UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT AND CHARACTERIZATION OF PARENTERAL NANO-DELIVERY SYSTEM LOADED WITH AZITHROMYCIN

GHAIDAA S. DAOOD

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By

GHAIDAA S. DAOOD

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

June 2016

It is my genuine gratefulness and warmest regard that I dedicate this work

To

My Father and my Mother
"If I have seen further, it is by standing on the shoulder of giants"

Sir Isaac Newton

My brother Manhal and my sister Dabyaa
"The happiest moments of my life have been the few which I have passed at home in the bosom of my family"

Thomas Jefferson

My children Mayar, Fatimah, Eethar, and Yaman
You are the light in my life

And

My awesome husband Auday
For a debt I can never repay

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

DEVELOPMENT AND CHARACTERIZATION OF PARENTERAL NANO-DELIVERY SYSTEM LOADED WITH AZITHROMYCIN

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June 2016

Chairman : Professor Hamidon Bin Basri, PhD
Faculty : Medicine and Health Science

The blood brain barrier (BBB) is established by brain microvascular endothelial cells (BMEC) working simultaneously with pericytes and astrocytes, in which tight junctions and several transporters strictly regulate the penetration of various bioactive compounds into the brain including antibiotics. This has significant consequences for the treatment of Central Nervous System (CNS) infections, as antibiotics have to gain access to the brain through the BBB in adequate concentrations to exhibit their antibacterial activity. Azithromycin (AZO) is a broad spectrum antibiotic with a unique pharmacokinetic profile. However, its role in treatment of CNS infections is limited because it does not possess the appropriate physicochemical properties that enables it to achieve sufficient concentrations in brain tissue.

Nanoemulsion system is one of the potential strategies for efficient delivery of lipophilic actives across the BBB owing to their nano-sized, biocompatible, biodegradable, physical stability and relatively easy to produce on a large scale. With the aim of brain targeting, AZO-loaded nanoemulsions were developed utilizing high pressure homogenization with a homogenization pressure of 1000 bar for 8 cycles. The formulated nanoemulsions were optimized utilizing artificial neural network (ANN) as a multivariate statistical technique. In order to achieve the optimum topologies, ANN was trained by Incremental Back-Propagation (IBP), Batch Back-Propagation (BBP), Quick Propagation (QP), and Levenberg-Marquardt (LM) algorithms for testing data set. The topologies were confirmed by the indicator of minimized root mean squared error (RMSE) for each. Based on that indicator, the BBP-5-14-1 was selected as the optimum topology to be used as a final model to predict the desirable particle size and relative importance of the formulation's effective variables. The ANN analysis showed that with optimum compositions of soya bean oil 6%, oleic acid 2%, AZO 1.4%, lecithin 2%, Tween

80 2%, glycerol 2.5%, vitamin E 0.25%, and water 83.85%, minimum particle size can be obtained.

The optimized nanoemulsions were evaluated for the various physicochemical properties. The characterization revealed particle size of 54.67 ± 0.81 nm, polydispersity index (PDI) of 0.218 ± 0.023, zeta potential of -34.65 ± 0.78 mV, pH of 7.82 ± 0.07, viscosity of 1.77 ± 0.05 cps, and osmolality of 288 ± 1.00 mOsm/kg, suggesting their compatibility for intravenous administration. AZO was successfully incorporated into nanoemulsion system with an average encapsulation efficiency of 98.21 ± 1.97% and a relatively high drug loading of 91.19 ± 5.93%. Morphological analysis with Transmission Electron Microscopy (TEM) confirmed the formation of almost spherical shaped uniformly distributed nano-sized oil droplets.

In vitro drug release study of the selected formulation demonstrated a release profile similar to that of AZO standard solution, both exhibited a biphasic behavior characterized by a fast initial release of the encapsulated drug followed by a slower sustained release till the optimized formulation achieved a total accumulative release of the drug of 84.94 ± 4.76% within 48 h. Kinetically, AZO release profile from nanoemulsion system *in vitro* appeared to fit best with the Higuchi model. Stability of nanoemulsion prepared with the optimized formula was mainly evaluated in term of preserving its physical integrity, namely particle size and polydispersity index (PDI). The formulation maintained its properties in a satisfactory range up to 12 months of storage at 4˚C and 25˚C, which demonstrated sufficient physical stability upon long-term storage.

