Evaluation of Tissue Permeability of novel Copper based Anti-arthritic Drugs

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MASTER OF SCIENCE

By

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Your rewards in life are determined by the kinds of problems you are willing to solve for others (Dr. Mike Murdock).

Dedication

I dedicate this entire dissertation to my mother (Georgette Tsumbu), Father (Umba Makaba), Aunt (Fidelise Ngoma) for their love and support during my study at University of Cape Town, and to everyone suffering with Rheumatoid Arthritis.

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I would like to express my sincere gratitude to the following:

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Conferences

Parts of this thesis have been presented at the following conferences:

1. South African Chemical Institute (SACI): Conference on Inorganic Chemistry, INORG2009, University of Free State and Bains Game Lodge, Bloemfontein. 13-17 September 2009, Poster presentation.

2. Democratic Republic of Congo, University of Kinshasa, Seminar on Rheumatoid Arthritis, 04-05 May 2010, Talk.

Abstract

The present study has been designed to investigate the effect of different ligands on the dermal absorption of copper.

Eight copper chelating agents, ethylenediaminetetraacetate (EDTA), glycine and alanine, diethylenetriaminepentaacetic acid (DTPA), homopiperazine, N,N'-di (aminoethylene)-2,6-pyridine-dicarbonylamine (PrDH), N-[2-(2-aminoethylamino) ethyl] picolinamide (H(555)-N) and N,N'-bis[aminoethyl] propanediamide (6UH) were selected and permeability coefficient K_p of copper complexes at room temperature through a Cerasome 9005 measured using a modified Franz cell. The order of permeability coefficient was found to be Gly > H(555-N) > EDTA > 6UH > Homop. > Alan. > PrDH > DTPA > *$CuCl_2.2H_2O$ (Copper(II) Chloride Dihydrate)

The permeability coefficient (K_p) values of complexes with amino-acids were comparable to those found in the literature. All the ligands were found to enhance the diffusion of copper when compared to $CuCl_2$, with enhancement factors ranging from 1.6 to 6.1.

In order to explain the diffusion enhancement, octanol/water partition coefficients (log $K_{o/w}$) of seven complexes at pH 7.00 were measured. The order of lipohilicity of the copper complex was found to be Gly > CuH(555-N) > 6UH > EDTA > PrDH > Homop > DTPA. However, the complexes were still lipophilic with log $K_{o/w}$ = -3.

Linear regression of the measured log K_p, with both MW and log $K_{o/w}$ failed. However, non-linear regression analysis of log K_p, log $K_{o/w}$ and MW was successful.

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Abbreviations

AAS	Atomic Absorption Spectroscopy
CAT	Enzymes catalase
CuEDTA	Copper(II)-Ethylenediaminetetraacetate
CuDTPA	Copper(II)-Diethylenetriaminepentaacetic acid
CuGlyc	Copper(II)-Glycine
CuAlan	Copper(II)-Alanine
CuBIDPAP	Copper(II)-1,15-bis(N,N-dimethyl)-5,11-dioxo-8-(N-benzyl)-1,4,8,12,15-pentaazapentadecane
CuPrDH	Copper(II)-N,N'-di (aminoethylene)-2,6-pyridine-dicarbonylamine
CuAcet.	Copper(II)-Acetylsalicylate
CuHomop.	Copper(II)-Homopiperazine or 2H-1,4-Diazepane
CuPrDPr	Copper(II)-N,N'-bis[2-(2-pyridyl)-methyl]pyridine-2,6-dicarboxamide
Cu[555-N]	Copper(II)-N^1-(2-aminoethyl)-N^2-(pyridin-2-ylmethyl) ethane-1,2-diamine
Cu[H(555)-N]	Copper(II)-N-[2-(2-aminoethylamino) ethyl] picolinamide
Cu[H_2(555)-N]	Copper(II)-N,N'-[2,2'-azanediylbis (ethane-2,1-dyil)] dipicolinamide
Cu(6UH)	Copper(II)-N,N'-bis [aminoethyl] propanediamide
CuDMDM	Copper(II)-N,N' bis [2-(dimethylamino) ethyl] propanediamide
DMARDs	Disease Modifying Antirheumatic Drugs
ESTA	Equilibrium Simulation for Titration Analysis
GSSH	Glutathione-peroxidase
HSA	Human Serum Albumin

HCL	Hollow-cathode lamp
H_{-1}	Hydroxide ion (OH^-)
ML	Metal-ligand
ML_2	1 Metal coordinated to 2 ligands
MQ-water	Milli-Q water
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PAMPA	Parallel Artificial Membrane Permeability Assay
PBS	Phosphate buffered saline
PDMS	Poly (demethylsiloxane)
PMT	Photomultiplier tube
QSARs	Quantitative structure-activity relationships
RA	Rheumatoid Arthritis
SAAS	Slow-acting anti-arthritic drugs
SOD	Superoxide dismutase
SA	Serum Albumin

Symbols

J Flux in g/cm^2s

K_p Permeability coefficient in cm/s

$K_{o/w}$ Octanol/water partition coefficient

ΔP Hydrostatic pressure in Pa

Δh Height fluid in m

φ Fluid density in Kg/m^3

g Acceleration due to gravity in m/s^2

β_{pqr} Formation constant of complex

I_o Incident light

I_t Transmitted light

SE Standard Error

SD Standard Deviation

R^2 Coefficient of correlation

MW Molecular weight in g/mol

D^0 The diffusivity of hypothetical molecule having zero molecular weight

h The membrane thickness

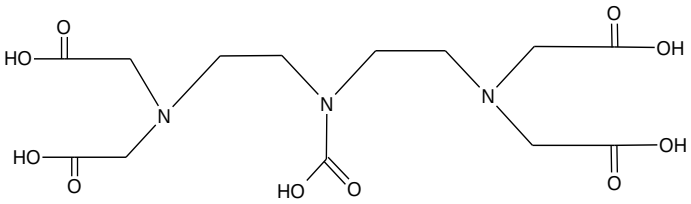
f Constant which accounts for the difference between the partitioning domain presented by octanol and that presented by the membrane lipids

β' Constant which includes a conversion factor for the substitution of molecular weight for molecular volume.

K_m Membrane/donor vehicle partition coefficient of the drug

H_b Hydrogen bonding

Structural formulae of ligands discussed in this work



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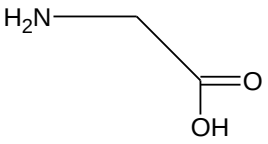

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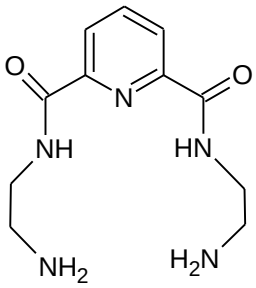
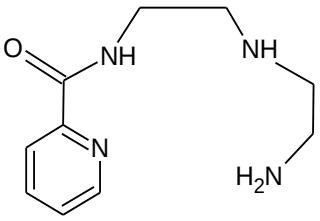
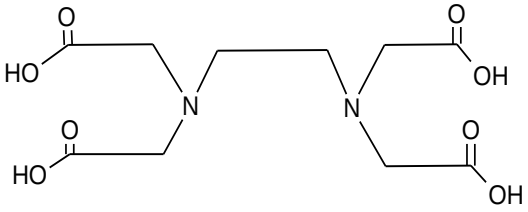


PrDH



[H(555)-N]



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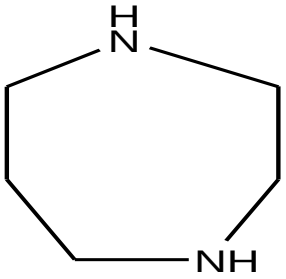
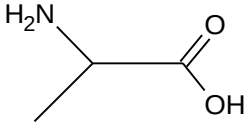
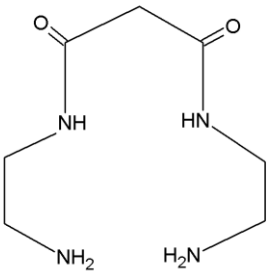


Homop



Alanine



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CHAPTER ONE

INTRODUCTION

1.1 Inflammatory Disease

An inflammation is a protective response elicited by injury (or destruction of tissues) and is characterised by increasing blood supply, activation of defence mechanisms, pain (dolor), heat (calor), redness (rubor), swelling (tumour) and loss of function (arthritis). The beginning of the tissue repair process is required to re-establish normal function but if the inflammation persists, the result will be a lack of normal function which is recognised as "chronic inflammation" (e.g. Rheumatoid Arthritis).

The inflammatory response can be provoked by physical, chemical and biological agents, including exposure to excessive amounts of sunlight, x-rays and radioactive materials, corrosive chemicals, extremes of heat and cold and infectious agents such as bacteria, viruses and other pathogenic microorganisms.[1]

1.2 Rheumatoid Arthritis

The first written reference to arthritis sounds very similar to what is known now as rheumatoid arthritis. In 123 AD, a text from India called Caraka Samhita describes a disease where swollen, painful joints initially strike the hands and feet, then spread to the body, causing loss of appetite, and occasionally fever. Year 1939 brought the first autoimmune theory into the picture. Sr. McFarlane Burnet (head of Research Institute of Melbourne, Australia) proposed that autoimmunity, the process by which the body's defence system malfunctions and attacks its own tissues, causes many arthritis conditions. This theory holds true today.[2]

Rheumatoid arthritis (RA) is therefore defined as a chronic inflammation, systemic autoimmune disorder that causes the immune system to attack the joints, tissues around joints and other organs in the body as well, where it causes inflammation (arthritis) and destruction. The concept "arthritis" means joint disorder featuring by inflammation (a basic way in which the body reacts to infection, irritation or other injury) and Rheumatoid (from Rheumatic: pains caused by rheumatism which is any of several pathological conditions of the muscles, tendons, joints, bones, or nerves, characterized by discomfort and disability). It's a progressive inflammatory disease that has the potential to cause joint destruction, functional disability and can lead to substantial loss of mobility. It has long been suspected that an infection, various bacteria and/or viruses (antigen) may be the cause of the disease process but there is no evidence for this.[3]

RA affects approximately 5% of the World's population, with women being affected two to three times more often than men. Its can occur at any age but is more common between 40-60 years old. There's no cure.[4]

1.3 Immune System Response

The immune system response is a normal and essential response of the body to a harmful stimulus (antigen, bacteria…) characterized by painful inflammation of synovial joints which along with angiogenesis leads to the formation of a tissue called the "pannus".[4-7] The development of pannus causes erosion of cartilage and bone as shown in Figure 1.1.

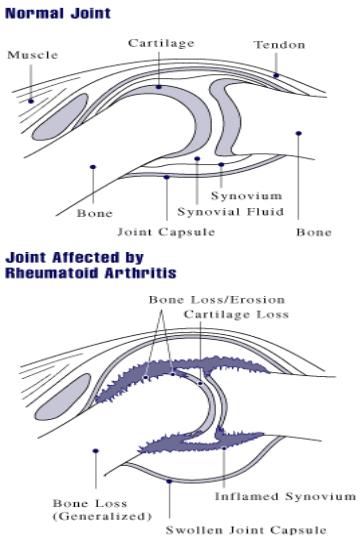

Figure 1.1 Normal and Arthritis joint[8]

The development of bone and cartilage erosion will be followed by swelling, stiffness and loss of joint function (destruction of connective tissue). Progressive degeneration of the joint appears as a result of unrestrained inflammation until the joint becomes deformed[9] as shown in Figure 1.2.[10]

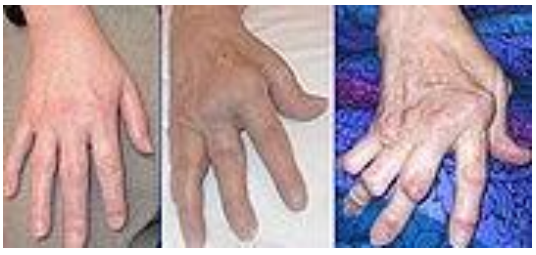

Figure 1.2.1 Early stage | Figure 1.2.2 Later stage | Figure 1.2.3 Chronic stage

1.4 Treatment of Symptoms associated with RA

The medical approach to the treatment of any disease requires the removal of the causal agent(s), interruption of pathological mechanism and alleviation of symptoms or remission of disease. Since the cause of RA still unknown, the primary requirement is not included in therapeutic strategy but the second and third requirements are included by alleviation of symptoms through anti-inflammatory agents.[4]

The following are some of the most important agents currently used by most rheumatologists according to a recent review by Sanz and Alboukrek.[11]

1. Non-Steroidal anti-inflammatory drugs (NSAIDs)
2. Disease modifying anti-rheumatic drugs (DMARDs)
3. Glucocorticoids.

1.4.1 NSAIDs

NSAIDs are the most commonly used because they are well tolerated by patients and decrease the inflammation resulting from disease or injury. They include salicylates such as aspirin (acetylsalicylic acid), the first commercially available NSAID introduced into medicine by Frederick Bayer and Company in 1889.[12] It is the most useful drug for treating RA. If taken in high dosage it can suppress the inflammation[1] but has a complex dosing schedule and dangerous side effects (e.g. gastropathy, renal toxicity and renal insufficiency) if overdoses are taken.[12-13] There are also a series of non-salicylates which are generally weak carboxylic or enolic acid derivatives.[9] Ibuprofen and acetaminophen are widely used for their ability to decrease inflammation and pain. NSAIDs have also analgesic and antipyretic properties but cannot prevent progression of joint destruction or organ damage.[1]

In 1971, J. R. Vane proposed the hypothesis that the anti-inflammatory effects of NSAIDs were due to their ability to inhibit cyclooxygenese (COX) enzymes pathway of prostaglandin (PG) synthesis.[1] A mechanism was proposed by McCarthy D.J (1989) and validated by Weismann M.H (1995) enouncing that: a variety of free radicals derived from molecular oxygen, including superoxide, hydroxyl and perhydroxyl radicals are involved in biosynthesis of prostaglandins and provoke cell injury. The NSAIDs may act as free radical foragers (antioxidants) by preventing the activity of various facilitators of inflammation such as bradykinins, prostaglandins and oxygen radicals.[14-15]

Examples of NSAIDs are shown in Table 1.1 below.

