compatibility for parenteral application.

Cefuroxime loaded nanoemulsion (CLN) was subjected to in vitro and in vivo studies. A humanized in vitro model of blood brain barrier based on cocultures of human microvascular endothelial cells (hCMEC/D3) and normal human astrocyte (NHA) was developed. This model was validated to ensure it closely resemble the microenvironment condition of blood brain barrier. This model was used to evaluate the penetration efficiency of cefuroxime loaded

nanoemulsion. The in vitro study showed that the formulated CLN has higher apparent permeability (0.04 ± 0.01 cm/h) when compared to cefuroxime solution (0.02 ± 0.02 cm/h). The pharmacokinetic profile generated from in vivo study revealed that CLN was successfully improved the plasma and brain concentration of cefuroxime when compared to cefuroxime solution. From the results obtained, drug loaded nanoemulsion could be an effective carrier for drug delivery across the brain.

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

FABRIKASI NANOEMULSI MENGANDUNGI CEFUROXIME UNTUK TRANSLOKASI CEKAP PENGHALANG DARAH OTAK

Oleh

SITI NORHAWANI BINTI HARUN

Januari 2018

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Meningitis adalah salah satu infeksi saraf akut yang biasa berlaku. Ubat yang berkesan terhadap penyakit pada sistem saraf pusat sampai ke otak melalui darah perlu melepasi penghalang darah otak (BBB), iaitu permukaan perlindungan unik terhadap molekul yang mungkin berbahaya. Dos antibiotik yang tinggi digunakan untuk merawat penyakit ini dan hal ini telah meningkatkan kesan sampingan yang ketara. Nanoemulsi adalah pembawa dadah yang berkesan disebabkan ia boleh serasi secara bio, stabil, mempunyai dos pemuatan yang tinggi, kadar kesitotoksikan yang rendah dan berupaya melindungi dadah daripada hidrolisis dan kemusnahan kemusnahan enzim secara fisiologi.

Dalam penyelidikan ini, sistem nano untuk translokasi penghalang darah otak menggunakan nanoemulsi yang mengandungi cefuroxime telah dibangunkan. Sistem penyampaian dadah yang baru ini boleh mengurangkan kesan sampingan periferi cefuroxime dan pada masa yang sama meningkatkan penembusan ke seluruh halangan darah otak. Pengoptimuman, pencirian dan penilaian kestabilan telah dilakukan untuk memastikan nanoemulsi yang diformulasikan memenuhi spesiifikasi untuk penyampaian dadah secara parenteral. Pencirian menunjukkan saiz zarah adalah 100.32 ± 0.75 nm, indeks kepoliserakan adalah 0.18 ± 0.01, potensi zeta adalah -46.9 ± 1.39 mV, kelikatan adalah 1.24 ± 0.34 cps dan osmolaliti adalah 285.33 ± 0.58 mOsm/kg, yang menunjukkan keserasian nanoemulsion untuk aplikasi parenteral.

Nanoemulsi yang mengandungi cefuroxime (CLN) juga dikaji secara in vitro dan dan in vivo. Model in vitro halangan darah otak menggunakan sel endothelial mikrovaskular (hCMEC / D3) dan sel astrosit manusia (NHA) telah dikaji. Model ini telah menjalani proses penentusahan untuk memastikan ia

menyerupai keadaan mikro alam sekitar penghalang otak darah. Model ini digunakan untuk menilai kecekapan penembusan nanoemulsi yang mengandungi cefuroxime. Kajian in vitro menunjukkan bahawa formulasi CLN mempunyai kebolehtelapan yang jelas lebih tinggi (0.04 ± 0.01 cm/h) berbanding cairan cefuroxime (0.02 ± 0.02 cm/h). Profil farmakokinetik dijana dari kajian in vivo mendedahkan bahawa CLN telah berjaya meningkatkan kandungan cefuroxime di dalam plasma dan otak berbanding cairan cefuroxime. Daripada data yang diperolehi, nanoemulsi yang mengandungi dadah boleh menjadi pembawa yang berkesan untuk penghantaran dadah di seluruh otak.

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I certify that a Thesis Examination Committee has met on 4 January 2018 to conduct the final examination of Siti Norhawani binti Harun on her thesis entitled "Fabrication of Nanoemulsion Loaded with Cefuroxime for Efficient Translocation Across the Blood Brain Barrier" 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 Doctor of Philosophy.