A linear relationship between the particle size (cube of the radius of dispersed phase droplets (r^3)) and time (t) was obtained identifying Ostwald ripening (OR) as the dominant destabilization mechanism of AZO-loaded nanoemulsion from a 12-months shelf-life study. OR rate (ω) was extrapolated graphically from the slope and was found to be 0.232×10^{-8} nm^3/s. Optimized nanoemulsion was analyzed for its drug content to monitor its chemical stability. The shelf-life ($t_{0.9}$) of optimized nanoemulsion formulation was estimated to be 4.85 years at 25˚C which reflected the ability of nanoemulsion formulation to maintain the drug and efficiently protected it against degradation.

To summarize, the studies conducted indicate the utility and potential advantage of AZO-loaded nanoemulsion system as a promising delivery carrier worth to explore further for its parenteral applicability in the treatment of bacterial meningitis.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBENTUKAN DAN PENCIRIAN SISTEM NANOPENGHANTARAN SECARA PARENTERAL YANG MENGANDUNGI AZITROMISIN

Oleh

GHAIDAA S. DAOOD

Jun 2016

Pengerusi : Profesor Hamidon Bin Basri, PhD
Fakulti : Perubatan dan Sains Kesihatan

Sawar darah otak (BBB) terhasil oleh sel-sel endotelial mikrovaskular otak (BMEC) yang bekerja serentak dengan perisit dan astrosit, di mana simpang sempit dan beberapa pengangkut mengawal ketat penembusan pelbagai sebatian bioaktif ke dalam otak termasuk antibiotik. Ini mempunyai kesan yang signifikan untuk rawatan jangkitan sistem saraf utama (CNS), kerana ubat perlu mendapat akses kepada otak melalui BBB dalam kepekatan yang mencukupi untuk menjalankan aktiviti terapeutiknya. Azitromisin (AZO) adalah agen anti-bakteria yang boleh dipercayai dengan spektrum antimikrob yang luas dan profil farmakokinetik unik, namun, peranannya dalam rawatan jangkitan CNS adalah terhad kerana ia tidak mempunyai sifat-sifat fizikokimia yang sesuai yang membolehkan ia mencapai kuantiti yang mencukupi dalam tisu otak.

Sistem nanoemulsi adalah salah satu strategi yang berpotensi untuk penghantaran cekap lipofilik aktif ke seluruh BBB kerana mereka bersaiz nano, boleh serasi secara bio, mesra alam, mempunyai kestabilan fizikal yang unggul dan agak mudah untuk dihasilkan secara besar-besaran. Dengan tujuan untuk mensasarkan otak, AZO yang dimuatkan dengan nanoemulsi telah dibangunkan menggunakan homogenisasi tekanan tinggi dengan tekanan homogenisasi 1000 bar untuk 8 kitaran.

AZO yang dimuatkan dengan nanoemulsi telah dioptimumkan menggunakan rangkaian neural tiruan (ANN) sebagai teknik statistik multivariat. Bagi mencapai topologi yang optimum, ANN telah dilatih oleh Incremental Back-Propagation (IBP), Batch Back- Propagation (BBP), Quick Propagation (QP), dan algoritma Levenberg-Marquardt (LM) untuk menguji set data. Topologi telah disahkan oleh petunjuk kepada ralat punca purata kuasa dua (RMSE) minimum bagi setiap satu. Berdasarkan petunjuk itu, BBP-5-14-1 telah dipilih sebagai topologi yang optimum

untuk digunakan sebagai model akhir untuk meramalkan saiz zarah yang dikehendaki dan kepentingan relatif pembolehubah formulasi yang berkesan. Analisis ANN menunjukkan bahawa dengan komposisi optimum minyak kacang soya 6%, asid oleic 2%, AZO 1.4%, lesitin 2%, Tween 80 2%, gliserol 2.5%, vitamin E 0.25% dan air 83.85% menghasilkan saiz zarah yang minimum.
Nanoemulsi yang dioptimumkan itu telah dinilai mengenai pelbagai sifat fizikokimia. Pencirian itu mendedahkan saiz zarah 54,67 ± 0.81 nm, indeks polydispersity (PDI) daripada 0.218 ± 0.023, keupayaan potensi zeta daripada -34.65 ± 0.78 mV, pH 7.82 ± 0.07, kelikatan 1.77 ± 0.05 cps, dan osmolaliti 288 ± 1.00 mOsm / kg, menunjukkan keserasiannya bagi administrasi intravena. AZO telah berjaya dicampur ke dalam sistem nanoemulsi dengan kecekapan purata pengkapsulan 98.21 ± 1.97% dan kandungan dadah agak tinggi 91.19 ± 5.93%. Analisis morfologi dengan transmisi mikroskop elektron (TEM) mengesahkan pembentukan titisan minyak bersaiz nano berbentuk hampir sfera yang teragih secara seragam.