Table 1.1 Non steroidal anti-inflammatory drugs [1]

ACTION			
Anti-inflammatory, Analgesic, Antipyretic	Anti-inflammatory, Analgesic	Anti-inflammatory	Analgesic
Aspirin	Diflunisal	Choline-Mg-Trisilicate	Fenoprofen
Salsate	Azapropazone	Piroxicam	Isoxicam
Phenylbutazone	Tolmetin	Orgotein	Etodolac
Indomethacin	Fenbufen		
Mefenamic acid	Tiaprofenic		
Diclofenac	Meclofenamate Sodium		
Suprofen	Flufenamic acid		
	Tenoxicam		
	Ibuprofen		
	Naproxen		
	Ketoprofen		
	Flurbiprofen		

1.4.2 DMARDs

Diseases modifying anti-rheumatic drugs (DMARDs) are known to have some effect on changing progression of RA. However, they are toxic and patients need to be re-evaluated by their physicians. They are also slow-acting drugs and have been classified as slow-acting anti-arthritis drugs (SAARDs). Therefore, they must be administered over a long period of time (4 – 6 months) before a therapeutic benefit can be achieved.[1,9,15-19] Their dosage, assimilation, side effects and toxicity are given in Table 1.2

Table 1.2 Disease modifying anti-rheumatic drugs[1]

Drug	Dosage	Assimilation	Side effects	Toxicity
<u>Gold salt:</u> Sodium auriothio-maleate (Autm or Myochrysine) Sodium aurothio-sulphate (Auts or Solganal)	50 mg. 1x Week	Injected into muscles and excreted in urine and faeces	Skin rash, proteinuria and blood dyscrasia Inhibit the oxidative burst of polymorphonuclear leukocytes (PMN) by about 30% at concentration above 5µM.	Mild toxicity include stomatitis, pruritis and local rash. Serious toxicity include bone marrow suppression, thrombocytopenia and leucopoenia
Penicillamine	125 - 250 mg per day	Orally administered, well absorbed in stomach.	Low marrow, cell count, dermatitis, nausea and anaemia	Retinal toxicity up to 60%

Sulfasalazine			Fatal conditions Such as haemolytic anaemia, aplastic anaemia and leucopoenia	Retinal toxicity up to 20% Gastrointesti-nal intolerance and musculo-cutaneous toxicity
Azathioprine	100mg/day In 3 doses. 150mg/day if response is slow	Orally Administered And excreted in Urine and faeces		Less toxic than other Immunosup-pressive agents.
<u>Anti-malarial:</u> Chloroquine and Hydroxychloroquine	<u>Adults</u>: 200-250 mg /day ChlQ. 400-600 mg /day HChlQ <u>Children</u>: 3mg/dayChlQ 5 mg/day HChlQ	Orally administered, absorbed in the stomach and excreted in the urine.	Visual impairment due to retinal damage Cutaneous (rash, Change in pig-mentation), cramps, abdominal distension, nausea and diarrheal	

There is also another series of drugs called “synthetic corticosteroids” which occur naturally in the body and take part in many physiological chemical reactions. An example of such drugs in relevance to RA is glucocorticosteroids.[20]

1.4.3 Glucocorticosteroids

The glucocorticosteroids are considered to be potent, fast-acting anti-inflammatory agents such as cortisone, prednisone and dexamethasone. They act to reduce heat, swelling and tenderness at the inflamed joint and have proved to be the best for combating inflammation. However, their mode of anti-inflammatory action is unclear. They are neither antiviral nor antibacterial.[1,9,21]

The corticosteroids on the other side are not considered to have disease-remitting potential, although this has been reported recently to protect against joint erosion but the mode of action has not yet been determined.[11]

The glucocorticosteroids can be administered orally, intra-articularly or at peri-articular sites of local inflammation and their side effects on patients can be dramatic depending on dose and duration of use. Side effects on patients are gastritis, ulceration and severe myopathy. Other complications including effect on bone, gastrointestinal tract, glucose intolerance, increased susceptibility to infections and impaired wound healing. It is also difficult to stop their use once patients have developed a steroid-dependence.[8,15-19,22-24]

It was said previously that the treatment of disease requires: removal of the causal agent(s), interruption of pathological mechanism and alleviation of symptoms or remission of disease. Unfortunately none of the current drugs (NSAIDs, DMARDs and glucocorticosteroids) have managed to treat the disease efficiently but they have tried to interrupt the pathology mechanism, alleviate symptoms and stop the side effects:

- Gastropathy, Renal toxicity and Renal insufficiency (NSAIDs)
- Skin rash, proteinuria and blood dyscrasia (DMARDs)
- Dermatitis, nausea, anaemia, aplastic anaemia and leucopoenia (DMARDs)
- Cramps, abdominal distension and diarrhea (DMARDs)
- Suppression of the pituitary-adrenal axis, gastritis, ulceration and severe myopathy (glucocorticosteroids).
- Gastrointestinal tract, glucose intolerance, increased susceptibility to infections and impaired wound healing (glucocorticosteroids).

This inefficiency of current drugs against arthritis has challenged scientists to develop an anti-inflammatory drug having very few side effects. In 1960, Bonta showed that copper compounds possess anti-inflammatory activities in animals with lower toxicity and Sorenson confirmed this findings.[1,9,25-27] Therefore, copper compounds have been proposed for further research as potential anti-arthritic agents.

1.5 Motivation for study

1.5.1 Copper and Rheumatoid Arthritis

Copper occurs as the metal in oxidation states (0), (I), (II) and unstable (III). Copper occurs as Cu(II) ion in aqueous solution.[9]

Copper is an essential trace element that acts as a cofactor for a variety of enzymes by virtue of its ability to accept and donate electrons under physiologic conditions.[2] It is an essential component of the following enzymes;[4,9,28]

1. The free radical scavenger- superoxide dismutase (SOD) – required for the destruction of superoxide radicals.

2. Cytochrome c oxidase – involved in oxidative metabolism, brain functioning, haem and phospholipids synthesis.

3. Tyrosine – for the synthesis of dihydroxyphenyl-alanine which is subsequently transformed to melanin which is required for pigmentation.

4. Lysyl oxidase - required for connective tissue in the lungs and bones.

Copper is one of the most abundant metallic elements in the human body following iron, calcium, potassium, magnesium and sodium. The body of a healthy male (70 kg) contains 110 mg of copper, much as skeleton (46 mg), skeletal muscle (26 mg), liver (10 mg), brain (8.8 mg) and blood (6 mg). The normal human body contains 80-120 mg of copper compared to Fe 4-5 mg and Zn 1.4-2.3 mg. Metabolic balance studies show that people with daily intakes of 2-5 mg of copper absorb 0.6 - 1.6 mg (32%).[9]

The richest dietary sources of copper are animal liver, crustacean, shell fish, dried fruits, nuts and chocolate. Dietary copper is generally absorbed in the stomach and small intestine, from where it is transported to the liver by the blood as a serum albumin complex. It is in the liver that copper is processed and stored as a metallothionein complex or converted into ceuroplasmin which is released into the blood to meet normal metabolic needs.[7,9,29]

Table 1.3 shows normal human copper concentrations in various biological media and daily excretion levels.[30]

Table 1.3 Copper concentrations in various biological fluids and daily excretion in humans[31]

Tissue	Humans	Daily excretion (μg)
Whole body	800 - 1300 μg L^{-1}	
Serum/plasma	800 - 1750 μg L^{-1}	
Urine	12 - 80 μg L^{-1}	30 – 70
Bile	4.0 μg L^{-1}	2500
Duodenal fluid	0.17 μg L^{-1}	400 – 2200
Synovial fluid	0.2 – 0.5 μg L^{-1}	

Most of the ingested copper is excreted via the bile, the major excretory route, thus preventing tissue toxicity. Trace amounts of copper are excreted via urine except in cases of copper overload. Excess copper in the tissue leads to the production of damaging free radicals and subsequent DNA cleavage.[28]

In blood plasma, at least 90% of copper is irreversibly bound to ceruloplasmin in a non-exchangeable form, while about 10% is reversibly bound to serum albumin and less than 1% is distributed amongst low molecular weight (l.m.w) complexes, predominantly [Cu(histidinate)(cystinate)] as the exchangeable copper fraction in the blood.[31-32]

Serum albumin (SA) has been considered to facilitate the transport of trace metals between tissues and blood and it is a major metal binding protein in the body, with about 40 μg of copper able to bind to the albumin contained in 1ml of human plasma. Albumin appears to be the primary copper carrier protein in the body.[33-34]

The proposed structure of major Cu(II) binding site in HSA is shown in Figure 1.3. The metal ion is bound to the α-NH_2 nitrogen, two peptide nitrogens and the imidazole nitrogen of the N-terminal Asp-Ala-His residue.[35]

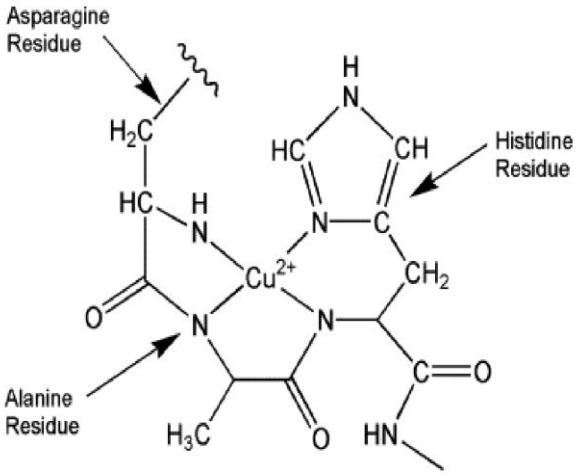

Figure 1.3 Copper binding sites in Human Serum Albumin (HSA).

It has been observed that serum levels of copper are significantly increased during acute phases of RA inflammation, returning to normal with remission. This rise in serum copper is due to an increase in ceruloplasmin concentration and represents a physiological response to inflammation. It has however been shown that ceruloplasmin is a powerful antioxidant and could thus provide protection against cellular destruction, which may be the reason for its increased synthesis. This would however bring about decrease in the concentration of serum albumin copper, as well as of the labile low molecular weight copper complexes.[9]

The mechanism of development of Rheumatoid Arthritis disease can be summarized by the following Figure 1.4.

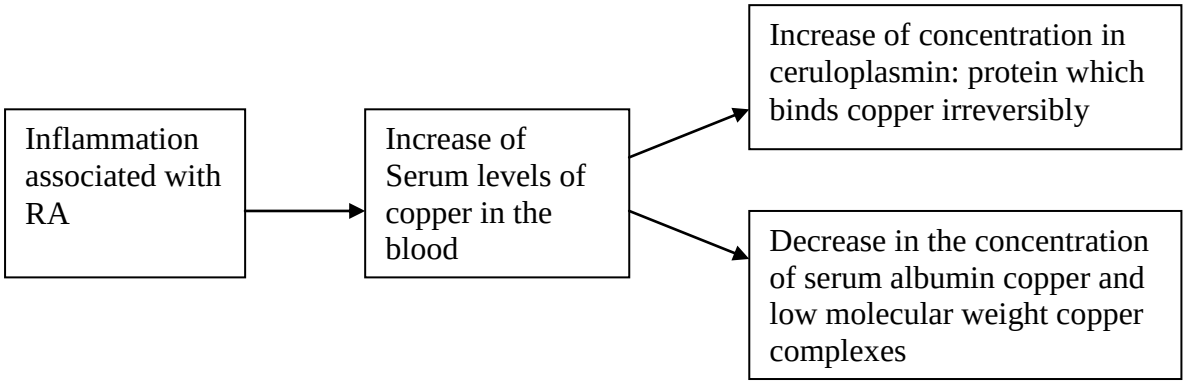

Figure 1.4 Biochemical mechanism of Rheumatoid Arthritis

Therefore, two ways have been proposed by which copper concentration can be adjusted; either from serum albumin by direct complexation using a powerful ligand or from ceruloplasmin using destructive chelators (e.g. penicillamine).

1.5.2 The Anti-inflammatory role of copper

Historically copper has been used therapeutically for over 300 years, and the copper bracelet specifically has long been used as a folk remedy for the treatment of arthritis. Cupriphores from sweat solubilises and promotes copper dermal absorption into the blood stream.[36-37]

Sorenson[25] and Jackson *et al.*[32-33] have shown that Cu(II) complexes are effective in reducing the inflammation associated with RA, enhancing bio-availability of copper and reducing their toxicity.[38-39]

The beneficial role of copper in minimizing inflammation has been attributed to its redox activity, particularly the ability of copper in such enzymes as SOD to remove the highly pro-inflammatory superoxide radical anion O_2^-. The superoxide radical O_2^- has been implicated in the promotion of arthritis due to its ability to degrade hyaluronic acid (HA) which is an important component of the synovial fluid maintaining internal joint connection by acting as lubricant.[9]

The dismutation of O_2^- by copper in SOD is summarized as follows;

$$Cu(II) + O_2^- \longrightarrow Cu(I) + O_2 \quad (1.1)$$

$$Cu(I) + O_2^- + 2H^+ \longrightarrow Cu(II) + H_2O_2 \quad (1.2)$$

$$\text{Net: } 2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (1.3)$$

Equations 1.1, 1.2 and 1.3 explain the antioxidant role of copper in SOD by reducing the radical O_2^- to hydrogen peroxide (H_2O_2) which in cells will be converted to water by the enzymes catalase (CAT) and glutathione-peroxidase (GSSH).[40]

1.6 Aims and Objectives of the research

Cu(II) complexes can be administered orally or by intravenous injection. Although these two methods are easy drug administration routes injection is painful and orally the complex would have to tolerate the harsh conditions in the stomach. Therefore, dermal absorption is proposed as the best route of administration because it is slow (no acute response), tolerable and painless. However, the efficacy of this route depends on the ability of the drug to pass through the skin.[9] The aim of this study then was to investigate the effect of different ligands on the dermal absorption of copper. This was done using the following steps:

- Selecting model systems whereby the permeability of copper can be studied, either by an artificial or biological membrane.
- Building and testing an appropriate apparatus to measure dermal flux (Franz Cell).
- Selecting a membrane.
- Measuring passive transport through the membrane as a function of time.
- Calculating permeability coefficient.
- Measuring octanol/water partition coefficient.
- Studying correlation between permeability coefficient and partition coefficient.

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CHAPTER TWO

TISSUE PERMEABILITY OF COPPER COMPLEXES

2.1 Dermal Absorption study

2.1.1 Skin[1-4]

The skin is the outermost tissue and the largest organ of the body. Its major function is to act as a barrier to the exterior environment (bacteria, harmful chemicals, ultraviolet light and temperature). It also prevents water loss and regulates body temperature.

The skin is essentially composed of three layers: epidermis, dermis and hypodermis (or Subcutis) as shown in Figure 2.1.

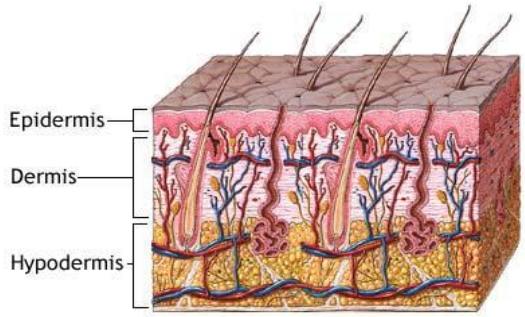


Figure 2.1 Diagram of skin layers[5]

The epidermis contains 5 layers named respectively from top to bottom: stratum corneum, stratum licidum, stratum granulosum, stratum spinosum and stratum basale as shown in Figure 2.2

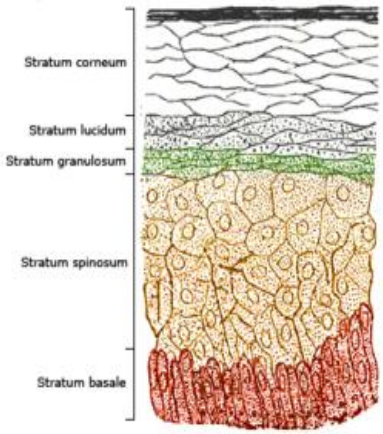


Figure 2.2 Diagram of Epidermis layers[6]

The stratum corneum (in Latin "stratum" = "layer" and "corneum" = "horny" so stratum corneum = horny layer) is essentially composed of 15 to 20 cells layers thick (keratin, protein), water and intercellular lipids as shown in Figure 2.3:

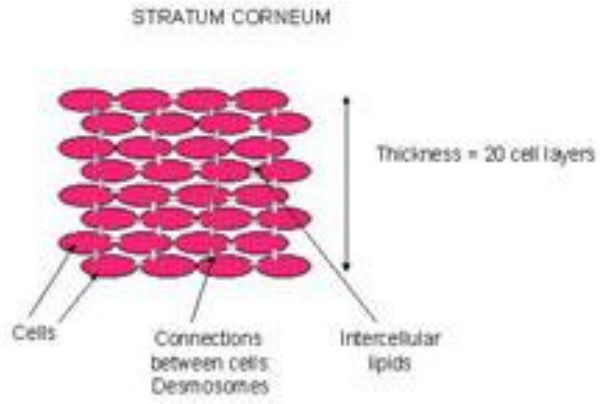


Figure 2.3 Diagram of Stratum corneum[7]

The lipids consist of neutral lipids (60-80%) and sphingolipids (15-35%). The neutral lipids are composed of free sterols, free fatty acids, triglycerides, n-alkanes of C_{19} to C_{34} and squalene. Sphingolipids are composed of 80% ceramides and 20% of glycosphingolipids. The presence of an important mass of lipids gives the stratum corneum its lipophilic nature. This top layer of the skin is extremely important for the skin barrier function.