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LIST OF ABBREVIATIONS

ABC	ATP binding casette
ABM	astrocytes basal medium
AC	adenylate cyclases
ALP	alkaline phosphatase
AMT	adsorptive mediated transcytosis
ANOVA	analysis of variance
ATCC	American Type Culture Condition
ATP	Adenosine triphosphate
AUC_{0-x}	area under the curve from time 0-x, where x is the last time point
API	active pharmaceutical ingredients
BBB	blood brain barrier
BCRP	breast cancer resistance protein
BSA	bovine serum albumin
cAMP	adenosine 3', 5'-cyclic monophosphate
CI	total clearance
CCL5	cytokine receptors
CLN	cefuroxime loaded nanoemulsion
C_{max}	the maximum concentration was taken directly from the concentration course
CNS	central nervous system
CO_2	carbon dioxide
CS	corticosteroids
CSF	cerebrospinal fluid
DEX	dexamethasone
DIV-BBB	dynamic in vitro model of BBB
DLS	dynamic laser scattering
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBM-2	endothelial basal medium-2
ECM	extracellular matrices
ECs	endothelial cells
EE	entrapment efficiency
EGM-2	endothelial growth medium-2
EVOM2	voltohmeter
FBS	fetal bovine serum
FDA	U.S. Food and Drug Administration
GAGs	glycosaminoglycans
GPI	gycerophosphoinosotol
HPH	high pressure homogenizer
HPLC	High performance liquid chromatography
HRP	goat polyclonal horseradish peroxidase
hTERT/SV40	human telomerase reverse transcriptase and Simian vacuolating virus 40
Hib	Haemophilus influenzae type B
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use

IV	intravenous
K_{el}	elimination rate constant
K_p	brain to plasma ratio
K_{pu}	unbound brain-to-plasma concentration ratio
LCT	long chain triglycerides
LDL	low density lipoprotein
MCT	middle chain triglyceride
MELAS	Mitochondrial encephalopathy, lactic acidosis and stroke-likeepisodes
MIC	minimum inhibitory concentration
MRPs	multidrug resistance associated-proteins
MS	multiple sclerosis
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NHA	normal human astrocytes
O/W	oil in water
O_2	oxygen
OFAT	one factor-at-a-time
P_{app}	apparent permeability
PBP	penicillin-binding proteins
PBS	phosphate buffer solution
PDE	phosphodiesterases
PDI	polydispersity index
PET	polyethelene
Pgp	P-glycoprotein
PHS	plasma derived serum
PK	pharmacokinetic
PKOEs	palm kernel oil esters
PLA2	phospholipase A2
pNP	p-nitrophenol
PUFAs	poly-unsaturated fatty acids
RMT	receptor mediated transcytosis
SAFO	safflower oil
SBO	soybean oil
SCT	short chain triglycerides
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEO	sesame oil
SLCs	solute carriers
SSO	safflower seed oil
SUO	sunflower oil
$T_{1/2}$	elimination half life
TEER	transendothelial electrical resistance
TEM	transmission electron microscopy
Tfr	transferin receptor
TJ	tight junction
T_{max}	time to achive C_{max}
TB	tuberculous
UPLC	Ultra performance Liquid Chromatography
Vd	apparent volume of distribution

w/o	water in oil
WBC	white blood cells
ZO-1	zona accludens-1
ZP	zeta potential
γ-GTP	gamma-glutamyl transpeptidase

LIST OF UNITS

cm	Centimeter
cP	Centipoise
Da	Dalton
˚C	Degree Celsius
h	Hour
µg	Microgram
µm	Micrometer
µl	Microliter
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
mOsm/kg	Milliosmole per kilogram
mV	Millivolt
min	Minute
m	Month
nm	Nanometer
%	Percentage
Pa	Pascal

CHAPTER 1

INTRODUCTION

1.1 Background of Research

The central nervous system (CNS) is the most critical and sensitive system in the human body. The CNS is also extremely sensitive to wide range of neurotoxic chemicals that are mostly consumed in our diet although the substances are readily metabolized and excreted without harm to peripheral organ systems (Hawkins & Davis, 2005). Thus, the presence of interface between the CNS and peripheral circulatory systems is essential as a dynamic regulator of ion balance, a facilitator of nutrient transport and a barrier to potentially harmful molecules.

The three main barrier sites in the CNS include the brain endothelium forming the blood brain barrier (BBB), the arachnoid epithelium forming the middle layer of the meninges and the choroid plexus epithelium which secretes cerebrospinal fluid (CSF). Fan et al., 2010 reported that BBB is the most important barrier between systemic circulation and the central nervous system due to its substantially larger surface area, and it presents a major challenge to the treatment of most brain disorders. A basic BBB function is to eliminate the passage of macromolecules, microbial pathogens, and circulating leukocytes into the central nervous system. The BBB has long been regarded as the gate keeper of the CNS (Marsala et al., 2004).

