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A STUDY TOWARDS THE SYNTHESIS OF A HYBRID BIOARTIFICIAL LIVER

Tasneem Vally

A STUDY TOWARDS THE SYNTHESIS OF A HYBRID BIOARTIFICIAL LIVER

Tasneem Vally

A thesis submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, 2004

DECLARATION

I declare that this thesis is my own, unaided work, except where otherwise acknowledged. The thesis is being submitted for the degree of Doctor of Philosophy at the University of Witwatersrand, Johannesburg. This thesis has not been submitted before for any degree or examination at any other university.

Signed this _____ day of _____ 20____

_____

Tasneem Vally

ABSTRACT

If one were to imagine the body as a chemical processing plant, then the liver would be equated to the reactor responsible for almost all the metabolic activities of the body. There is hardly any chemical produced, secreted or regulated that the liver is not directly or indirectly responsible for, making the design of an artificial liver a daunting task for any researcher. It is currently impossible to replicate the multitude of functions of a single liver cell, even given all the knowledge and technological advances of the 21st century.

An artificial liver should be able to supplement the failing functions, especially those of a detoxification nature, of an injured or diseased liver. This can be achieved by harnessing the natural capabilities of transformed hepatocytes for use in a hybrid artificial liver. Today, even with all the research currently taking place, liver transplantation is still the most common response to acute or chronic liver failure. The aim of this study was to develop a feasible theoretical design for a hybrid artificial liver reactor. This study draws on various disciplines such as biochemistry, medicine and engineering. A high-level systems approach was employed to the Process Synthesis. Process Synthesis methodology ensures efficiency in the design process which is achieved by conducting the laboratory experiments in parallel with the reactor or process design such that only those experiments or parameters that require optimisation need to be performed.

The capability of the selected transformed hepatocyte cell lines, HuH7 and HepG2 for specific liver functions; the intrinsic cells kinetics for the two cell lines and the sensitivity of the reactor design to the cell line incorporated were determined. The three liver function tests selected were: ammonia metabolism, lignocaine uptake and ^{99m}Tc-DISIDA uptake. Our laboratory data demonstrate that for all three functions, both the cell lines exhibit liver functionality and that their kinetics are fairly similar. This finding suggests that the type of cell line incorporated in the reactor does not

appear to significantly impact on the reactor design. Hence, it would appear from the preliminary screening tests that the choice of cell line incorporated is not a key parameter. Since Chang's method of immuno-isolation by microencapsulation was employed, the kinetics of external mass transfer was compared to the intrinsic cell kinetics to determine the rate-limiting step. Results indicate that the capsular membrane does not significantly impede mass transfer and that intrinsic cell kinetics is the rate-limiting step. The research has demonstrated that a packed bed configuration is a feasible reactor type capable of including the number of cells neccesary to effect the reaction rate essential for the adequate removal of substances required for artificial liver support.

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LIST OF SYMBOLS AND ABBREVIATIONS

^{99m}Tc-DISIDA	Technetium DISIDA (radioactive bilirubin analogue)
^{o}C	Temperature measure, Degree Celcius
APA	Alginate-Poly-L-lysine-Alginate, (microcapsule membrane structure)
BAL	Bioartificial liver
Ci	Activity measure, Curies
C_{max}	Maximum concentration
e	Voidage, fraction of the bed not occupied by solid material
$e_{\min}$	Minimum bed voidage
ECS	Extra Capillary Space in a Hollow fibre device
FCS	Fetal Calf Serum
FHF	Fulminant Hepatic Failure
h	Hill coefficient, Parameter in Hill Kinetic Model
HPLC	High Performance Liquid Chromatography
HALR	Hybrid Artificial Liver Reactor
HFD	Hollow fibre device
ICS	Intra Capillary Space in a Hollow fibre device
K_m	Hill Model Coefficient, Concentration at half the maximum reaction rate
L/D	Refers to reactor geometry, Length to Diameter ratio
ΔP	Pressure drop across the bed of microcapsules in a reactor
Q	Flow Rate through the reactor, Volume per unit time
R_{max}	Maximum rate of reaction

$t_{½}$	Half-life, Time taken for substrate concentration to decrease to half its initial concentration
$t_{½biol}$	Biological Half-life
V_{beads}	Volume of microcapsules in the reactor
V_{blood}	Volume of blood in the reactor
$V_{reactor}$	Volume of the reactor
V_{min}	Minimum volume of the reactor
V_{max}	Maximum volume of the reactor
u_{mf}	Minimum fluidisation velocity