In terms of the passage of chemicals through skin, the stratum corneum is essentially a lipidic layer, which interfaces with an aqueous medium beneath it. The transport of lipophilic chemicals is through the stratum corneum, and as these compounds must transfer directly into an aqueous medium, a highly lipophilic compound will remain in the stratum corneum.[8]

Thus, for the development of QSARs (Quantitative structure-activity relationships), descriptors of hydrophobicity are likely to be highly influential. However, descriptors for molecular size and hydrogen bonding (which may describe non-covalent interactions with skin proteins) may affect its passage through stratum corneum.[9]

2.1.2 Selection of model system for permeability study

The in vitro study of copper(II) absorption through the skin is generally studied by measuring passive transport through, either a biological membrane or an artificial membrane. An excellent membrane selected for permeability studies must allow the diffusion of the metal, be obtained and conserved easily, not limited to species (age, sex, weight, colour, size…) and give results close to the reality of diffusion through human skin

Two types of membranes have been studied, biological and artificial membranes.

2.1.2.1 Biological membrane[10-15]

Samples of biological membranes are generally obtained from human or animal skins and are characterised by age, sex, site targeted and origin. They are stored in a freezer or frozen in liquid nitrogen.

The tape-stripping technique of human stratum corneum is widely used as method for studying the kinetics and penetration depth of drugs. Its consists of stripping off most of the horny layer (corneocytes) of stratum corneum from adhesive tape.[14]

Note that hairs and all fatty material must be removed before skin extraction and after being frozen, the skin membrane must be thawed at room temperature before the experiment.

2.1.2.2 Artificial membrane

A variety of polymers have been selected as artificial membrane such as poly (dimethylsiloxane) PDMS, liposome (modelled stratum corneum) which is composed of horny layer lipids (liquid crystalline system), silastic (silicone), phospholipids and hydrophobic filter material (phosphatidylcholine in dodecane) coupled with phospholipids.[16-18]

2.1.2.3 Difference between model membranes

A biological membrane is dependent on the time they were obtained, the conditions of conservation and treatment. The age, sex and weight have to be specified for a biological membrane. These considerations are necessary for artificial membranes.

2.1.2.4 Selection of model membrane

Because of the reasons above, an artificial membrane was selected for the permeability study.

The artificial membrane used in this study was "Cerasome 9005". This membrane is a lipid solution which mimics human stratum corneum. Cerasome 9005 was purchased from Germany (Lipoid GmbH, Frigenstr.4, D-67065 Ludwigshafen, 2008) and possess the following characteristics: Consistency (aqueous, transparent to opaque dispersion), colour (off-white), solid matter (10%), Phosphorus (0.11%), pH (7.1) and mean particle size (200 nm).

2.1.3 Diffusion

A drug can across a membrane either by passive or active diffusion. Passive diffusion occurs from a region of high concentration to a region of low concentration and does not require an external energy source while active diffusion occurs from a region of low concentration to a region of high concentration and requires energy and a biochemical carrier to ferry the drug across the membrane.

For passive molecular diffusion, Fick's law of diffusion states that; the amount of material (M) flowing through a unit cross-section (S) of a barrier in unit time (t), is known as the flux (J) and can be described by the following equation[19-20] :

$$J = -\frac{dM}{S.dt} \qquad (1)$$

J= flux in g/cm^{2}s; S= cross section of barrier in cm^2; dM/dt = rate of diffusion in g/s; (M= mass in g; t = time in sec)

The flux is also proportional to the concentration gradient dC/dx, which is described in Equation 2:

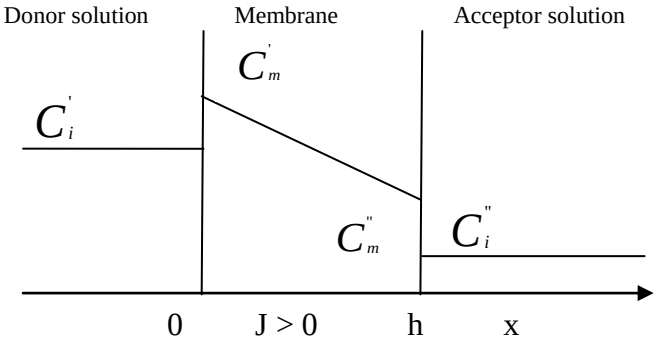
$$J = -D\frac{dC}{dX} \qquad (2)$$

D=diffusion coefficient of a drug in cm^2/s; C=concentration of drug in g/cm^3;

X =distance in centimetres of movement perpendicular to the surface of the barrier.

The diffusion coefficient D is a physical property of the drug molecule which is dependent on temperature, pressure, solvent properties, and chemical nature of the diffusant (drug).

The theoretical relation existing between the flow of diffusion (J), the permeability coefficient (K_p) and the partition coefficient ($K_{o/w}$) can be demonstrated from Fick's law giving the flow of diffusion of a solute through a membrane:



$$J = -D\frac{dC_m}{dX} \qquad (3)$$

This equation can be integrated (considering the membrane as a thin and homogeneous layer):

$$J\int dX = -D\int dC_m$$

$$J = \frac{D}{h}\left(C'_m - C''_m\right) \qquad (4)$$

As the relation between the concentrations of solute in membrane (C_m) and in aqueous solution (C_i) is given by the equation:

$$C_m = K_{o/w}\, C_i \qquad (5)$$

Assuming that the partition equilibrium of the complexes at each membrane interface is faster that its diffusion through the membrane (that means $K'_{ow} = K''_{ow} = K_{o/w}$):

$$J = \frac{D}{h}\left(C'_m - C''_m\right) = \frac{D}{h} K_{ow}\left(C'_i - C''_i\right) \quad (6)$$

Where

$$K_p = \frac{K_{ow} D}{h} \quad (7)$$

Then,

$$J = K_p\left(C'_i - C''_i\right) \quad (8)$$

Considering the initial concentration only, the relation between permeability coefficient (K_p, cm/s) and steady-state flux is given by the equation:

$$K_p = \frac{J}{C'_i} \quad (9)$$

Whereby C'_i is the initial permeate concentration in donor solution of drug and J is its mass passing through a unit area S of the membrane in unit time t.

2.1.4 Partition Coefficient

Partition coefficient is a measures hydrophobicity of a compound. The measure of hydrophobicity can be expressed as the logarithm of partition coefficient between 1-octanol and an aqueous phase.[21-22, 27] For Cu(II)-complex, this may be given as;

$$\text{Log } P_{oct/wat.} = \text{Log} \left| \frac{[Cu^{2+}]_{oct.}}{[Cu^{2+}]_{wat}} \right| \quad (10)$$

Where $[Cu^{2+}]_{org}$ and $[Cu^{2+}]_{wat}$ represent respectively the total concentrations of Cu(II) in 1-octanol and aqueous phases.

2.2 Method and Equipment

Amongst the most widely used methods for studying in *vitro* permeation are the Franz diffusion cell and PAMPA method.

2.2.1 Franz Diffusion Cells

The Franz diffusion cell is the common method used in permeation study. It is based on vertical diffusion between donor and acceptor phase. The following figure (Figure 2.4) shows a typical Franz diffusion cell.[23]

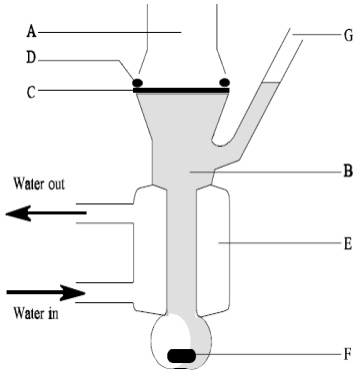


Figure 2.4 Normal Franz cell apparatus

A = donor compartment; B = acceptor compartment; C = membrane; D = O-ring;
E = water jacket; F = stirring bar; G = sampling port.

Note that the membrane is generally mounted between the cell compartments; an O-ring is used to position and seal the membrane and the clamp serves to hold together the two cell compartments. The receiver phase is thermostated at 37°C by circulating water through an external water jacket and the diffusion is done vertically under the influence of gravity.

A. Advantages

Industry standard, acceptor phase can be stirred and thermostated.

Can use artificial or natural membranes and is reusable.

B. Disadvantages

Much larger so need more sample and only one sample at a time.

2.2.2 Parallel Artificial Membrane Permeability Assay (PAMPA)

The Parallel Artificial Membrane Permeability Assay (PAMPA) is a rapid technique for studying many compounds at once.[24]

The method consists of a 96-well filter plate placed in a receiver plate (used as permeation acceptor) and pre-coated with a lipid membrane (used as permeation donor).

The two wells are coupled together, incubated for 5 hours at room temperature, separated afterwards and the concentrations of compounds from both compartments measured. Figure 2.5 shows an example of PAMPA apparatus[25]:

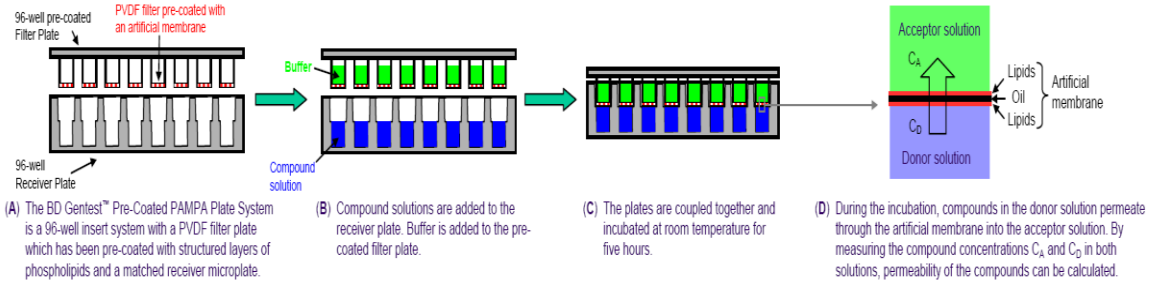


Figure 2.5 Parallel Artificial Membrane Permeability Assay apparatus

A. Advantages

Fast, 96 samples concentrated at a time, can use lipid as artificial membrane.

Small, can use small amounts of sample, cheap and dispensable equipment.

B. Disadvantages

Cannot stir receiver phase and cannot thermostat only one phase.

Cannot use biomembranes.

2.2.3 Choice of Method and Equipment

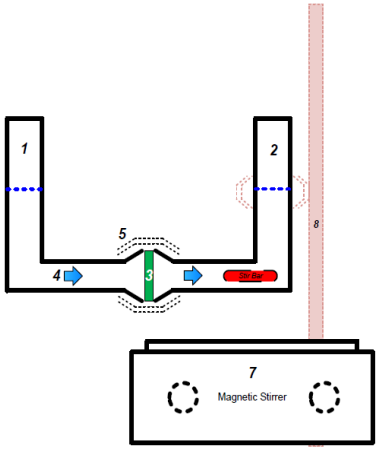
After analysing both methods the Franz diffusion cell was chosen because it is cheap and 17 compounds were planned to be analysed in this study. Also the solution needed for AAS analysis meant that a larger receiver phase was needed. However, the method is still slow and only one compound or concentration could be studied at a time.

2.2.4 Modified Franz Diffusion Cell

One of the problems with the Franz cell is that it is difficult to make, however it is commercially available. Also because of the vertical arrangement, care has to be taken so as not to trap air between the two phases. For this reason a modified Franz diffusion cell was designed and built.

Again to simplify construction the water jacket around the receiving vessel was eliminated. The horizontal design meant that assembly was easy and no air pockets formed. It was also possible to stir both cells. The whole system could be thermostated in a temperature controlled environment, but it was not possible to thermostat only the receiver phase.

Figure 2.6 below shows details of the modified Franz diffusion cell made in our laboratory.



1. Donor phase filled with copper complex
2. Receptor phase filled with MQ-Water
3. Membrane (Green): Filter Paper soaked in cerasome 9005
4. Diffusion Direction
5. Clamp
6. Stirrer Bar
7. Magnetic Stirrer
8. Burette Stand with Clamp

Figure 2.6 Modified Franz cell apparatus

The modified Franz diffusion cell is based on Pascal's law or the Principle of transmission of fluid-pressure which states that "pressure exerted anywhere in a confined fluid is transmitted equally in all directions throughout the fluid.[26]

$$\Delta P = \varphi g (\Delta h) \quad (11)$$

Where:

ΔP is the hydrostatic pressure given in Pascal in the International System of Units (SI system), or the difference in pressure at two points within a fluid column, due to the weight of the fluid;

φ is the fluid density (in kilograms per cubic meter in the SI system);

g is acceleration due to gravity (normally using the sea level acceleration due to Earth's gravity in meters per second squared);

Δh is the height of fluid above the point of measurement, or the difference in elevation between the two points within the fluid column (in meters in SI).

In addition to Pascal's law,

$$\Delta P_{donor} = \Delta P_{receptor}, \text{ means } \Delta h_{donor} = \Delta h_{receptor} \quad (12)$$

Note that the difference of height between acceptor and donor phase will affect the **"Steady state of diffusion".** Therefore, it's recommended to keep the level on the two cylinders constant.

2.3 Selection of Ligands for permeability study

2.3.1 Introduction

A series of anti-inflammatory drugs found on the market, some common ligands and synthetic ligands have been studied which are listed as follows:

- **Anti-inflammatory drugs:** diclofenac sodium salt, propionic acid, acetylsalicylic acid and salicylic acid.

- **Some common ligands:** ethylenediaminetetraacetate (EDTA), glycine and alanine (Amino-acids), diethylenetriaminepentaacetic acid (DTPA) and homopiperazine.

- **Synthetic ligands:** N,N'-di(aminoethylene)-2,6-pyridine-dicarbonylamine (PrDH), 1,15-bis(N,N-dimethyl)-5,11-dioxo-8-(N-benzyl)-1,4,8,12,15-pentaazapentadecane (BIDPAP), N,N'-bis[2-(2-pyridyl)-methyl]pyridine-2,6-dicarboxamide (PrDPr), N^1-(2-aminoethyl)-N^2-(pyridin-2-ylmethyl) ethane-1,2-diamine [555-N], N-[2-(2-aminoethylamino)ethyl]picolinamide [H(555)-N], N,N'-[2,2'-azanediylbis(ethane-2,1-dyil)]dipicolinamide [H_2(555)-N], N,N'-bis[amino ethyl] propanediamide (6UH) and N,N'-bis[2-(dimethylamino)ethyl] propanediamide (DMDM).