kajian pelepasan dadah *In vitro* bagi beberapa formulasi terpilih menunjukkan profil pelepasan sama dengan larutan AZO piawai, kedua-duanya mempamerkan ciri-ciri tingkah laku dwifasa, pelepasan awal dadah pengkapsulan yang cepat diikuti oleh pelepasan yang lebih perlahan yang berterusan sehingga ia mencapai pelepasan terkumpul jumlah dadah 84.94 ± 4.76% dalam tempoh 48 jam. Secara kinetiknya, profil pelepasan AZO daripada sistem nanoemulsi *in vitro* muncul terbaik sesuai dengan model Higuchi itu. Kestabilan nanoemulsi yang disediakan dengan formula optimum itu dinilai terutamanya dari segi pemeliharaan integriti fizikal, iaitu saiz zarah dan taburan saiz (PDI). Formulasi mengekalkan sifat-sifatnya dalam julat yang memuaskan sehingga 12 bulan penyimpanan pada 4˚C dan 25˚C, yang menunjukkan kestabilan fizikal yang mencukupi untuk penyimpanan jangka panjang.

Hubungan linear antara saiz zarah jejari kuasa tiga fasa tersebar (r^3) dan masa (t) telah diperolehi mengenal pasti Ostwald ripening (OR) sebagai mekanisme ketidakstabilan dominan bagi nanoemulsi termuat AZO dari kajian jangka hayat 12 bulan. Kadar OR (ω) ekstrapolasi ditentukan secara graf dari kecerunan dan didapati ialah 0.232×10^{-8} nm^3 /s. kandungan dadah nanoemulsi optimum telah dianalisis untuk memantau kestabilan kimia. Jangka hayat ($t_{0.9}$) formulasi nanoemulsi optimum dianggarkan 4.85 tahun pada suhu 25˚C yang menunjukkan keupayaan formulasi nanoemulsi untuk mengekalkan dadah dan cekap melindunginya terhadap pereputan.

Sebagai ringkasan, kajian yang dijalankan menunjukkan utiliti dan kelebihan potensi sistem nanoemulsi termuat AZO boleh dipercayai sebagai pembawa penghantaran yang bernilai untuk diterokai lebih untuk kegunaan parenteral dalam rawatan bakteria meningitis.

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I certify that a Thesis Examination Committee has met on 28 June 2016 to conduct the final examination of Ghaidaa S. Daood on her thesis entitled "Development and Characterization of Parenteral Nano-Delivery System Loaded with Azithromycin" 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

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Professor
Faculty of Medicine and Health science
Universiti Putra Malaysia
(Chairman)

Mohd Zaizi bin Desa, PhD
Associate Professor
Faculty of Science
Universiti Putra Malaysia
(Internal Examiner)

Mohd Cairul Iqbal Mohd Amin, PhD
Professor
National University of Malaysia
Malaysia
(External Examiner)

ZULKARNAIN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 23 August 2016

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

Hamidon bin Basri, PhD
Professor
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Chairman)

Johnson Stanslas, PhD
Professor
Faculty of Medicine and Health Science
Universiti Putra Malaysia
(Member)

BUJANG BIN KIM HUAT, PhD
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Supervisory Committee: Professor Dr. Hamidon bin Basri

Signature: _____
Name of Member of
Supervisory Committee: Professor Dr. Johnson Stanslas

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LIST OF ABBREVIATIONS

AAD	Absolute average deviation
AUC	Area under the curve
ABC	ATP- binding cassette transporters
ABM	Acute bacterial meningitis
AMT	Adsorptive-mediated transcytosis
ANN	Artificial neural network
AZO	Azithromycin
BBB	Blood brain barrier
BBP	Batch Back-Propagation
BCSFB	Blood–cerebrospinal fluid barrier
CMC	Critical micelle concentration
CNS	Central nervous system
CSF	Cerebrospinal fluid
CV	Coefficient of variation
CYP3A4	Cytochrome P450 3A4
DLS	Dynamic Light Scattering
DMSO	Dimethyl sulfoxide
ECs	Endothelial cells
ECM	Extracellular matrix proteins
FDA	Food and Drug Administration
GA	Genetic algorithm
hCMEC/D_3	Immortalized human cerebral microvascular endothelial cells
Hib	*Haemophilus influenza type b*
HLB	Hydrophilic-lipophilic balance
HPH	High Pressure Homogenization
HS	Human serum