1. INTRODUCTION

1.1 INTRODUCTION TO THE THESIS

As a young naïve graduate student the choice of topic for pursuing postgraduate research studies at the University of the Witwatersrand, Johannesburg seemed easy. (The gravity of the decision was endured much later into the studies). The appeal of being a pioneer, researching in a fairly new field at the University was all too tempting to resist. Biomedical engineering appeared as an exciting alternative to distillation. Since the university did not have a Biomedical Engineering department, expert supervision had to be sourced from various avenues. Within this context, expertise was sourced in the form of my supervisors: Professor Diane Hildebrandt and Professor David Glasser from the field of Chemical Engineering; Dr David Rubin, a medical doctor with a background in Electrical Engineering and Biomedical Engineering; and Professor Nigel J. Crowther, Associate Professor of Chemical Pathology at the University of the Witwatersrand Medical School.

Developing a bioartificial hybrid liver reactor required input from each of these disciplines. It was of paramount importance that the questions raised in each of these areas were addressed by the research. We had the daunting task of consolidating this hybrid mass of work in such a way that it would satisfy both the engineering and medical realms. To this end, a high-level systems approach was employed to the Process Synthesis.

Process Synthesis methodology ensures efficiency in the design process. This is achieved by conducting the laboratory experiments in parallel with the reactor or process design. This implies that only those experiments or parameters that require optimisation need to be performed, saving the researcher the time and costs of unnecessary laboratory work.

The literature review elucidated the various approaches that have been pursued in terms of developing artificial liver support. Since the late 1950s' work in this particular area has been ongoing, with not a single device achieving widespread clinical application. Questions such as the best way to introduce liver functionality into the reactor without the need for immunosuppression; or the sensitivity of the reactor design to the biological component; the rate limiting reaction; how the rate-limiting step influences or affects the reactor design; and what reactor type is most feasible for a Hybrid Artificial Liver Reactor (HALR) forged the direction of the research.

Many more questions were raised in each of the avenues investigated, and further research could have been carried out in order to take the arguments further, but only those issues that had a direct impact on the reactor design were investigated.

All research conducted was consolidated into three independent research papers, submitted or accepted for publication in a peer reviewed journal and presented at various international conferences. Each of these papers appears as a chapter in this thesis, which can be read independently. Each chapter consists of an abstract, its own literature review, introduction and conclusions. Subsequently, the reader may find some repetition or redundancy. The author begs your patience in this regard.

1.2 OVERALL LITERATURE REVIEW

1.2.1 Liver Biochemistry

The liver is the largest gland in the body, weighing approximately 1500 grams in an adult. Owing to its great vascularity, wounds of the liver cause considerable haemorrhage. The liver is the only internal organ of the human body capable of regeneration (WILLIAMS *et al*., 1989).

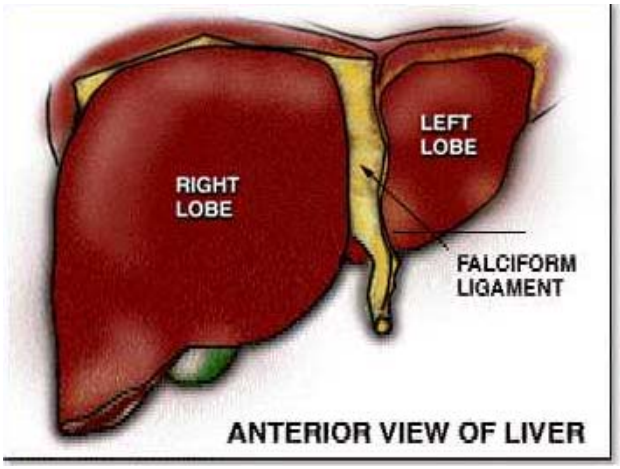

Figure 1.2.1.1: Anterior view of the liver reprinted from www.livercancer.com

Developing a bioartificial hybrid liver reactor required input from each of these disciplines. It was of paramount importance that the questions raised in each of these areas were addressed by the research. We had the daunting task of consolidating this hybrid mass of work in such a way that it would satisfy both the engineering and medical realms. To this end, a high-level systems approach was employed to the Process Synthesis.

Process Synthesis methodology ensures efficiency in the design process. This is achieved by conducting the laboratory experiments in parallel with the reactor or process design. This implies that only those experiments or parameters that require optimisation need to be performed, saving the researcher the time and costs of unnecessary laboratory work. It is commonly agreed, however, that the liver's main purpose is the maintenance of homeostasis (WILLIAMS et al., 1989).