2.3.2 Complexation of ligands with Copper at pH 7.00

The selection was based on complexation of ligands with Cu(II) at pH 7.00. Several ligands were rejected after screening because the copper precipitated as $Cu(OH)_2$ after their pH was adjusted to 7.00. These ligands were too weak to keep the copper in solution.

From all previous compounds cited above, only eight compounds did not form a precipitate, so they were selected. The ligands which were selected and studied in dermal absorption are presented as follows:

1. Ethylenediaminetetraacetate (EDTA)
2. Glycine and alanine
3. Diethylenetriaminepentaacetic acid (DTPA)
4. Homopiperazine (Homop)
5. *N,N'-di(aminoethylene)-2,6-pyridine-dicarbonylamine* (PrDH)
6. *N-[2-(2-aminoethylamino) ethyl] picolinamide* [H(555)-N]
7. *N,N'-bis[aminoethyl] propanediamide* (6UH).

2.3.3 ESTA (Equilibrium Simulation for Titration Analysis) software

The program ESTA calculates the distribution of species present in an equilibrium system as a function of the pH of the solution.[27]

ESTA software requires:

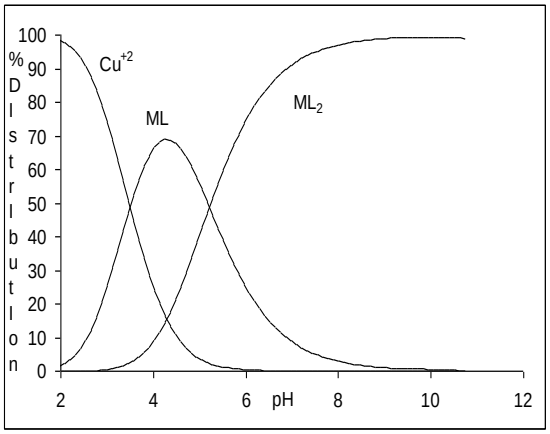
- Stability Constant or formation constant $\beta_{\mathbf{pqr}}$: constant for the formation of a complex from reagents at specific temperature and ionic strength. Refers to the equilibrium below, where p, q and r are the stoichiometric coefficients of the components in the complex

$$pM + qL + rH \rightleftharpoons MpLqHr \qquad (13)$$

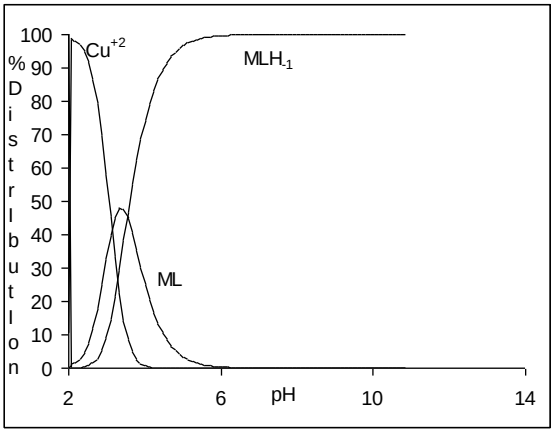
$$\beta_{\mathbf{pqr}} = \frac{[MpLqHr]}{[M]^p[L]^q[H]^r} \qquad (14)$$

- Ratio or concentrations of metal and ligand.
- The temperature and ionic strength are generally fixed.

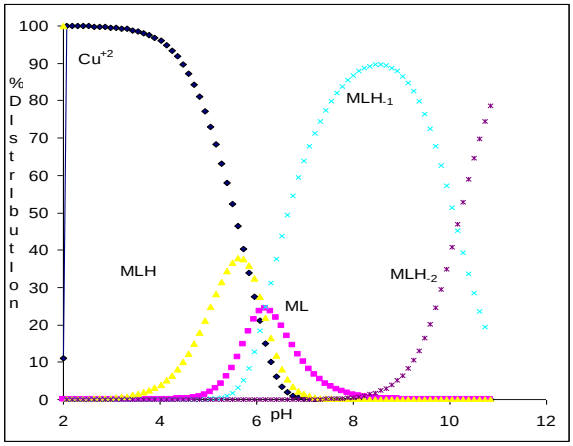
The distributions of copper complexes were determined in the pH range 2-11 by using the ESTA library of computer speciation programs at 298K and an ionic strength of 0.15M (NaCl). Figure 2.7 shows speciation curves for the ligands studied in the pH range 2-12.



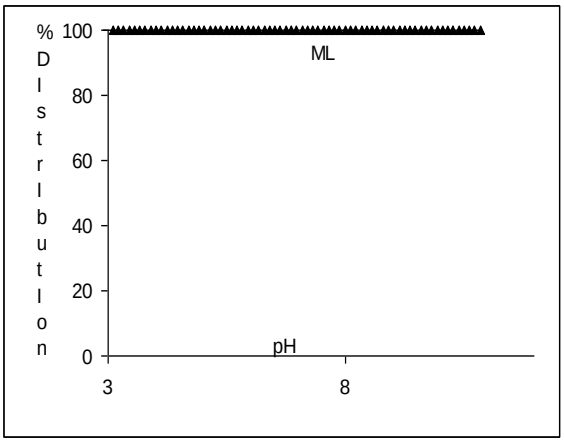
(a) % Distribution of Glycine-copper system (1:2 ratio) as function of pH



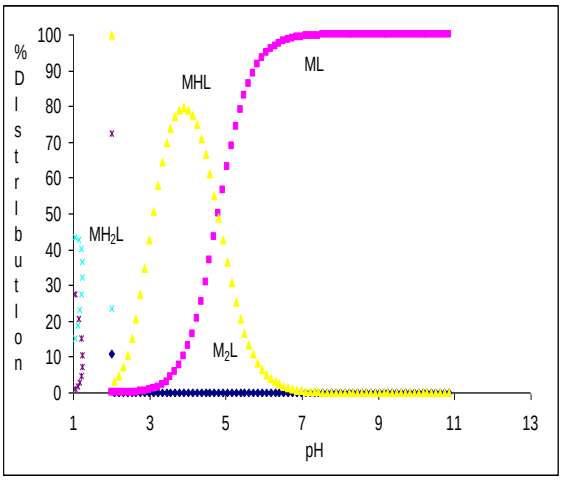
(b) % Distribution of H(555-N)-copper system (1:2 ratio) as function of pH



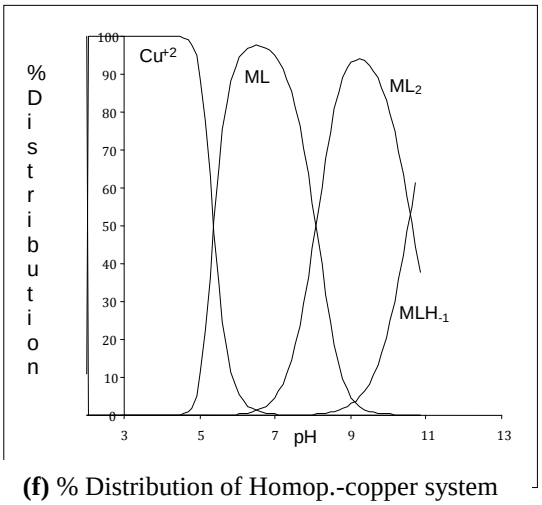
(c) % Distribution of PrDH-copper system (1:1 ratio) as function of pH



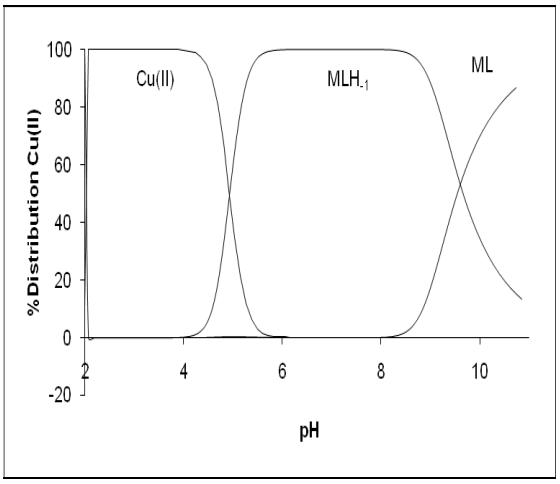
(d) % Distribution of EDTA-copper system (1:2 ratio) as function of pH



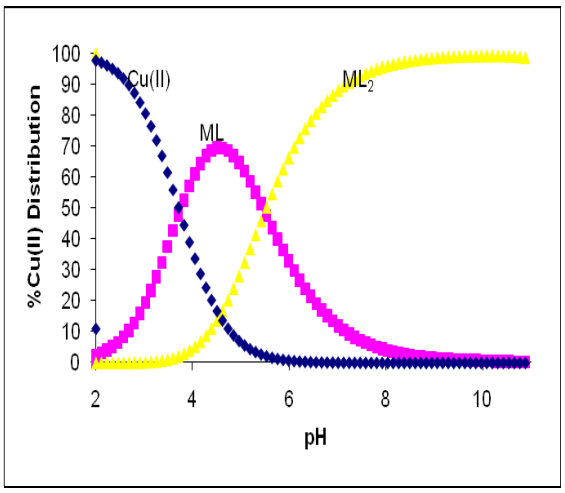
(e) % Distribution of DTPA-copper system (1:1 ratio) as function of pH



(f) % Distribution of Homop.-copper system (1:2 ratio) as function of pH



(g) % Distribution of 6UH.-copper system (1:2 ratio) as function of pH



(h) % Distribution of Alan.-copper system (1:2 ratio) as function of pH

Figures 2.7 shows that calculated speciation of Cu(II)-glycine (a), Cu(II)-H(555-N) (b), Cu(II)-PrDH (c), Cu(II)-EDTA (d), Cu(II)-DTPA (e), Cu(II)-Homop (f), Cu(II)-(6UH) (g) and Cu(II)-Alan.(h) systems as a function of pH. The normal convention of $M_pL_qH_r$ has been used to indicate the stoichiometry of the different species. Water of coordination is not specified.

2.4 Determination of Copper concentration

2.4.1 Atomic Absorption Spectroscopy[28-29]

2.4.1.1 Introduction

Spectroscopy is considered to have started in 1666, with Newton's discovery of the solar spectrum. Wollaston repeated Newton's experiment in 1802 and reported that the Sun's spectrum was intersected by a number of dark lines. Fraunhofer investigated these lines and in 1823 was able to measure their wavelengths. Kirchhoff (1859) and Fraunhofer were observing Atomic Absorption and Emission.

Atomic Absorption Spectroscopy (AAS) is the term used when the radiation absorbed by atoms is measured. The application of AAS to analytical problems was delayed because of the apparent need for very high resolution to make quantitative measurements. In 1953, Walsh overcame the obstacle by use of a line source which was pursued further and published by Alkemade in 1955.

Atomic Absorption Spectroscopy is summarized diagrammatically in Figure 2.8.

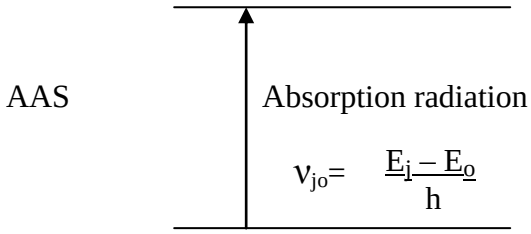


Figure 2.8 Atomic Absorption radiation diagram

Where;

- The horizontal lines represent different energy levels in an atom
- E_o is the term used for lowest energy level (ground state)
- E_j represents greater energy (higher) than E_o
- A solid vertical line refers to a transition involving the absorption of energy as radiation.

The energy of the radiation absorbed or emitted is quantized according to Planck's equation (equation 15),

$$E = h\nu \qquad (15)$$

Where;

- h is Plank's constant
- ν is the frequency of the radiation
- E is the energy difference between the two energy levels in the atom.

The frequency is related to wavelength by following formula,

$$\lambda = C/\nu \quad (16)$$

Where, λ is the wavelength and C as speed of light.

2.4.1.2 Principles and Instruments

AAS is a technique for determining the concentration of a particular metal element in a sample. Based on Beer-Lambert law of absorption of UV or visible light by gaseous atoms which states that the absorbance "A" is equal to the logarithm of the intensity of the incident light (I_o) over the intensity of the transmitted light(I_t) or is proportional to the thickness "l" through which the light is transmitted and to the concentration "c" of the absorbing substance. It's expressed by following equation 17

$$A = Log\frac{I_o}{I_t} = k.c.l \quad (17)$$

Where, A is the absorbance

I_o is the incident light and I_t the transmitted light

k is the absorption coefficient or molar absorptive (quantity of light absorbs by 1cm of a 1 molar solution).

l is the thickness or path length measured generally in centimetre.

c is the concentration of the substance or element (metal).

Atomic Absorption Spectroscopy operates with following steps:

- The liquid sample is evaporated and atomized by injection into a flame
- **Atomization of atoms in Flames**: the emission spectrum of the element under study produced in the light source is passed through an "absorption cell". The important function of this absorption cell is to produce metal atoms in the ground state from the ions or molecules present in the sample.

- This is the most difficult and critical process within the whole AA procedure, the success or failure of an analysis is virtually dependent upon the effectiveness of the atomization.
- The **Hollow-cathode lamp** (HCL) considered as source of light is used to determine the contained element, provide the analytical light line for the element of interest and a constant yet intense beam of the analytical line.
- The **nebulizer** sprays the sample into the flame and sucks up liquid sample at a controlled rate, create a fine aerosol for introduction into the flame and mix of aerosol, fuel (acetylene) and oxidant (compress air) thoroughly for introduction into flame.
- The **Monochromator** isolates analytical lines, photons passing through the flame and removes scattered light of other wavelength from the flame. In doing this, only a narrow spectral line impinges on the PMT.
- **Photomultiplier tube** (PMT) as a detector, PMT determines the intensity of the analytical line exiting the monochromator.
- Detection Limit is defined generally as the smallest concentration (in µg/l) or amount (in g) of the element to be determined that can be detected with 95% probability.

2.4.2 Linear Regression Analysis[30]

The aim of Linear Regression Analysis is to compare a linear equation of absorbance in terms of concentration for a particular analyst with the known reference equation.

2.4.2.1 Analysis aspects for linear calibration

It aims to determine the calibration curve and check its statistical significance with such required to be significant ($p < 0.05$) though highly significant ($p < 0.01$) is preferred.

2.4.2.2 Estimation of unknown concentration X

By re-expressing the fitted linear equation or calibration line $Y = a + bx$, the unknown X value can be estimated as

$$\vec{X} = (\vec{Y} - a)/b \qquad (18)$$

Where $\vec{Y}$ is the average of all absorbance ($\vec{Y}$) in calibration and $\vec{X}$ is the unknown concentration.

An estimate of the error associated with the predicted X value is called Standard Error SE($\vec{X}$) and given by:

$$SE(\vec{X}) = \frac{S_{y/x}}{b}\sqrt{1/m + 1/n + \frac{(\vec{Y} - \overline{Y})}{b^2 S_{xx}}} \qquad (19)$$

Where;

$S_{y/x}$ is the regression standard deviation

m is the number of replicate response measurements made on the unknown test material or number of readings

n is the number of data pairs in the calibration experiment

$\overline{Y}$ is the mean of the experimental responses.