IBP	Incremental Back-Propagation
IC_{50}	Inhibitory concentration for 50 % inhibition
IgM	Immunoglobulin-M
IL-1β	Inter leukin-1beta
IL-6	Inter leukin-6
ISF	Interstitial fluid
IV	Intravenous
LCT	Long chain triglyceride
LDL	Low density lipoprotein
LM	Levenberg-Marquardt
LSW	Lifshitz Slyozov Wagner
MBC	Minimal bactericidal concentration
MNS	Mononuclear phagocyte system
MRSA	Methicillin resistant *Staphylococcus aureus*
MTT	3-(4,5-Dimethlythiazol-2-yl)-2-5-diphenyltetrazolium bromide
MWCO	Molecular weight cutoff
NE	Nanoemulsion
NO	Nitric oxide
NVU	Neurovascular unit
OFAT	One-Factor-At-a-Time
O/W	Oil-in-water
OR	Ostwald ripening
PBS	Phosphate buffer solution
PCS	Photon Correlation Spectroscopy
PDI	Polydispersity index
pH	Negative logarithm of activity of hydronium ion
PMNS	Polymorph nuclear system
QC	Quality control

QP	Quick Propagation
RMT	Receptor-mediated transcytosis
rpm	Revolution per minute
RMSE	Root mean squared error
RSD	Relative standard deviation
SD	Standard deviation
TEM	Transmission Electron Microscopy
TJs	Tight junctions
TNF-α	Tumor necrosis factor-alpha
UPLC	Ultra-Performance Liquid Chromatography
VDW	Van Der Waals
VRE	Vancomycin resistant enterococci
W/O	Water-in-oil
W/O/W	Water-in-oil-in-water
w/w	Weight by weight

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 the Study

The pharmaceutical treatment of Central Nervous System (CNS) diseases is the second largest area of therapy following cardiovascular disorders. CNS disorders currently affect over 1.5 billion people worldwide and account for about one-third of the global disease burden being five of the top ten causes of disability. Many CNS disorders do not have satisfactory treatment and they are considered an important current and future priority for the pharmaceutical industry (Lewis et al., 2013). Nervous tissues are very soft and delicate and the irreplaceable neurons can be injured by even a slight pressure. Thus, the brain and the spinal cord have been protected by a bone enclosing them from the outside (the skull and the vertebral column), membranes (meninges), and a watery cushion (the cerebrospinal fluid (CSF)) from inside (Marieb & Hoehn, 2010). The protection of the brain from any harmful substances that could be circulating in the blood is provided by the blood brain barrier (BBB). The BBB acts very effectively to protect the brain from many common pathogens circulating in the systemic circulation, thus infections of the brain are not very common. However, since antibodies and antibiotics are too big to cross the BBB, infections of the brain that do occur are often very serious and too difficult to treat (Sandoval & Witt, 2008). One of these infections is meningitis.

Meningitis is an acute infection of the protective membranes surrounding the brain and spinal cord (meninges), usually due to the spread of an infection elsewhere in the body into the meninges and CSF, which will be followed by a CNS inflammatory reaction that causes coma, seizure, increased intracranial pressure and stroke. Meningitis occurs most commonly in young children under 5 years of old and people over 60 years (Alam et al., 2010) . The most common cause of meningitis is viral infections, but bacterial and fungal infections may also cause meningitis. Although bacterial meningitis affect fewer populations, it is considered the most serious type of meningitis. It can cause severe brain damage and is fatal in 50% of the cases if not treated. Even when the disease is diagnosed early and proper treatment is started, 5 - 10% of the patients die, typically within 24 - 48 h after the onset of symptoms. Bacterial meningitis may result in brain damage, hearing loss or learning disability in 10 - 20% of the survivors (Fitch & van de Beek, 2007). A range of antibiotics can be used to treat meningitis including, ceftriaxone, ampicillin, penicillin G, chloramphenicol, oxacillin, gentamycin, vancomycin. But for an antibiotic to be effective in the treatment of meningitis, it must not only be active against the pathogenic bacterium, but it should also be able to penetrate the BBB and reach the meninges in sufficient quantities and stay there long enough to produce its antibacterial activity.