Liver function is manifold, involving:

- Detoxification;
- Carbohydrate metabolism (glyconeogenesis and glycogenolysis);
- Fat and lipid metabolism;
- Synthesis of lipoproteins and cholesterol;

- Protein metabolism;
- Synthesis of plasma proteins (albumin, fibrinogen, coagulation factors, transferrin, globulin);
    - Albumin accounts for colloid osmotic pressure in the plasma,
    - Fibrinogen is crucial to blood clotting,
    - Globulins have enzymatic function in the plasma and enhance immunity;
- Coagulation of Bile Acids;
- Storage of Essential Nutrients and Vitamins;
- Biotransformation of Pharmaceuticals and Vitamins, and
- Immunity (WILLIAMS *et al*., 1989).

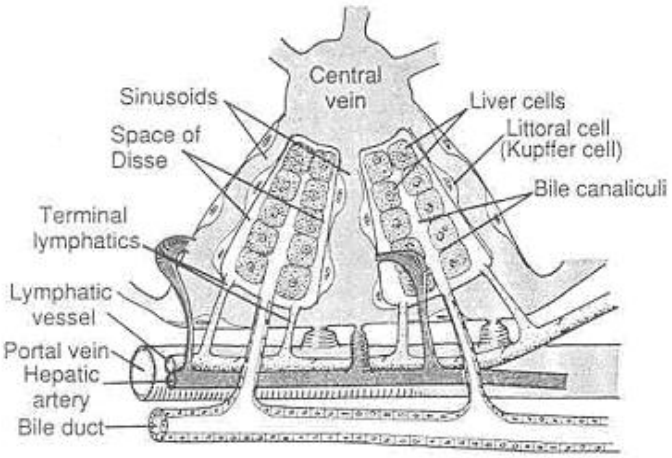

Figure 1.2.1.2 Basic structure of a liver lobule (Reprinted from GUYTON, 1960)

1.2.2 Need for Artificial Liver Support

Hepatic failure is either acute or chronic, depending on the amount of time the liver takes to fail. Liver failure is caused either by trauma, extensive injury or disease. Severe liver disease affects the body's ability to adequately sustain the various metabolic functions discussed previously. This prevents other organs in the body

from efficiently and adequately performing their functions. Fulminant hepatic failure (FHF) is defined as severe acute liver failure complicated by encephalopathy that develops within 8 weeks of the onset of symptoms. In FHF, the time course from onset of illness to death is approximately 10 days with a survival rate of 10-15% at best (DIXIT & GITNICK, 1996).

Hepatic encephalopathy occurs primarily as a consequence of a failure of the liver to adequately detoxify substances that are neurotoxic. Toxic substances include ammonia, short chain fatty acids, mercaptans and phenols (MATSUSHITA & NOSE, 1986). An artificial liver should have the ability to remove these toxins from circulation. The simplest means to artificially mimic liver function is to incorporate cells – an example would be transformed hepatocytes, which possess this inherent ability, into the design of the artificial liver reactor.

The regenerative capacity of the liver is enormous and is the foundation of recovery from FHF. Studies involving rats demonstrate that almost every hepatocyte undergoes a round of cell division about 20 hours after partial hepatectomy. Subsequent rounds of cell division are not as well coordinated, but the liver can double its hepatocyte mass in less than 1 day, which is critical in the treatment of FHF. Even patients with advanced disease may recover rapidly once regeneration begins, and those who recover have no residual liver disease and a normal life expectancy (SUSSMAN, 1996).

Since the first human cadaveric liver transplantation took place in 1963, there have been a number of advances in surgical technique, which has dramatically changed liver transplantation. These include venovenous bypass, caval anastomosis, arterial and venous grafts as well as the development of split, auxiliary (HEATON, 2002) and living donor liver transplantation (SAPA-AP, 2003).

Today, even with all the research currently taking place, liver transplantation is still the most common response to acute or chronic liver failure. Rejection of the transplanted tissue is a frequent outcome, and its control with suppression of the immune system opens the door to a long list of undesirable side effects. In 1988, the United States had 2329 patients on the UNOS (United Network of Organ Sharing) liver waiting list, while in 1995 this number increased 5 fold to 10 529. The number of deaths whilst awaiting orthotopic liver transplantation (OLT) had increased from 196 in 1988 to 254 in 1995 (MEMON, 2000). OLT means replacement of a potentially self-regenerating organ with a lifetime application of immunosuppression and risk of complications such as rejection of the organ. The shortage of viable donor livers and the cost, difficulty and trauma associated with the transplantation, add to the problems encountered with this approach (FOUAD, 1996).