$\vec{Y}$ is the average of all absorbance (Y) in calibration

S_{xx} is the correct sum of squares for X from equation 18

a is the intercept

b is the least squares estimate of (slope parameter) and it's given by equation 20

$$b = \frac{\sum_{i=1}^{n} x_i y_i - \sum_{i=1}^{n} x_i \sum_{i=1}^{n} y_i}{\sum_{i=1}^{n} (x_i)\text{^}2 - \frac{(\sum_{i=1}^{n} x_i)\text{^}2}{n}} = \frac{Sxy}{Sxx} \qquad (20)$$

$$a = \frac{\sum_{i=1}^{n} y_i - b\sum_{i=1}^{n} x_i}{n} \qquad (21)$$

2.4.2.3 Uncertainty in Measurement: Standard Deviation[31]

The broad aims of performing measurements in science are to increase our knowledge about some physical quantity, which is referred to as the measurand. We indicate this incomplete state of knowledge of measurand by a quantity known as the uncertainty. A measurement result in science is meaningless without a quantitative statement of the uncertainty associated with it.

One of the goals of measurement is to minimise the uncertainty associated with the measurand, this can be achieved by good experimental design as well as by collecting as much data as possible. Many books use the term "error" but there's a big difference between "error" and "uncertainty."

Error is an idealized concept that denotes the difference between the measured value and "a true value" of that quantity. Since the "true value" is never known, neither is the error. Uncertainty on the other hand is a well-defined term that can be calculated meaningfully.

The uncertainty associated with the spread in repeated measurements is referred to as the standard deviation of the mean, **S**, and is calculated using the equation 22 below:

$$\mathbf{S} = \sqrt{\sum \frac{d_i^2}{N-1}} \qquad (22)$$

Where Σ means "the sum of"

X_i = a particular value of the measurement

$\overline{X}$ = the mean value

$d_i = x_i - \overline{X}$

N = the number of the measurements.

Therefore, the final answer will be given by equation 23 below:

$$\boxed{\overline{\mathbf{X}} \pm \mathbf{S}} \qquad (23)$$

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CHAPTER THREE

EXPERIMENTAL

3.1 Preparation of Copper Complexes

10mM or 5mM of copper complexes were prepared from $CuCl_2.2H_2O$ (purchased from Merck) and the different ligand in MilliQ-water (MQ-water). The pH of the solutions was adjusting to 7.00 using concentrated NaOH or HCl.

Different metal/ligand ratios were used depending on the ligand so as to avoid formation of a precipitate. Table 3.1 lists the copper-ligand ratios used.

Table 3.1 Ratio of different Copper Complexes

Copper-Ligand	Ratio (M:L)
Cu(II)-PrDH	1:1
Cu(II)-DTPA	1:1
Cu(II)-Glyc.	1:2
Cu(II)-H(555-N)	1:2
Cu(II)-EDTA	1:2
Cu(II)-6UH	1:2
Cu(II)-Homop.	1:2
Cu(II)-Alan.	1:2

Ligands PrDH, H(555-N) and 6UH were synthesized in our laboratory.[1-3] Glycine and EDTA were purchased from Merck. Alanine, DTPA and Homopiperazine were purchased from SIGMA-ALDRICH.

3.2 Modified Franz Diffusion Cells

A modified Franz cells was designed based on a normal Franz cell.[4] Three modified Franz cells were made in which two had $50cm^3$ of volume cylinders and one had $25cm^3$ cylinders on both sides.

Figure 3.1 shows the apparatus used to study diffusion of copper complexes.

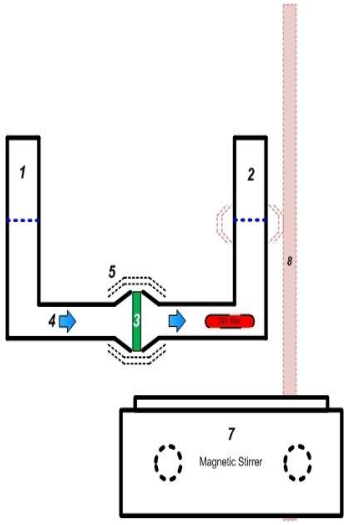

Figure 3.1 Modified Franz cells apparatus

Where;

1. Donor phase filled either with 25ml or 50ml of Copper complex
2. Acceptor phase filled with blank solution (MQ-water)
3. 0.0131g of artificial membrane.
4. Passive diffusion direction
5. Clamp
6. Stirrer bar
7. Magnetic Stirrer
8. Burette Stand with clamp.

The artificial membrane was made using filter paper (Macherey-Nagel) of 3.14 cm^2 discs and thickness 0.12cm. The filter paper was submerged in a Cerasome 9005 lipid solution purchased from Lipoid GmbH (Germany), dried for a few minutes at room temperature and then weighed. The amount of lipid absorbed was determined by mass difference and was typically 0.0131g.

The experiments were performed at room temperature with samples being collected at different times.

Note that PBS was not used to stabilize the pH as is normally done in biological measurements. This is because it coordinates to the copper(II) and hence disturbs the equilibrium being studied. Speciation calculation using ESTA and experiments using buffers confirmed that this interference was taking place.

3.3 Partition Coefficient

The shake flask method was used to measure partition coefficients where the organic phase was 1-octanol pre-saturated with water.[5] Figure 3.2 shows the different steps used to measure the partition coefficient of Cu(II).

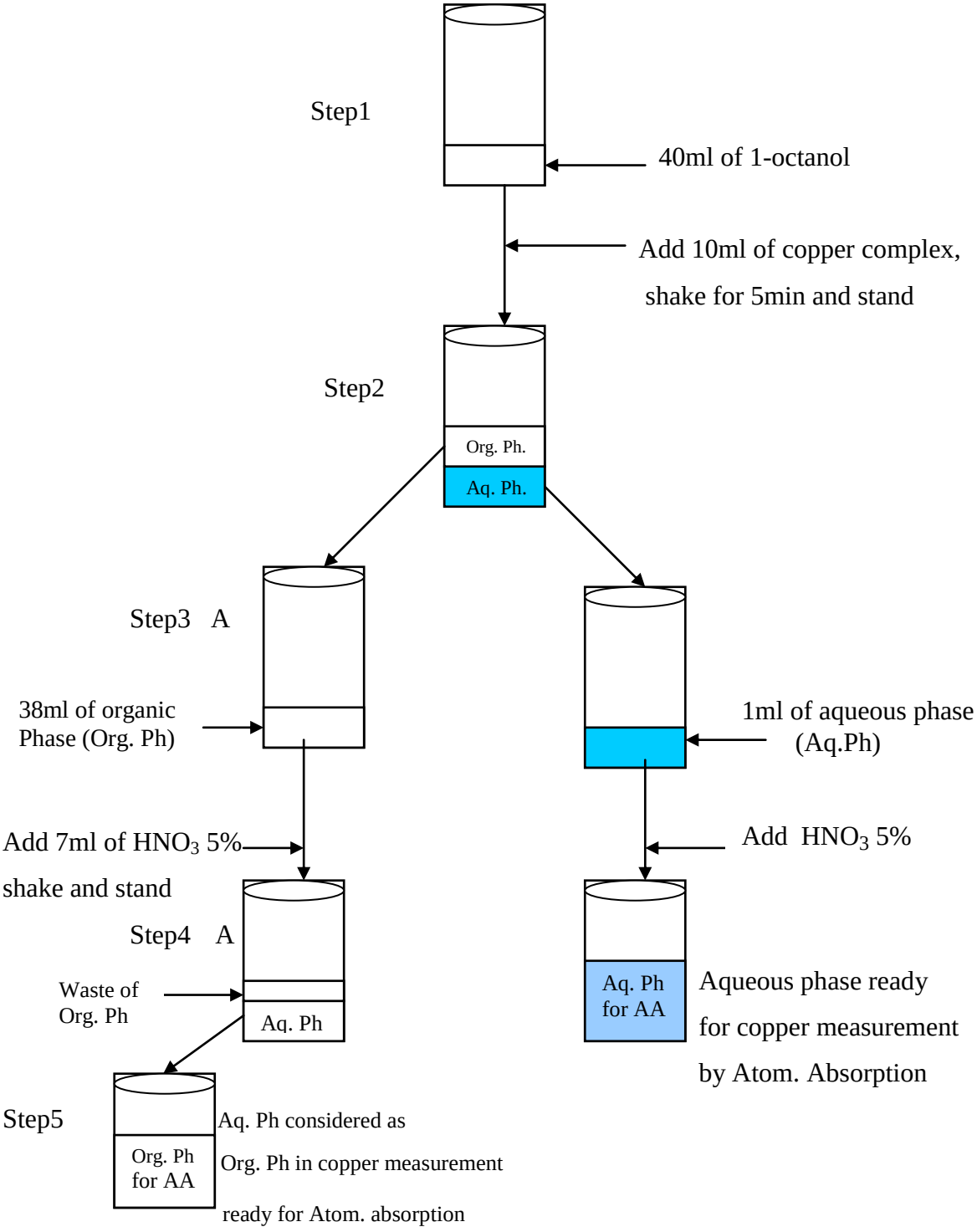

Figure 3.2 Process of measurement of partition coefficient of copper complex

Different steps present in Figure 3.2 are explained as follows: 40ml of 1-octanol, pre-saturated with water, were mixed with 10 ml of the aqueous complex solution and shaken for 5 min. After shaking, the bottle was allowed to stand for 5 min so that the two layers could separate. 1 ml of the aqueous layer and 38 ml of the organic layer were removed using micropipettes.

The aqueous layer was diluted to 10 ml using 5% v/v HNO_3. The organic layer could not be analysed directly using AAS and so the copper first had to be back extracted into an aqueous phase. This was done using 7 ml of 5% v/v HNO_3 solution. The concentrations of copper in the two layers were then measured using AAS.

Because of the very different concentrations of copper found in the two layers (1-octanol and water) after AAS analysis of different samples, the experiments were performed using different volumes until reasonable concentrations were obtained for AAS analysis. These volume differences were of course taken into account when calculating the partition coefficients.

Note that the AAS method used does not determine the concentration of the complexes species, but the total concentration of Cu(II) in each phase. Therefore, the calculation of partition coefficient is based on total concentration rather than concentration of any individual species. Where one species predominates under the conditions used, it is reasonable to assume that the partition coefficient of its species will be measured.

3.4 Atomic Absorption Spectroscopy

3.4.1 Working Conditions

Figure 3.3 is a picture of the Spectra AA-5 Varian used.

Figure 3.3 Atomic Absorption Spectrometer (Spectra AA-5 Varian)

Table 3.2 gives details of working conditions used.[6]

Table 3.2 Working Conditions

Copper Lamp	3mA	
Fuel	Acetylene	1.5units/70pka
Support	Compress air	350pka
Wavelength	324.7nm	
Time	2sec	
Abs. exp. Factor	1	
Slit Width	1nm	
Spectral Band Pass(Abs.)	50	
Detection limit	0.03-10µg/ml	

3.4.2 Calibration of Atomic Absorption Spectrometer

The calibration of the atomic absorption spectrometer was done using a Cu(II) standard (1000mg/l) purchased from SIGMA. Concentrations of 1ppm, 2ppm, 5ppm, 7ppm, 10ppm, 12ppm and 15ppm of copper standard solution were prepared.[6]

Figure 3.4 shows a typical AAS calibration curve.

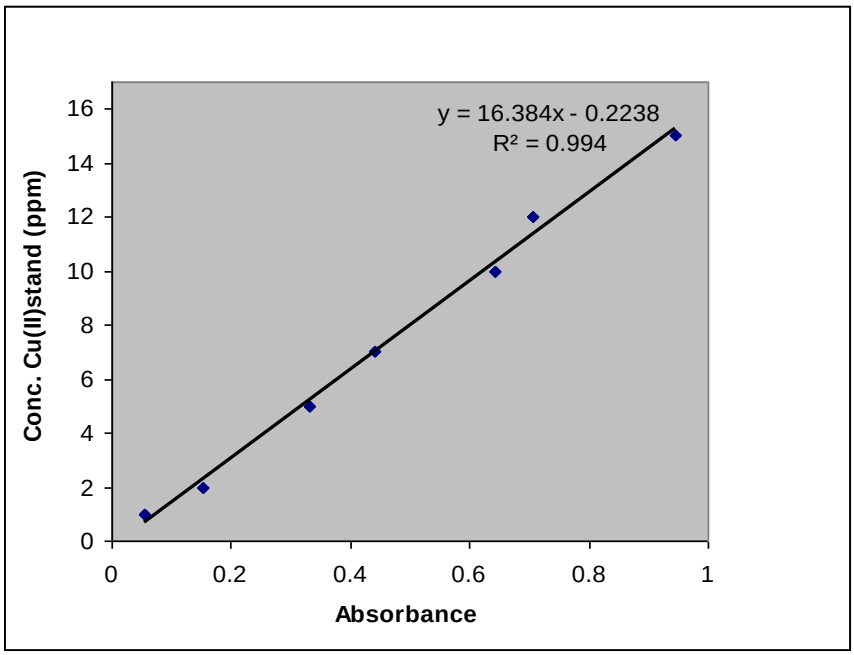

Figure 3.4 Typical Cu(II) calibration curve using Varian Spectra AA-5 Cu(II)

Figure 3.4 above shows a typical calibration curve. In the concentration range used, a linear curve was obtained with $R^2 = 0.99 \pm 0.02$. Linear regression analysis was used to determine the unknown concentration and their standard deviations (Equations 14 and 15, section 2.4.2.2).

3.4.3 Validation of Modified Franz cells

Literature results are not available for the diffusion of Cu(II) through the Cerasome membrane. For this reason the reproducibility of the experimental set up was tested using [CuDTPA] at pH 5.33. The experiment was repeated twice using a donor phase concentration of 635ppm giving a receptor phase concentration of 14.6 ± 1.1 ppm after 6hrs. The standard deviation in the copper AAS analysis (3 replicates) was much lower (0.01), most of the overall error coming from the reproducibility of membrane preparation. An overall error of ≈ 7% was deemed acceptable and is in line with literature measurements using a normal Franz cell. Note that the diffusion at this low pH is much higher than that reported later at pH 7.0.

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CHAPTER FOUR

RESULTS AND DISCUSSION

One of the objectives of this work was to establish if different ligands were able to promote the dermal absorption of Cu(II). This was done by measuring the effect of the ligands on the rate of diffusion of Cu(II) through a Cerasome membrane as a model for skin and comparing these results with partition coefficient determined in 1-octanol. The results are given below.

4.0 Dermal absorption

4.1 Diffusion in Franz cells

4.1.1 Effect of time on diffusion

The effect of time on the amount of copper diffusing through the membrane was studied using alanine and homopiperazine. The effect of these two ligands on the diffusion of Cu(II) was studied for 36hrs. The results are presented in Table 4.1 and Figure 4.1. From this it can be seen that there is a slow induction period of ~8hrs during which time an equilibrium is set up between the donor phase and the membrane. Thereafter, there is a steady state flux of copper into the receiver phase. This is shown by the straight line from 12hrs to 36hrs.