Delivery of drugs to the brain is a major challenge due to the presence of the BBB. Unlike the peripheral capillaries that allow relatively free exchange of substances across or between the cells, the BBB strictly limits the transport into the brain to maintain a stable environment for a proper function of the nervous tissues (Kaur et al., 2008). Thus the BBB is considered the rate limiting factor in determining permeation of therapeutic agents into the CNS (Patel et al., 2013). Many potential compounds, which are effective at their site of action, have failed during their development for the clinical use due to a failure in delivering them in an adequate quantities to the brain tissues to produce the therapeutic effect. It has been estimated that only 2% of the possible CNS therapeutic drugs can pass the BBB (Pardridge, 2002).

It has been generally anticipated that BBB disruption that occurs under inflammatory conditions may offer an opportunity for enhancement of drug transport into the brain via the paracellular route. This, however, has shown to be a more complex issue. Due to limited *in vivo* results obtained by different investigators under pathological conditions, the answer is not clear (Chen & Liu, 2012). Furthermore, several experimental models of bacterial meningitis in animals suggest that effective bacteriologic cure is associated with antibiotic concentration in CNS compartments that are 10 – 30 times the minimal bactericidal concentration (MBC) for a specific pathogen (Chávez-Bueno & McCracken, 2005). These bactericidal concentrations can only be achieved by administration of frequent and high systemic doses of antibiotics that might not be well tolerated by patients. Additionally, for some antibiotic families (such as aminoglycosides, glycopeptides), toxicity makes dose increase difficult to achieve.

As a broad-spectrum antibacterial, azithromycin (AZO) shares the same mechanism of action as other macrolide antibiotics and its range of activity is extended through inhibition of bacterial quorum-sensing and biofilm. Accumulating more effectively than other macrolides in cells, particularly circulating phagocytes, it is delivered in high concentrations to sites of infection. This important feature, combined with the extended plasma half-life of AZO, often allows effective single-dose administration for acute bacterial infections (Parnham et al., 2014). The antibacterial effects of AZO are facilitated by its ability to modulate inflammation and immunity in humans by influencing the production of cytokines, decreasing mucus hypersecretion, inhibiting chemotaxis of neutrophils, accelerating apoptosis (Bosnar et al., 2011). AZO is highly effective against two of the three main causative pathogens producing meningitis. Yet, its role in the treatment of CNS infections is very limited due to its relatively high molecular mass that restricts penetration through the BBB.

Numerous drug delivery and targeting strategies have been developed to circumvent the BBB. Disruption of the BBB, chemical modification, molecular antibody technology and various carrier systems have been tried to achieve the transport and the distribution of drugs into the brain (Burgess et al., 2014). One of the promising strategies to enhance the drug penetration to the brain is the

utilization of nanoemulsion as a drug carrier system. Nanoemulsion is a heterogeneous system composed of one immiscible liquid dispersed as droplets within another liquid, it is oil-in-water (O/W) or water-in-oil (W/O) emulsion where the average droplet diameter is between 20 - 200 nm (Solans et al., 2005).
Many drugs are hydrophobic, which limits their water solubility and consequently limiting their bioavailability, making the delivery of water-insoluble drugs to be a primary focus of delivery research. Emulsions provide a central oil core dispersed in water that can act as a reservoir for hydrophobic drugs. Emulsions have long been used for the topical administration, yet, the small size of nanoemulsions make them attractive for parenteral delivery. In addition to solubilization of hydrophobic drugs, nanoemulsions can reduce pain and irritation at the site of injection, improve pharmacokinetics, allow for new forms of administration and can provide a sustained or targeted release of the drug (Lovelyn, 2011).

1.2 Problem Statements

General

1. The presence of the BBB limits the penetration of a large number of pharmacologically active drugs aimed at treating CNS diseases, including meningitis. Several CNS-acting drugs are unable to cross the BBB and reach their site of action because they do not possess the appropriate physicochemical properties.
2. 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 systemic side effects of the drug.