The amount of liver tissue required providing adequate bioactive support is difficult to be determined. Anecdotal reports indicate that as little as 10% of total liver mass is compatible with preservation of life. Even in the most severe form of liver failure, there is always a certain amount of residual liver function and as little as 3-5% of additional liver mass in a liver support system could potentially make a difference (REDDY, 2001). Based on surgical resections, it is estimated that 20-40% of normal hepatocyte mass is necessary to sustain life. This corresponds to approximately 300g of hepatocytes if the patient weighs 70kg (LIU, 2002).

1.2.3 Advances in Hybrid Bioartificial Liver Devices

"Fegato artificiale" as a concept of liver support, was first introduced by Sorentino in 1956 (MATSUSHITA & NOSE, 1986). He demonstrated the detoxifying potential of fresh liver homogenate. Since then alternatives to whole organ transplantation as a possibility for treatment of liver dysfunction have been fervently investigated.

Early attempts used cross circulation of blood from one individual through the liver of another individual or mammal. These techniques were associated with several adverse events and have not been generally accepted (LIU *et al*, 2002). In 1995 a patient regained consciousness in the third hour of a four-hour ex vivo porcine liver perfusion (FOUAD, 1996). A similar incident occurred with a patient's blood being perfused through the livers of four dogs. Obviously the idea of dogs around a patient's bed is not the most practical approach, albeit an entertaining image.

The various methods of support treatments include:

1. *detoxification methods* [1]: extracorporeal blood or plasma treatment, dialysis, heamofiltration, charcoal or resin perfusion, heamodialysis;
2. *biological treatments which support the regulatory liver functions*:
    a. intracorporeal methods: hepatic cell transplantation, auxiliary partial liver transplantation, xenotransplantation,
    b. extracorporeal methods: human and xenogeneic liver perfusion and hybrid liver support (biochemically active liver cells cultivated in bioreactors and perfusion circuits.(IRGANG, 2002; KIMURA, 1980)

[1] Haemodialysis may however have an adverse effect on cardiac output and other haemodynamic parameters leading to increased cerebral oedema (LIU, 2002).

The reason most non-biological approaches have met with little success is because the synthetic and metabolic functions of the liver are absent. The important synthetic functions of the liver can be intravenously administrated. The only function of the liver that cannot be supported nor substituted is that of detoxification. Haemodialysis, Haemoperfusion over charcoal, resins and immobilised enzymes, plasmapheresis and plasma exchange have all been explored (ALLEN, 2001).

Bioartificial devices incorporate isolated cells into bioreactors to simultaneously promote cell survival and function as well as provide for a level of transport seen *in-vivo* (ALLEN, 2001). The biochemistry of the liver is enormous and the full complement of hepatocyte functions required in a BAL to effect positive clinical outcomes has not yet been completely determined. To address this problem, certain liver function tests believed to be essential to aid a failing liver have been employed as markers of adequate liver functionality and therefore of adequate cellular components. The implicit assumption is that hepatocytes capable of a wide array of known functions will also express those unknown functions that may perhaps be essential in liver metabolism (ALLEN, 2001).

Primary liver cells would be ideal, but like whole organs, they are limited in supply. They have been used for BAL application as well as for hepatocyte transplantation. The growth limitations of primary cells has spurred attempts to develop cell lines that can proliferate in culture while maintaining liver specific functions. Cell lines derived from hepatic tumours such as C3A (a subclone of HepG2), have already been used in clinical trials (ELAD®). In the case of tumour derived cell lines, filters preventing transmission have been implemented in the BAL design as extra precaution. Finally, stem cells are being considered for liver disease therapy. Potential sources include embryonic stem cells, adult liver progenitors and trans-differentiated non-hepatic cells (ALLEN, 2001).

Continued innovation in engineering and material science has greatly contributed to the development of extracorporeal liver assist devices. It was the amazing burst of creative engineering called artificial organ science that presented to the world the first working artificial kidney, the heart-lung machine, cardiac valves, pacemakers, and corneal, hip, and arterial prosthesis in only a decade. However the development of such devices, which are effective for liver support have proven more elusive. New discoveries in hepatocyte sourcing and stabilisation coupled with engineering innovation are quickly making BAL devices tailored for use with hepatocytes a reality.