Table 4.1 Diffusion of Cu(II) in the presence of Alanine and Homopiperazine through Cerasome 9005 membrane for 36hrs at pH 7.00

	Cu(II) conc.(ppm)	
Time (h)	**Homopiperazine**	**Alanine**
2	0.03 ± 0.64	0.03 ± 0.65
4	0.5 ± 0.6	1.0 ± 0.6
6	2.1 ± 0.6	2.8 ± 0.6
8	3.9 ± 0.5	5.8 ± 0.5
12	11.0 ± 0.5	15.6 ± 0.5
24	35.4 ± 1.2	57.6 ± 0.1
36	57.4 ± 1.5	98.6 ± 3.5

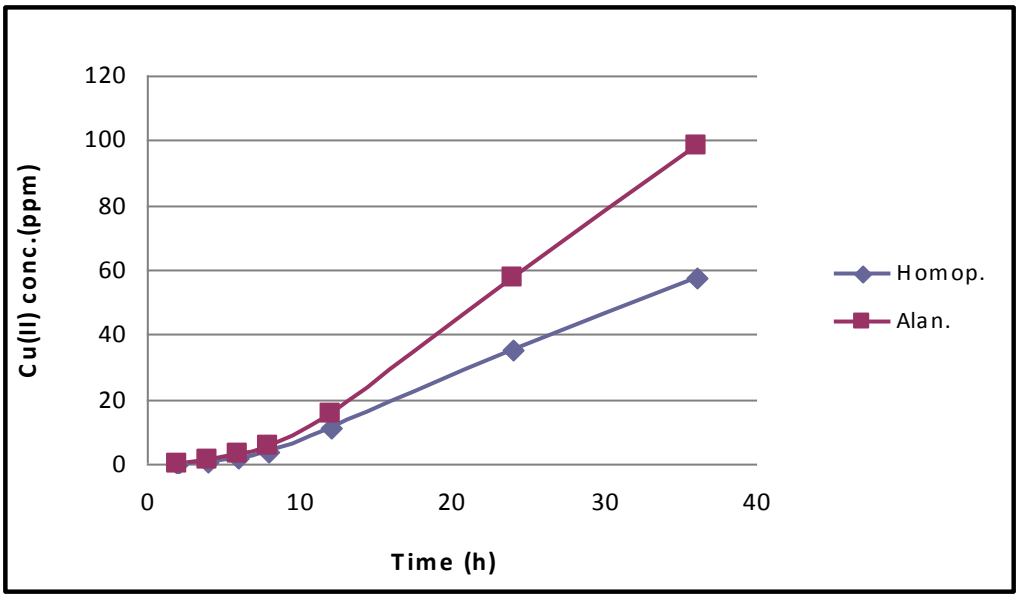

Figure 4.1 Copper diffusion of CuHomop and CuAlan through Cerasome 9005 membrane at pH 7.00

Having established that a steady-state flux was obtained between 8 and 36 hrs, the diffusion of other copper complexes was studied for 24hrs. The results are shown in Table 4.1 and Figure 4.2. Included in the Table 4.2 are results of $CuCl_2.2H_2O$ without any ligand added.

Table 4.2 Concentration (ppm) of Cu(II) in receiver phase in the presence of different ligands at pH 7.00 vs. time

Time (h)	H(555-N)	EDTA	6UH	Gly	Homop	PrDH	DTPA	Alan	*Cl^-
0	0	0	0	0	0	0	0	0	0
2	1.2 ± 0.6	0.2 ± 0.6	0.1 ± 0.6	1. 2 ± 0.6	0.03 ± 0.65	0.3 ± 0.6	0.2 ± 0.6	0.03 ± 0.01	0.6 ± 0.6
4	2.6 ± 0.5	0. 8 ± 0.6	1.0 ± 0.6	5.8 ± 0.5	0. 5 ± 0.6	1.2 ± 0.6	0.5 ± 0.6	1.04 ± 0.01	1.2 ± 0.6
6	4.9 ± 0.5	3.3 ± 0.5	2.8 ± 0.5	10.8 ± 0.5	2.1 ± 0.6	2.7 ± 0.5	0.9 ± 0.6	2.82 ± 0.02	1.9 ± 0.6
8	8.5 ± 0.5	7.2 ± 0.5	4.6 ± 0.5	17.7 ± 0.8	3.9 ± 0.5	4.8 ± 0.5	1.8 ± 0.6	5.79 ± 0.01	2.5 ± 0.6
24	63.0 ± 3.4	53.8 ± 2.8	39.3 ±1.9	84.4 ± 4.7	36. 3 ± 1.8	31.0 ± 1.5	22.5 ±1.0	57. 6 ± 0.1	13.8 ± 0.7

*pH 4.23

Note that the Varian spectra AA-5 was calibrated from 1ppm to 15ppm (ref. Fig.3.4), therefore all concentrations values found either less or close to 1ppm possessed higher standard error (detection limit equalled to 0.03-10μg/ml, Table 3.2).

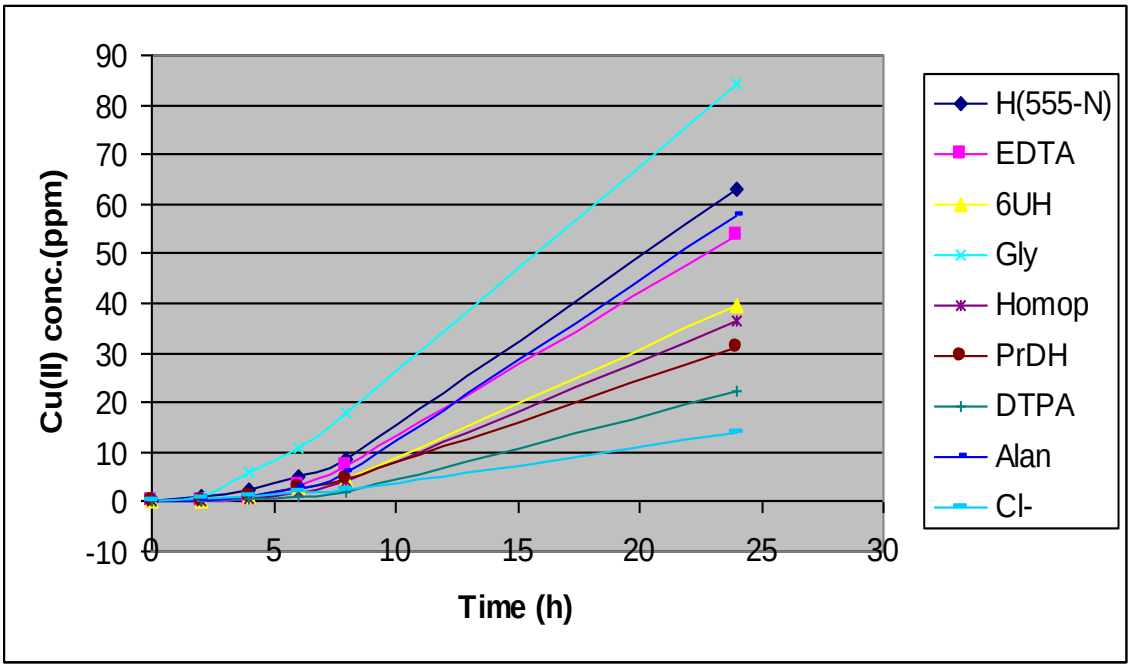

Figure 4.2 Variation of copper conc. of complexes vs. time through Cerasome 9005 membrane at pH 7.00

In Fig. 4.2 above, each complex has the same general trend. There's a slow induction period of 8h, where after there is a rapid increase in diffusion through the Cerasome 9005 membrane.

The reason for the slow induction period of 8h is probably due to the establishment of an equilibrium between the donor phase and the membrane.

Included in Table 4.2 and Fig.4.2 are the results for $CuCl_2.2H_2O$ without any coordinating ligand. At pH 7.00, at the concentration used, $Cu(OH)_2$ would precipitate. For this reason the pure $CuCl_2.2H_2O$ was done at pH 4.23. From Fig.4.2 it is clear that the ligands used are able to keep Cu(II) in solution at pH 7.00 and to promote the passage of Copper through the membrane. This effect is shown in Table 4.3 where the enhancement factor of the ligand after 24 hrs has been calculated. This ranges from 6.1 for Gly to 1.6 for DTPA.

Table 4.3 Enhancement factor of copper diffusion through Cerasome 9005 membrane by ligands after 24 hrs

Ligands	Factor
Gly	6.1
H(555-N)	4.6
Alan	4.1
EDTA	3.9
6UH	2.8
Homop	2.6
PrDH	2.2
DTPA	1.6

A better way of presenting the results is to calculate the flux and permeability coefficient as shown below.

4.1.2 Flux (J) and permeability coefficient (K_p) calculations

The steady state flux and permeability coefficients were calculated from the gradient of the curves[1] in Fig. 4.2. The values obtained for 8 ligands + $CuCl_2.2H_2O$ are given in Table 4.4, Fig.4.3 and Fig.4.4 where flux is expressed by J 10^{-9}g/cm^{2}s and permeability coefficient K_p 10^{-6}cm/s. While the measurements were repeated 3 times, effectively only 2 points are used (8hrs, 24hrs) to calculate the slope, hence calculations of standard deviation are not really meaningful. Also, given the variability in membrane preparation (≈ 7% error), too much emphasis should not be placed on the low analytical standard deviation (<1%).

Table 4.4 Flux of diffusion J (10^{-9}g/cm^{2}s) and permeability coefficient K_p (10^{-6}cm/s) of 8 copper complexes through Cerasome 9005 membrane

Complexes	J 10^{-9}g/cm^{2}s	K_p 10^{-6}cm/s
CuH(555-N)	3.4	10.7
CuEDTA	2.9	9.2
CuAlan	2.0	6.3
Cu(6UH)	2.2	6.8
CuGly	4.2	13.1
CuHomop.	2.0	6.4
CuPrDH	1.6	5.2
CuDTPA	1.3	4.1
$CuCl_2.2H_2O$ *pH 4.23	0.7	1.1

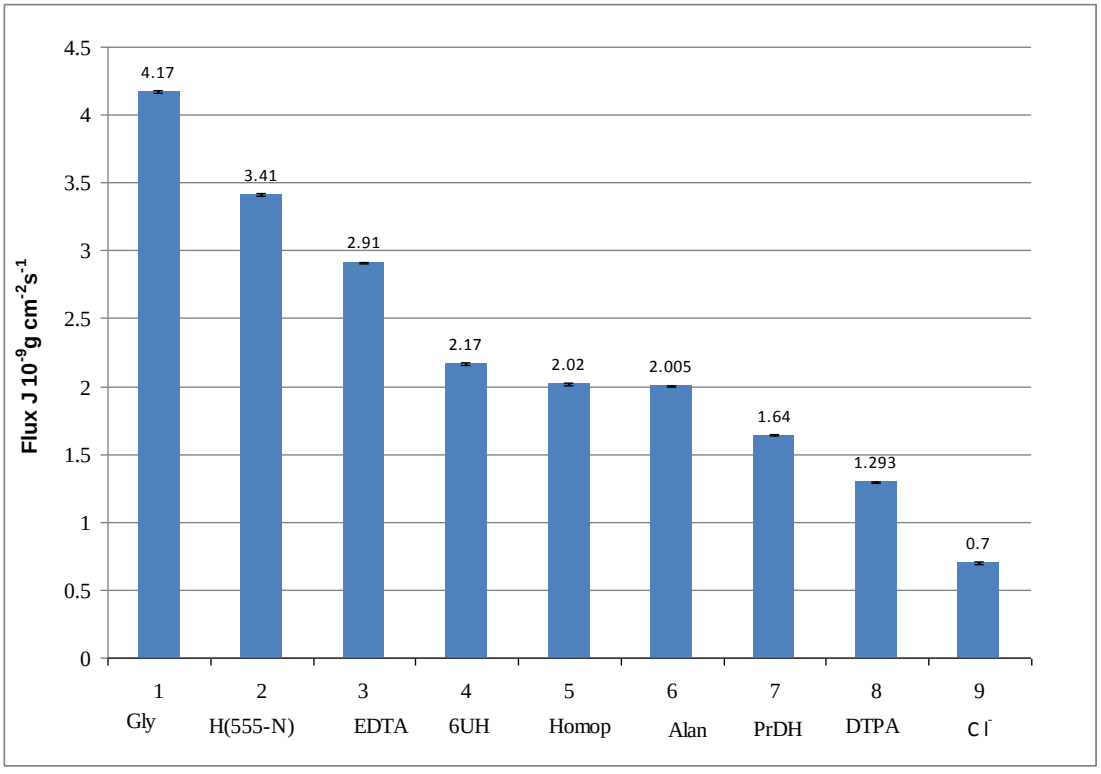

Figure 4.3 Effect of different ligands on the flux of copper through Cerasome 9005 membrane in modified Franz cell from 8-24hrs at pH 7.00

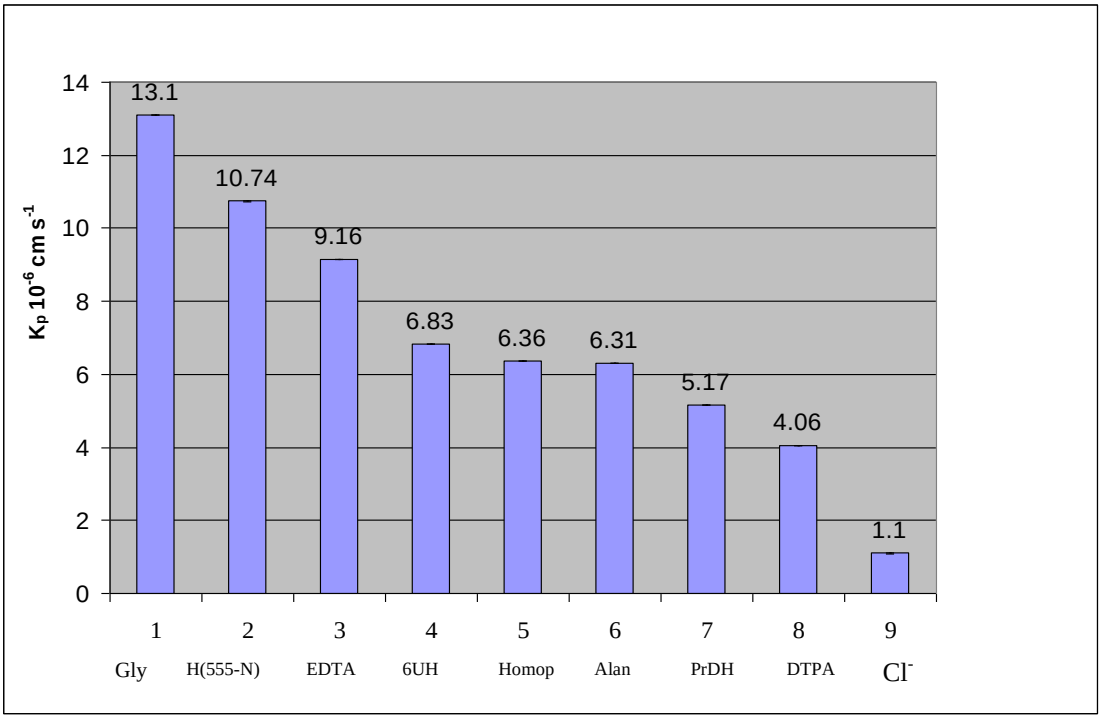

Figure 4.4 Influence of ligands on the permeability of copper complexes through Cerasome 9005 membrane at pH 7.00

Fig. 4.4 shows a decrease in permeability coefficient from Gly to Cl^- detailed as follows: Gly > H(555-N) > EDTA > 6UH > Homop > Alan. > PrDH > DTPA > Cl^-.

The permeability coefficient (K_p) of Cu(II) with amino-acids [Alan (6.310 ± 0.002)10^{-6}cm/s and Gly (13.100± 0.002)10^{-6}cm/s] in Fig. 4.4 are comparable to those found by Mazurowsky[1] [Alan (1.90 ± 0.16) 10^{-6}cm/s, Gly [(1.62 ± 0.06)10^{-6}cm/s]. Mazurowsky measured diffusion through liposomes and the acceptor phase was buffered with potassium phosphate (pH 7.4) which explains the slightly higher values obtained in this study.