Specific

1. AZO is a broad spectrum antibiotic that possesses a unique pharmacokinetic profile, however, it cannot be used for the treatment of meningitis because it does not have the suitable physicochemical properties that enable it to penetrate the BBB; though it is lipophilic but it is a large molecule with a molecular mass of 749 g/mol. Thus, incorporating the drug into a nano-carrier system seems to be a promising strategy to deliver the drug to the brain.
2. Optimal antibacterial activity for AZO is best achieved with high drug level at the site of infection thus loading the drug into nanoemulsion system can provide a good opportunity to increase the concentration of the drug delivered to the site of action in the brain.
3. The continuous increase in the incidence of infections caused by resistant bacteria poses a significant threat as it may lead to treatment failure and complications. Thus, using nanoemulsion as an antimicrobial drug delivery system has been found to be a promising strategy to overcome bacterial resistance.

1.3 Significance of the Study

Parenteral administration is the most effective route for drug application usually selected for actives with low bioavailability and narrow therapeutic index. Nanoemulsions with their capability to incorporate considerable quantities of lipophilic drugs, plus their combined biocompatibility and ability to protect drug from enzymatic hydrolysis and degradation make them best possible vehicles for parenteral administration. Moreover, the incidence and dosage of injections can be lessened throughout the period of drug therapy as these nanoemulsion systems offer a sustained and controlled release manner of the drug for extended periods of time (Thiagarajan, 2011). Additionally, the absence of flocculation, creaming, and sedimentation combined with a large interfacial area and free energy, provide additional advantages over traditional emulsions for this route of application.

In this study, nanoemulsion system loaded with AZO intended for parenteral administration was designed and developed. Several biocompatible compounds and various preparation methods were investigated to produce a nano-sized carrier system for this antibiotic. Formulation optimization was conducted using Artificial Neural Network (ANN) method. The formulated nanoemulsion was characterized with respect to particle size, polydispersity index, zeta potential, viscosity, osmolality, morphological study, entrapment efficacy, drug content, toxicity, and *in vitro* drug release kinetic. Long-term stability assessment of the formulated nanoemulsion was studied for the evaluation of both chemical and physical stability.

1.4 Objectives

General

The aim of this study was to formulate a nanoemulsion system that can successfully deliver large and highly localized concentrations of AZO to the brain efficiently so that it can decrease the administration dose and minimize the systemic side effects and drug toxicity.

Specific

1. To develop oil-in-water (O/W) nanoemulsion system loaded with AZO for parenteral drug delivery by means of low and high energy emulsification methods.
2. To optimize the compositions for formulating nanoemulsion system in nano-sized range and good stability utilizing ANN.
3. To characterize the physicochemical properties of the formulated nanoemulsion in terms of particle size, polydispersity index, zeta potential, viscosity, pH, osmolality, and morphology. And to assess drug content, entrapment efficacy, *in vitro* drug release, and toxicity of the formulated nanoemulsion.
4. To evaluate the long-term stability of the formulated nanoemulsion with respect to physical and chemical stability with time at various temperatures and storage conditions.

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BIODATA OF STUDENT

Ghaidaa Saad Daood was born in Baghdad/Iraq. She was awarded her Bachelor of Science from Collage of Pharmacy/Baghdad University in 1993. She spent one year as 'Pharmacist under Training' in Ghazi Hariri Hospital for Specialized Surgery/Shahid Adnan Surgical hospital, Medical City, Ministry of Health, Republic of Iraq. From 1994- 1997, she served as 'Pharmacist Practitioner' in Hemedat Health Center/Ninawa. From 1997- 2007, she was appointed as 'Permanent full time Clinical Pharmacist' in Teaching Hospital Al Zahrawi for Surgery Teaching (Al-Jamhuri Teaching Hospital) serving in plastic surgery ward. On 2007, she was transferred to Specialized Hospital Al Khansaa Maternity & Children, Ministry of Health/Ninawa. On 2010, she obtained an unpaid leave to accompany her husband who joined Universiti Putra Malaysia (UPM) to further his PhD study in Faculty of Computer Science and Information Technology. Starting on September 2011, she enrolled her Master of Science programmer at Department of Medicine, Faculty of Medicine and Health Science, UPM under the supervision of Professor Dr. Hamidon Bin Basri.

LIST OF PUBLICATIONS

Daood, G. S., Basri, H., Stanslas, J., Fard Masoumi, H. R., & Basri, M. (2015). Predicting the optimum compositions of a parenteral nanoemulsion system loaded with azithromycin antibiotic utilizing the artificial neural network model. *Royal Society of Chemistry Advances*, *5*, 82654–82665.

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