The four main types of BAL devices each with their inherent advantages and disadvantages are:

	Hollow Fibre Device	**Flat Plate and Monolayer**	**Perfused Beds and Scaffolds**	**Encapsulation and Suspension**
Advantages	❑ Well characterised from dialysis application ❑ Attachment surface ❑ Potential for immunoisolation	❑ Uniform cell distribution and microenvironment	❑ Ease of scale up ❑ Promotes 3D architecture ❑ Minimal transport barrier	❑ Minimal transport barrier-Better mass transfer ❑ Best surface area to volume ratio ❑ Scalability ❑ Potential to mimic in vivo microenvironment ❑ Use of whole blood perfusion
Disadvantages	❑ Non uniform cell distribution ❑ Mass transfer limitations imposed by membranes ❑ Scale up, design constraints volume of cells ❑ Plasma instead of whole blood perfusion	❑ Complex scale up ❑ Potential large dead volume ❑ Cells exposed to shear ❑ Low surface area to volume ratio	❑ Non uniform perfusion ❑ Clogging ❑ Cells exposed to sheer forces	❑ Degradation of microcapsules over time ❑ Potential exposure to shear forces if excessive superficial velocities are used.

Table 1.2.3.1: Advantages and disadvantages of current BAL designs

A successful and clinically effective BAL device should satisfy a few key criteria:

- Adequate bi-directional mass transport;
- Maintained cell viability and function;
- Potential for scale up to therapeutic levels.

Bidirectional Mass Transfer

In BAL devices, bi-directional mass transfer is required to provide nutrients to sustain cell viability and allow removal of metabolic products.

Cell viability and function

One of the major obstacles to BAL offering long-term treatment is the inability to maintain highly functional hepatocytes *in-vitro*. Current device designs do little to mimic *in-vivo* microenvironment of normal liver cells.

Scale up

For a device to become a clinical reality it must be scaled up to a size that provides effective therapy. Studies indicate that between 10 and 30% of liver cell mass is required to sustain life. This translates into 150 to 450g of cells needed in a device.

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2. CHAPTER ONE

Determining the Sensitivity of a Hybrid Artificial Liver Reactor Design to the Choice of Cell Line/s Incorporated

This chapter was written in the form of a paper for publication. Certain sections of this paper were also presented at the World Congress on Medical Physics and Biomedical Engineering (WC2003) held under the auspices of the International Federation for Medical and Biological Engineering, held in Sydney, Australia, August 24-29 2003.

Determining the Sensitivity of a Hybrid Artificial Liver Reactor Design to the Choice of Cell Line/s Incorporated

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Abstract

An artificial liver should be able to supplement the failing functions, especially those of a detoxification nature, of an injured or diseased liver. This can be achieved by harnessing the natural capabilities of transformed hepatocytes for use in a hybrid artificial liver. The aims of this study were to determine the capability of the selected transformed hepatocyte cell lines, HuH7 and HepG2 for specific liver functions; the intrinsic cells kinetics for the two cell lines and the sensitivity of the reactor design to the cell line incorporated. The three liver function tests selected were: ammonia metabolism, lignocaine uptake and ^{99m}Tc-DISIDA uptake. This was achieved by characterising the metabolic activities of both cell lines. Data demonstrate that for all three functions, both the cell lines exhibit liver functionality with respect to the initially mentioned tests and that their kinetics are fairly similar. The implication of this result is that the type of cell line incorporated in the reactor does not appear to significantly impact on the reactor design as the reaction rates are of the same order of magnitude. Hence, it would appear from the preliminary screening tests that the choice of cell line incorporated is not a key parameter. If the reaction rates obtained for the two cell lines with respect to the three tests differed significantly, more cell lines might have been tested for application in a Hybrid Artificial Liver Reactor (HALR).

2.1 INTRODUCTION

The biological complexity of the liver is immense. Many of the specific processes involved with this organ's multiple functions are not yet fully understood. However, it is commonly agreed that the liver's main purpose is the maintenance of homeostasis (WILLIAMS *et al*., 1989). As the liver has a substantial regenerative capacity, an artificial liver could provide invaluable time to both patients awaiting liver transplantation and those who would benefit from the reduced hepatic load, such a device would provide, to a recovering liver. Our hybrid artificial liver design is a chimera of both biological and synthetic components. The biological component that needs to be considered is determining a suitable cell line to incorporate into the reactor.

2.1.1 Choosing a Cell Line/s

A suitable reactor should be capable of providing and supplementing essential liver functions. This would provide reprieve to the diseased liver to recover and regenerate or in the case of a bridge to transplantation, mitigate the pathological effects of liver failure until a donor liver becomes available. The consensus among researchers evolving from the charcoal Haemoperfusion era has been to employ transformed hepatocytes (hepatoma cell lines) in their various reactor configurations (MATSUURA, *et al*, 1998). It must be added that the possible risks of using transformed cells still needs to be thoroughly investigated before any consideration of clinical trials can be envisaged, however, this aspect is not addressed in these *in vitro* studies.