Diseases of the CNS such as meningitis, schizophrenia, migraine, Parkinson's disease and Alzheimer's disease require delivery of the drug to the brain for treatment. Meningitis (infection of the meningeal layer) is one of the commonest and most debilitating acute neurological conditions (Mistry et al., 2009; Pardridge, 1999). Antibiotics in high doses had been used to treat this illness however, with significant increase in side effects. Drugs that are effective against diseases in the CNS and reach the brain via the blood compartment must across the BBB. Many drugs are not useful in CNS because they are insufficiently lipid soluble to penetrate the BBB. The entry of drugs, including antibiotics, into the cerebrospinal fluid and extracellular space of the brain is governed by the molecular size, lipophilicity and plasma binding protein.

1.2 Problem statements

The conventional parenteral delivery of cefuroxime exhibit burst release to produce desirable therapeutic effect. This type of release produces rapid response and short drug action on targeted site. The drug delivery through nanoemulsion system guarantee the release of cefuroxime in a sustained and controlled mode over long periods of time that improve bioavailability and cause the extended drug action on targeted site (Agrawal et al., 2012). Thus, formulating cefuroxime as nanoemulsion lipid carrier can prolong its bioavailability efficiency when compared to similar dosage that administrated via conventional delivery. The capability of lipids emulsion to dissolve hydrophobics drugs and protect the drugs from hydrolysis and enzymatic degradation are the criteria that make nanoemulsion as an ideal vehicles for the purpose of parenteral transport. Another attractive features offer by lipid emulsions delivery systems are the stability as well as their safety record due to their accepted regulatory status of the raw materials used and the safe metabolism (Hormann & Zimmer, 2016).

The presence of the BBB limits the penetration of a large number of pharmacologically active drugs aimed at treating CNS diseases because they do not possess the appropriate physicochemical properties that enable them to cross the BBB. Thus, higher doses of the drug are needed in order to exhibit a therapeutically effective CNS concentrations and this might lead to a significant increase in the side effects of the drug. The submicron size of droplets were created through nanoemulsion technology have higher probability of crossing the BBB that assist in improving the therapeutic efficiency in the brain (Jain, 2007; Sutradhar & Amin, 2013). Hence, this new form of drug delivery will be able to reduce the peripheral side effects of the cefuroxime due to the reduction of dosage and frequency of injections throughout the drug therapy period and at the same time increase the penetration across the BBB. Even parenteral administration of cefuroxime was well tolerated, the common reported side effects include fever, skin rash, neutropenia, thrombophlebitis and intramuscular injection reaction (Brogden et al., 1979; Olaisoo & Alestig, 1990).

1.3 Scope of study

Cefuroxime loaded nanoemulsion (CLN) was evaluated based on particle size, zeta potential, polydispersity index (PDI), viscosity, morphology, osmolality, drug assay, entrapment efficiency, in vitro drug release and cytotoxicity study to ensure the formulation meets the requirements for parenteral administration. The in vitro model of blood brain barrier which involved the manipulation of brain endothelial cells and astrocytes is developed to test the penetration of CLN across the barrier system. The morphology study, transendothelial electrical resistance (TEER) measurements, enzyme activity assay, permeability test and western blot were among the characterizations

performed to validate the model. The efficiency of CLN in living subjects was explored by conducting the animal study. Through the in vivo study, the pharmacokinetic profile of CLN was compared with the marketed cefuroxime.

1.4 Objectives

The main objective of this study is to develop novel nanoemulsions incorporated with cefuroxime for efficient delivery across blood brain barrier. In this research, it was hypothesised that the developed cefuroxime loaded nanoemulsion improved penetration of cefuroxime across the brain compared to marketed cefuroxime solution that may lead to the decrease of administration dose. As the consequence, the drug side effects and toxicity is minimized through the reduction of administration dosage. In order to successfully achieve the main objective, following objectives have to be accomplished:

i. To develop nanosystem efficient in blood-brain barrier translocation utilizing emulsions loaded with cefuroxime
ii. To characterize the formulated nanoemulsions system and to evaluate the long-term stability of the formulated nanoemulsion with respect to physical and chemical stability
iii. To develop in humanized in vitro model of blood brain barrier using hCMEC/D3 cells and validate the model
iv. To study the delivery potential of cefuroxime loaded nanoemulsion (CLN) using in vitro and in vivo approaches across blood brain barrier

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