Having established that different ligands were able to enhance the passage of Cu(II) through an artificial membrane, the question that has to be asked is why. In the design of new ligands used to promote Cu(II) absorption, it is necessary to identify those ligand factors which are important. Previous studies done by Potts and Guy have established that for organic compounds, molecular weight and partition coefficient (lipophilicity) are important.[7] A correlation between these factors and the measured flux was therefore investigated.

4.2 Partition coefficient

The Shake-flask method was used in octanol/water mixtures to determine the partition coefficient of the Cu(II) complexes.[2]

The octanol/water partition coefficient ($K_{o/w}$) is a quantitative measure of lipophilicity of a substance and Table 4.5, along with Figure 4.5, summarize the partition coefficients measured in 1-octanol/water mixtures.

Table 4.5 Logarithm of partition coefficient - Log $K_{o/w}$ (mean ± SD) of Cu(II) in the presence of different ligands in octanol/water mixtures

Order of lipophilicity	ligand	- Log $K_{o/w}$
1	Gly	2.66 ± 0.12
2	H(555-N)	3.00 ± 0.14
3	6UH	3.02 ± 0.14
4	EDTA	3.07 ± 0.12
5	PrDH	3.45 ± 0.12
6	Homop	3.49 ± 0.14
7	DTPA	3.62 ± 0.12

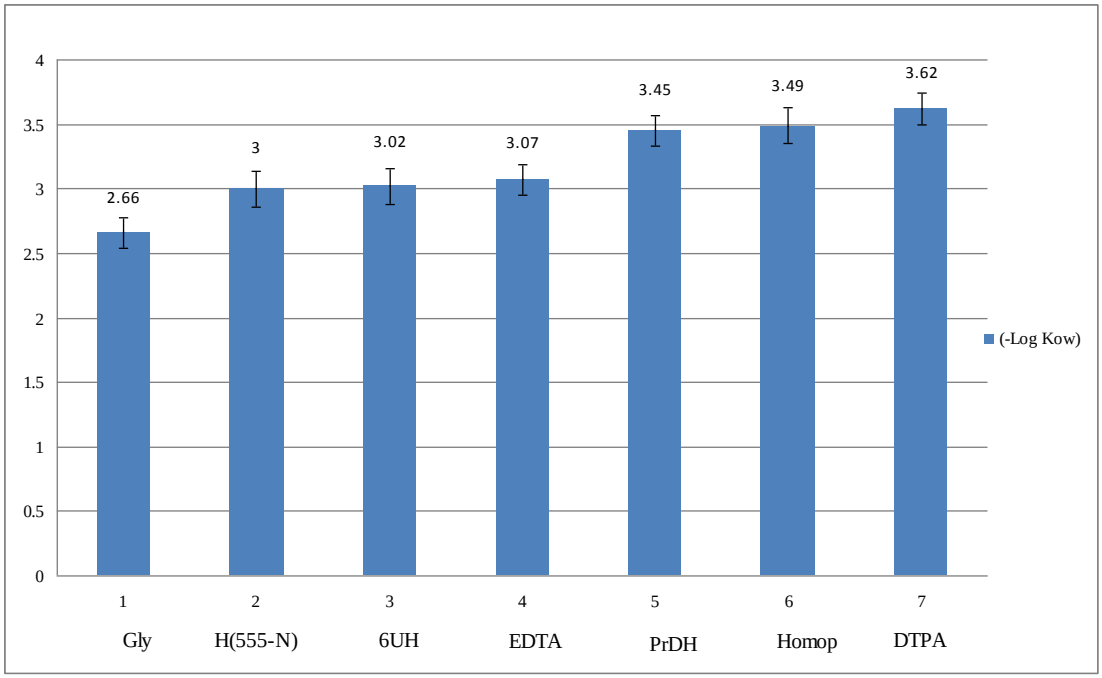

Figure 4.5 Logarithm of partition coefficient (- Log $K_{o/w}$) in 1-octanol/water mixtures of copper with different ligands

Table 4.5 and Figure 4.5 show the effect of different ligands on the partition coefficients (log $K_{o/w}$) of Cu(II) determined at room temperature and pH 7.00.

The principle of like dissolves like (lipophile-lipophile) along with the lipophilic nature of 1-octanol is the main cause of different partition coefficient values observed.

It was suggested that for a drug to be reasonably lipophilic its log $K_{o/w}$ value must be at least 0.6.[4] Negative values of log $K_{o/w}$ indicate that a drug is weakly lipophilic and largely hydrophilic.[3] The negative values of log $K_{o/w}$ found in this study indicate that these complexes are largely hydrophilic and CuGly and CuH(555-N) have higher lipophilicity compared to the other 5 complexes. Similar values have also been found for other copper(II) amino acid complexes.[3,4]

The results of Table 4.5 and Fig. 4.5 are very similar to those found in Fig. 4.4 of permeability coefficient, where Gly and H(555-N) are higher and very close to each other.

CuPrDH and CuH(555-N) have lower log $K_{o/w}$ values [(-3.45± 0.12 and -3.00± 0.14) respectively] compared to those found by Odisitse and Zvimba at pH 7.4 [^{64}CuPrDH, log $K_{o/w}$ = -2.35; ^{64}CuH(555-N), log $K_{o/w}$ = -1.39]. This difference can be explained by the higher pH used in their study. At pH 10, the partition coefficients are: ^{64}CuPrDH, log $K_{o/w}$ = -1.72; ^{64}CuH(555-N), log $K_{o/w}$ = -1.14.

Note that the main purpose of including CuAlan was to compare permeability values found in this work with literature values (Mazurowsky[1]). CuAlan partition coefficients were not determined.

4.3 Data Analysis

Potts and Guy analysed permeability data using a model which depends only upon the size of the drug (MW) and its octanol/water partition coefficient.[7] Therefore, the aim of this analysis was to investigate if there was a correlation between permeability coefficient (K_p), octanol/water partition coefficient ($K_{o/w}$) and molecular weight (MW) of the copper complexes. So far the different parameters have been assigned to the ligand but of course it is the copper complexes of the ligand that are diffusing through the membrane or partitioning between octanol and water.

For this reason the speciation of the copper, in the presence of the different ligands, was calculated. This is done in the next section.

4.3.1 Copper speciation in the presence of different ligands

Table 4.6 gives a list of the speciation of copper in the presence of different ligands at pH 7.0.

Table 4.6 Copper speciation in the presence of different ligands at pH 7.0

Ligand	**Predominant Species formed (pH 7.00)**	**% Cu(II)**
DTPA	$[Cu(DTPA)]^{-3}$	100
PrDH	$[Cu(PrDH)H_{-1}]^{+}$ $[Cu(PrDH)]^{+2}$	70 30
Homop.	$[Cu(Homop)]^{+2}$	95
CuGly	$[Cu(Gly)_2]$	100
CuEDTA	$[Cu(EDTA)]^{-2}$	100
CuH(555-N)	$[CuH(555\text{-}N)H_{-1}]^{+}$	100
Cu(6UH)	$[Cu(6UH)H_{-1}]^{+}$	100

The results in Table 4.6 show that in most cases only one copper species is present in solution and so it is reasonable to assume that the measured parameters refer to this species. In the case of PrDH, two species predominate.

4.3.2 Relationship between logarithm of permeability coefficient (Log K_p) and molecular weight (MW) of copper complexes

Table 4.6 and Fig.4.6 show the investigation of a possible correlation between logarithm of permeability coefficient (log K_p) and molecular weight (MW) of the copper complexes. One difficulty with this analysis is knowing which species are present in solution. The copper speciation is dependent on the concentration of ligand used and the pH of the solution. For this reason speciation calculation were done on all the systems, using experimental conditions and literature equilibrium constants.

The results are given in Table 4.6. A second difficulty is knowing the structure of the species in solution. Thermodynamics will tell us the stoichiometry and equilibrium constant of the different species but not their structure.

For this reason we have to either infer the structure form a knowledge of the ligand and the complex stoichiometry or obtain the structure from the original publication where these have been done. This is necessary because the waters of coordination are generally not specified in the stochiometry but need to be inferred from the structure. This has been done in Table 4.7. In some instances it was not possible to unambiguously decide on the number of coordinated water molecules as the ligand my be bidentate or tridentate and so two alternatives are given. In calculating, the molecular weight it is assumed that the metal is octahedral (tetragonally distorted) with free coordination sites occupied by water.

Table 4.7 Permeability Coefficient (Log K_p), Molecular Weight (MW) and % distribution of Cu(II) in copper-ligands species for 7 Copper complexes

Complexes	MW (g/mol)	- Log K_p	% Cu(II)
CuGly $[Cu(Gly)_2(H_2O)_2]$	250	4.88	100
CuHomop $[Cu(Homop)(H_2O)_4]^{+2}$	236	5.2	95
Cu(6UH) $[Cu(6UH)H_{-1}(H_2O)_2]^{+}$ $[Cu(6UH)H_{-1}(H_2O)_3]^{+}$	304 322	5.16	100
CuH(555-N) $[Cu\{H(555\text{-}N)H_{-1}(H_2O)_2]^{+}$ $[CuH(555\text{-}N)H_{-1}(H_2O)_3]^{+}$	324 342	4.97	100
CuPrDH $[Cu\{(PrDH)H_{-1}(H_2O)_2]^{+}$ $[Cu\{(PrDH)H_{-1}(H_2O)_3]^{+}$	367 385	5.29	70
CuEDTA $[Cu(EDTA)]^{-2}$	356	5.19	100
CuDTPA $[Cu(DTPA)]^{-3}$	457	5.04	100

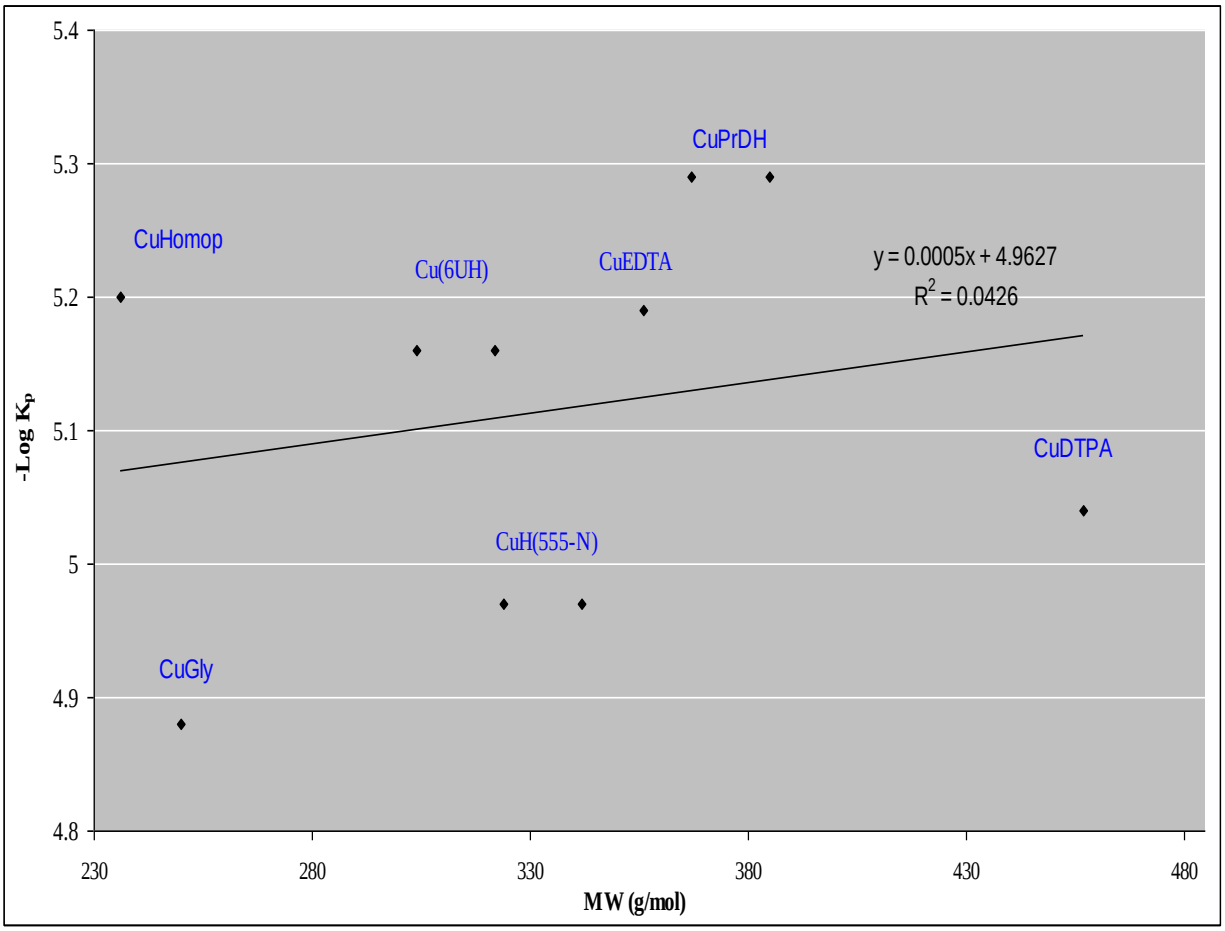

Figure 4.6 Logarithm of permeability coefficient (-Log K_p) plotted against molecular weight (MW) of 7 copper complexes

The coefficient of correlation obtained from Fig.4.6 is $R^2 = 0.0426 \pm 0.005$,which is far from 1 demonstrating that there is no correlation between logarithm of partition coefficient and molecular weight (MW) of all 7 complexes. This conclude that logarithm of permeability coefficient is independent of molecular weight (MW) of the copper complexes

4.3.3 Relationship between permeability coefficient (Kp) and octanol/water partition coefficient (Log $K_{o/w}$)

A correlation was investigated between permeability coefficient (K_p) and octanol/water partition coefficient **(**Log $K_{o/w}$**)**.

A plot of Log K_p against Log $K_{o/w}$ of 7 copper complexes is shown in Figure 4.7.