The rationale behind using hepatoma cell lines is multifaceted. Firstly, a steady supply of very large quantities of hepatocytes can be made available. Secondly, the use of hepatoma cell lines allows us to harness the natural capabilities of liver cells, instead of attempting to artificially duplicate complex liver functions. Furthermore, it is not possible to keep long-term primary cell cultures of hepatocytes because hepatocytes start to dedifferentiate within a week of culture (DIXIT & GITNICK, 1996). Thirdly, hepatoma cells can be maintained *in vitro* indefinitely without loss of functionality. Primary cells have a limited *in vitro* life span even though they may have important hepatic functions. A steady supply of fresh cells is required, and cell isolation is technically challenging and time consuming. A human immortalised cell line is an attractive alternative as cell division is practically unlimited.

What factors should be considered when choosing a cell line to be used in a bioartificial liver device?

A workshop sponsored by the National Digestive Disease Advisory Board concluded that the ideal hepatocyte for cell-based extracorporeal devices should be human in origin, rapidly and easily grown in culture at high densities and of normal (non-malignant) phenotype. These cells should remain in a well-differentiated state for days or weeks, and consequently, they must display synthetic and detoxifying features of mature hepatocytes (JAUREGUI, 1999). As with most differentiated cells, pure populations of hepatocytes exhibit phenotypic changes resulting in alterations of specific cell functions early in their life span *in vitro* (CLEMENT *et al.*, 1984; NAKABAYASHI, *et al.*, 1984).

Isolated porcine hepatocytes have been used in certain bioartificial liver reactor configurations (MIYASHITA, 2000). Swine has been exploited as a source of hepatocytes due to the availability and the accumulation of biological knowledge of the species (MIYASHITA, 2000). However, they do not provide a viable alternative to primary cells due to the problems associated with porcine cells (MIYASHITA, 2000):

1. functional hepatocytes are only cultured for several days,
2. viability and function are affected by cryopreservation,
3. risk of porcine endogenous retroviral infection.

Research in developing the "perfect cell line" with extended culture capability for incorporation in a liver device is ongoing (TALBOT, 1999).

The cells incorporated in our reactor design were chosen by virtue of their ability to mimic selected liver functions both qualitatively and quantitatively. The functional integrity of two cells lines, HuH-7 and HepG2, were determined based on three labile liver function tests. These were ammonia metabolism, lignocaine uptake by the P450 hepatic system and ^{99m}Tc-DISIDA uptake by the bilirubin pathway.

Research by Nakabayashi *et al* (1984) demonstrated that the HuH-7 cell line exhibits a stable phenotypical expression of some differentiated liver functions in long term cell culture. These cells produced a number of plasma proteins, liver specific enzymes and bile acids. The HuH-7 cell line is reported to produce the following human plasma proteins: albumin, prealbumin, α_1-antitrypsin, ceruloplasmin, fibrinogen, fibronectin, haptoglobin, hemopexin, β_2-microglobulin, transferrin, complement components 3 and 4 and α_1-fetoprotein. The HuH-7 cell line does not contain the hepatitis B surface antigen (NAKABAYASHI, *et al.*, 1982). The HuH-7 cell line has been reported to produce the highest amount of AFP when compared to other human hepatoma cell lines (NAKABAYASHI, *et al.*, 1984).

HepG2 retains hepatic functions well compared to other hepatic cell lines (MIYASHITA, 2000). Studies by NAGAKI, *et al*., (2000) indicate that HepG2 cells are capable of ureagenesis. The following HepG2 products have been reported by the ATCC [14]: α_1-fetoprotein, albumin, α_1-antichymotrypsin, α_1-antitrypsin, complement components 3 and 4 and fibrinogen, among others. C3A is a clonal derivative of HepG2, which exhibits strong contact inhibition of growth and high albumin production. The cells display gluconeogenesis activity and have nitrogen-metabolizing activity comparable to perfused rat livers [14]. HepG2 and C3A (a clonal derivative of HepG2) have been included as the preferred cell lines of choice in Kelly's liver assist device (LAD) (Kelly, 1994).

2.1.2 Liver Function Tests

The aim of this study was not to determine or develop the best cell line to be employed, but to investigate whether a significant difference exists in the metabolic and the uptake rates between different cell lines. Initial kinetic determination was aimed at establishing the sensitivity of the reactor design to the choice of cell line incorporated. Therefore, only two cell lines were initially tested, namely, HuH-7 and HepG2. If the rates determined differed significantly between the cell lines, for the selected liver tests, then more cell lines might have been tested. Liver function comprises many diverse reactions all of which relate to the maintenance of homeostasis (WILLIAMS, 1989). The functions we chose to test include ammonia metabolism, which is a stringent test of hepatocyte integrity; P450 activity (in the form of lignocaine uptake), which relates to the detoxification capability of the hepatocytes and bilirubin removal using an analogue, ^{99m}Tc-DISIDA, shown (Okuda, 1986) to employ the same uptake pathway as bilirubin.

2.2 METHOD AND MATERIALS

Hepatocyte Sub-Culturing Procedure:

Hepatocytes (HuH-7, HepG2) were cultured in Dulbecco's Modified Eagle's Medium DMEM (1.0 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1250 μL penicillin/ streptomycin) supplemented with 5-10% fetal calf serum. *(All chemicals purchased from Sterilab Services, Kempton Park, South Africa)*. Spent medium was replaced every four days. 1.0ml of trypsin *(Sterilab Services, Kempton Park, South Africa)* solution was added to the flask and cells observed under an inverted light microscope until cell layer was dispersed (usually 5 – 15min). **Note:** Clumping was avoided by not agitating the cells by hitting or shaking while waiting for cells to detach. Cells that were difficult to detach were placed at 37°C to facilitate dispersal. 3ml of complete tissue culture medium were added and mixed by gently pipetting. Appropriate aliquots of the cell suspension were added to new culture vessels, at dilutions ranging from 3 to 20 fold. Cell cultures were incubated at 37°C in a 95% air and 5% CO_2 atmosphere.

Method for Cryopreserving Cells:

The cryoprotectant medium consists of Fetal Calf Serum (FCS) supplemented with 10% Dimethyl Sulphoxide (DMSO). 1 ml of trypsin was added to detach cells from the flask. 3 ml of tissue culture medium was added after cell detachment to inhibit trypsin. Cells were counted using a haemocytometer. Cells were spun down for 5 min at 400 G. Supernatant was removed and discarded. Cells were suspended in cryoprotectant medium to yield 10^{6}cells/ ml and 1 ml aliquots were added to cryovials. Cells were frozen overnight at –20°C. Frozen cells were transferred to liquid nitrogen.

Hepatocyte Viability:

Cells were trypsinised and then stained with trypan blue *(Sterilab Services, Kempton Park, South Africa)*. Live hepatocytes exclude trypan blue. Both stained and unstained cells were counted and viability was calculated as follows:

$$Viability(\%) = \frac{(N_{Total} - a)}{N_{Total}} \times 100$$

where N_{Total} is the total number of cells and *a* represents the number of dead (stained) cells.

2.2.1 Ammonia Metabolism

Ammonia has been implicated in hepatic coma (GERLACH, *et al,* 1989; HANKER, 1993). We chose the metabolism of ammonia to urea as a test of the metabolic integrity of hepatocytes, because urea synthesis involves both cytoplasmic and mitochondrial cell functions. The determination of urea kinetics has been used as a measurable index of functionality both in the clinical and investigative realms (KAPLAN *et al*., 1999). Normal liver cells *in vivo* synthesise urea at a rate of 2-3 μmol/ 10^6cells/ 24hrs (YOON *et al*., 1999; ZUCKER *et al*., 1995).

2.2.1.1 Materials and Methods

HepG2 and HuH-7 cell lines were grown in 50mL culture flasks to approximately 95% confluency. Spent medium was replaced by glutamine (60mM) supplemented DMEM. FCS was specifically excluded from the cell culture medium cocktail for its urea content. 100µl aliquots were removed at specific time intervals (10min, 20 min, 30 min, 1hr, 2hr, 24 hours, 40 hours) for urea analysis. Cell growth rate during the course of the experiment was assumed to be negligible. At the end of the experiment, medium was removed, cells trypsinised and counted under the haemocytometer. Cell viability was determined as per method described above. Urea concentration in the sampled medium was determined colorimetrically (Cintra 5 UV-VIS Spectrometer) at 340nm using the Merck Ecoline 25 ® Urea Kit (1.14855.0001), employing the GIDH-method, kinetic UV test.