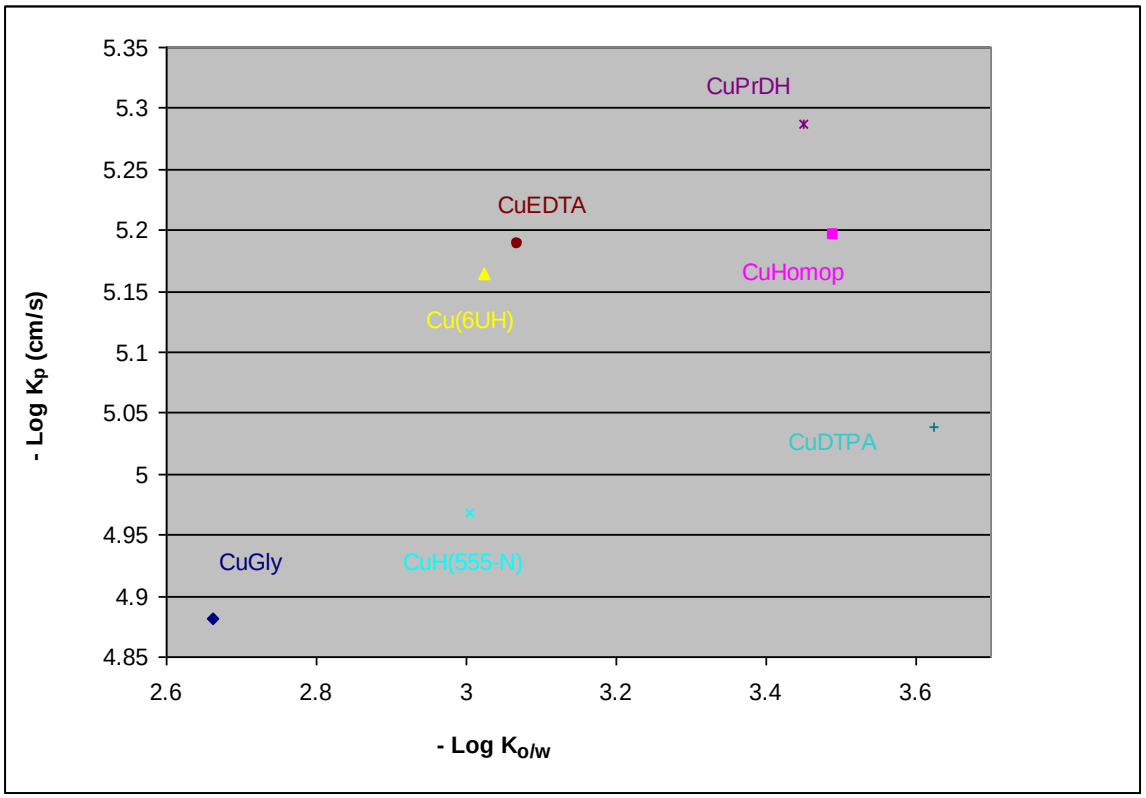

Figure 4.7 Logarithm of permeability coefficient Log K_p plotted against logarithm partition coefficient (Log $K_{o/w}$) of 7 copper complexes

The coefficient of correlation obtained from Fig.4.10 is $R^2 = 0.32 \pm 0.01$, which is again far from 1, demonstrating that there is no correlation between permeability coefficient and partition coefficient of all complexes. This to conclude that permeability coefficient does not linearly depend on octanol/water partition coefficient for all copper complexes.

4.3.3 Relationship between permeability coefficient (Log K_p), octanol/water partition coefficient (Log $K_{o/w}$) and molecular weight (MW) of copper complexes

Various models, based on Fick's first law of diffusion, have been used to predict the relationship between permeability coefficient and partition coefficient of drugs.[4] As said previously (section 4.3), Potts and Guy analysed permeability data using a model which depends only upon the size of the drug (MW) and its octanol/water partition coefficient[7]. The following equation was used to predict skin permeability[8]:

$$\text{Log } K_p = \text{Log } (D^0/h) + f \text{ Log } K_{o/w} - \beta' \text{ MW} \qquad (24)$$

Where,

K_p= the permeability coefficient; D^0= the diffusivity of hypothetical molecule having zero molecular weight; h = the membrane thickness; f = constant which accounts for the difference between the partitioning domain presented by octanol and that presented by the membrane lipids; $K_{o/w}$ = the octanol/water partition coefficient; MW = molecular weight, β' = a constant which includes a conversion factor for the substitution of molecular weight for molecular volume. The Cerasome 9005 membrane thickness was (h=0.12cm)

Multiple linear regression analysis of log K_p upon log $K_{o/w}$ and MW was used to obtain values for **β'**, **Log (D^0/h)** and **f**.

Table 4.8 Relationship between permeability coefficient Log Kp, partition coefficient Log $K_{o/w}$(mean ± SD) and molecular weight (MW) of the copper complexes

Complex	- Log Kp (cm/s)	- Log $K_{o/w}$	MW (g/mol)
CuGly $[Cu(Gly)_2(H_2O)_2]$	4.88	2.66 ± 0.12	250
CuHomop $[Cu(Homop)(H_2O)_4]^{+2}$	5.2	3.49 ± 0.14	236
Cu(6UH) $[Cu(6UH)H_{-1}(H_2O)_2]^+$ $[Cu(6UH)H_{-1}(H_2O)_3]^+$	5.16	3.02 ± 0.14	304 322
CuH(555-N) $[Cu\{H(555\text{-}N)H_{-1}(H_2O)_2]^+$ $[CuH(555\text{-}N)H_{-1}(H_2O)_3]^+$	4.97	3.00 ± 0.14	324 342
CuPrDH $[Cu\{(PrDH)H_{-1}(H_2O)_2]^+$ $[Cu\{(PrDH)H_{-1}(H_2O)_3]^+$	5.29	3.45 ± 0.12	367 385
CuEDTA	5.19	3.07 ± 0.12	355.79
CuDTPA	5.04	3.62 ± 0.12	456.89

The average of **intercept Log (D^0/h), slope (β') and f found after considering species solvated either with $2H_2O$ or $3H_2O$ for Cu(6UH), CuH(555-N) and CuPrDH were**: **Log (D^0/h) = -4.32 ± 0.03**, **β' = - 0.00037 ± 0.00001** and **f = 0.28 ± 0.01.** Equation 25 can be written as follows:

$$\text{Log } K_p = -4.32 + 0.28 \text{ Log } K_{o/w} + 0.00037 \text{ MW} \qquad (25)$$

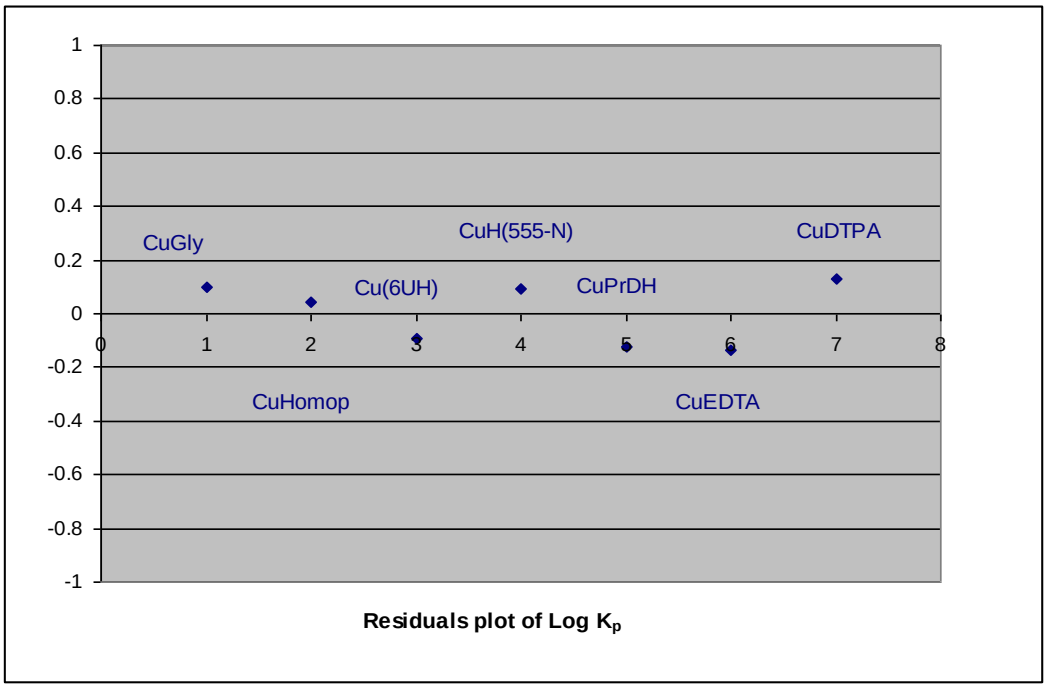

Figure 4.8 Residuals plot of permeability coefficient (Log K_p) measured vs. Log K_p predicted

Fig.4.8 shows a residuals plot of the multiple linear regression analysis. The biggest error is 0.13 or ≈ 2.5% of the measured values of log K_p. This gives us confidence that equation 25 is a good representation of copper diffusion through the Cerasome 9005 membrane.

The values of Log K_p ≈ -5 of our copper-ligands systems are in agreement with values of Log K_p = -5 predicted by Flynn[5] (1990). Flynn used a number of algorithms to predict skin permeability and proposed that if Log $K_{o/w}$ < 0.5 and MW > 150, consequently Log K_p = -5.

The value of f = 0.28 is in agreement with literature which states that f values should be greater than zero[6]. However, using human nail plates, Kobayashi *et al*[6] found f to be - 0.160. Potts and Guy (1992) for a data range [18 < MW < 765; -3 < Log $K_{o/w}$ < 6][7] found f to be 0.71. Yi-Bo Liou *et al* used f = 0.71 in their studies of diffusion through the skin of nude mice (buffer solution, pH 7.4).[9]

The reason why our value of f is < 0.71 may be due to the range of $K_{o/w}$ values for our complexes [-3 < Log $K_{o/w}$ < -2] as opposed to the literature range, and the different membrane used.

The value of f says something about the lipophilic nature of a membrane via K_m. K_m is the membrane/donor vehicle partition coefficient of the drug and is given by:

$$K_m = [K_{o/w}]^f \qquad (26)$$

Equation 26 shows that the lipophilic nature (K_m) increases with an increase of f value. A comparison of lipophilic nature of membranes was studied through f values and equation 26: K_m = human nail plates (f = -0.16) < Cerasome 9005 (f = 0.28) < human skin (f = 0.71).

These results suggest that Cerasome 9005 behaves as a lipophilic partition membrane and human nail plates as a hydrophilic gel membrane.

The values of Log (D^0/h), β' and f found by Flynn were respectively – 2.72, 0.0061 and 0.71 but our values were -4.32 for Log (D^0/h), 0.00037 for β' and 0.28 for f. The difference can be due to the nature of membrane used. Flynn used human skin and our study used a membrane mimic of human skin.

Lien and Gao (1995)[10] analysed a subset of the Flynn data set. They demonstrated that the number of hydrogen bonds that may be formed by a compound (H_b), in addition to molecular weight and biphasic response to hydrophobicity, could model skin permeability (unit cm/h) very well:

$$\text{Log } K_p = 0.84 \log K_{o/w} - 0.07 (\log K_{o/w})^2 - 0.27H_b - 1.84 \log MW + 4.39 \qquad (28)$$

n = 22 and $r^2 = 0.96$

Where, n is the number of compounds analysed and r^2 coefficient of correlation.

This model does emphasise the possible importance of hydrogen bonding. Our study investigated the correlation between permeability coefficient, partition coefficient and molecular weight only but did not study influence of hydrogen bonding. A further investigation based on the Lien and Gao equation will be useful.

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CHAPTER FIVE

CONLUSION AND SUGGESTIONS

Sorenson and Jackson *et al.* have shown that Cu(II) complexes are effective in reducing the inflammation associated with RA[1], enhancing bio-availability of copper and reducing their toxicity.[2-3]

Several studies have been carried out to design stable Cu(II) complexes and evaluate their dermal absorption through the octanol/water mixture partition coefficient.[9,12] The present study has been designed to investigate a new method of studying dermal absorption and to compare these results with partition coefficient measurements.

The Franz diffusion cell was chosen and modified as a method of dermal absorption study because it is cheap, reliable, easy, quick to diffuse samples, and there is no need to analyse the composition of both phases but only receptor phase.

The initial concentration of copper(II) used in the donor phase of modifiez Franz cell was constantly 317.5 ppm.

Cerasome 9005, purchased from Germany (Lipoid company), was chosen as an artificial membrane because it mimics stratum corneum, gives reproducible results, is easy to prepare and maintain. Using an artificial membrane does not require ethics permission.[4]

Ligands EDTA, glycine, alanine, DTPA, PrDH, H(555-N), 6UH and homopiperazine were selected for permeability study after a screening of sixteen ligands at pH 7.00.

Atomic absorption spectroscopy and linear regression analysis software were used to determine copper concentration.[5-6]

Fick's law of diffusion for a steady-state flux (J) and octanol/water mixtures have been used to determine the permeability coefficient (K_p) and partition coefficient ($K_{o/w}$).[8]

Potts and Guy data analysis was used to determine the constants which accounts for the difference between the partitioning domain presented by octanol and that presented by Cerasome 9005 membrane (f). This relationship includes a conversion factor for the substitution of molecular weight for molecular volume (β') and the diffusivity of hypothetical molecule having zero molecular weight (D^0).[7,9]

The diffusion of the copper complexes was studied for 24hrs after establishing a steady state flux that was achieved after 8 hrs using alanine and homopiperazine as ligands. This steady state validated the modified Franz cells used.

Phosphate buffered saline (PBS) in donor and acceptor phase of modified Franz cells was replaced by water (MQ-water) because PBS would compete with the ligands and affecting the steady state of flux.[10]

The permeability coefficient (K_p) values of complexes with amino-acids were comparable to those found by Mazurowsky through nail-plates.[11] This comparison again validated the modified Franz cells used in this study.

It was observed that the ligand affects dermal absorption of copper complexes. In fact, Gly and H(555-N) with K_p respectively equalled to 13.1 10^{-6}cm/s and 10.7 10^{-6}cm/s were found to enhance the most copper diffusion through Cerasome 9005 membrane.

The lipophilicity of CuH(555-N) through its partition coefficient (Log $K_{o/w}$) = -3.00 was found to be less than CuGly and higher than the rest of complexes. The negative values of log $K_{o/w}$ found in this study indicate that these complexes are largely hydrophilic.

In terms of correlation between parameters, no correlation was found between molecular weight and logarithm of partition coefficient, permeability coefficient and molecular weight, permeability coefficient and formation constant Log β_{110} and permeability coefficient and % distribution of Cu(II) in copper-ligands systems.

Multiple linear regression analysis of log K_p upon log $K_{o/w}$ and MW provided values of slope **β'= -** 0.00037 ± 0.00001, intercept **Log (D^0/h) = -** 4.32 ± 0.03 **and f** = 0.28 ± 0.01.

D^0 and **β'** were considered constant and the value of f = 0.28 was in agreement with literature which states that f value should be greater than zero.[6] A value of f = -0.16 was found by Kobayashi *et al*[13] using human nail plates and f = 0.71 was found by Potts and Guy using human skin.[8]

Cerasome 9005 membrane used in this research was confirmed to be lipophilic through positive value of f.

Potts and Guy model did not study the influence of hydrogen bonding. Therefore, a further investigation based on the equation of Lien and Gao[14] will be useful.

The octanol/water partition coefficient makes a greater contribution to the permeability coefficient than molecular weight. Permeability coefficient of the copper complexes increases when partition coefficient increases, this was observed only for CuGly, CuH(555-N), CuPrDH and CuHomop.

The ligand H(555-N) [N-2[2-(2-aminoethylamino)ethyl]picolinamide] will require particular attention for further dermal absorption studies because of permeability coefficient and partition coefficient values found in this research.

CuH(555-N) concentration value after 24 hrs (19.85 ± 0.22)% was found to be very close to those found by Zvimba[12] with ^{64}CuH(555-N) *in vivo* with white mice (albino) 24 hrs post-injection in carcass (19.12 ± 3.44) %, 10.65 % less through urine (30.50 ± 2.30) and 19.31 % higher through blood organ. This difference can be explained *in vitro* study done with artificial membrane Cerasome 9005 in this research.

The partition coefficient values with radioactive copper were found by Zvimba and Odisitse to be a function of pH. Therefore, a further investigation on the effect of pH upon partition coefficient of copper will be useful.

Speciation calculations show that the speciation is very dependent on pH. It would therefore be interesting to see the effect of pH upon copper flux. By varying the pH it may be possible to estimate K_p for individuals species at specific pH.

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