2.2.1.2 Results

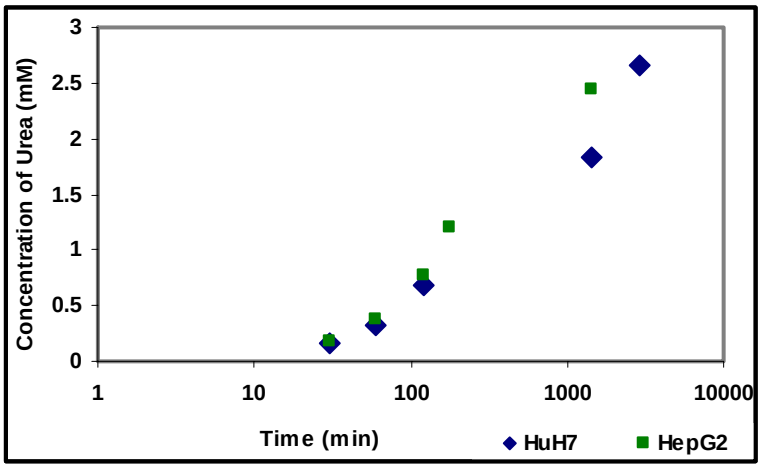

Figure 2.2.1.2.1: Graph of urea concentration (mM) as a function of time for transformed cell lines HuH-7 and HepG2.

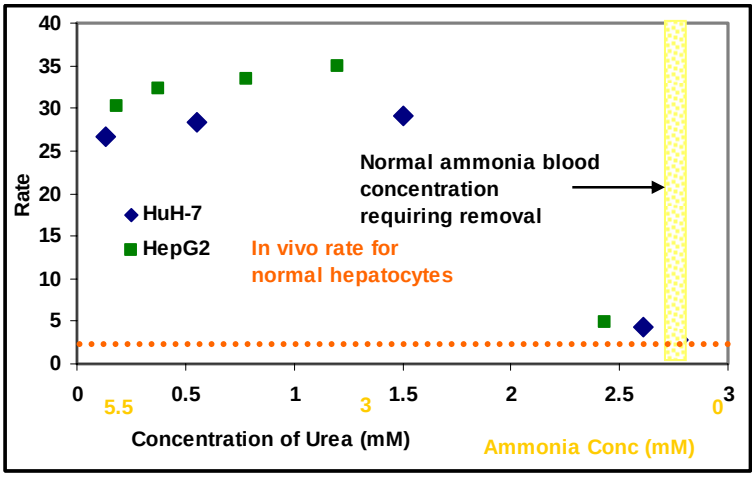

Figure 2.2.1.2.2: Rate of urea production (nmol/10^6cells/min) as a function of concentration for HuH-7 and HepG2. (The dotted line indicates the average rate obtained *in vivo*).

The concentration of urea in the culture medium of the incubated cells was measured over a 24hr period. All experiments were conducted in triplicate. Data in Figure 2.2.1.2.1 indicate that both cells lines exhibit similar ureagenesis functionality, i.e. the ability to metabolise ammonia into urea. The rate at which urea is initially produced increases as the concentration of urea increases until the maximum reaction rate is reached after which the rate starts decreasing probably corresponding to the decreasing reactant concentration (ammonia) (Figure 2.2.1.2.2).

Based on the reaction stoichiometry, two ammonia molecules are required to produce a single urea molecule. Thus, if we assume that when the rate of urea production has reached zero, at approximately 2.5-3.0 mM urea, the ammonia has all been used up. We can assume an initial ammonia concentration of about 5-6 mM (say 5.5mM). Using this value, we can assign proposed values of ammonia concentration on the same axis as the urea concentration.

As previously mentioned, the urea production rate for normal liver cells is quoted in the literature (YOON *et al*., 1999; ZUCKER *et al*., 1995) as 2-3 μmol/10^6cells/24hrs, which translates into 2.08 nmol/10^6cells/min. The rate at which normal cells synthesise urea is an order of magnitude less then the *maximum* rate of reaction (29nmol/10^6cells/min for HuH-7 and 35nmol/10^6cells/min for HepG2) achievable by the transformed hepatoma cell lines tested. The *average* rate for normal cells is illustrated on Figure 2.2.1.2.2 by the rust-coloured dotted line.

The normal concentration range of ammonia in the blood is approximately 18-72 μmol/l (HAWKER, 1993). So if one had to approximate, the maximum concentration (C_{max}) that a normal liver would have to remove, 100 μmol/l is a reasonable initial estimate (illustrated on Figure 2.2.1.2.2. by the yellow shaded region). This concentration is substantially lower than what the hepatoma cells are able to metabolise, as is demonstrated by the range of rates measured over ammonia concentrations, which are higher than 100 μmol/l. Note however, the reaction rates achieved by these hepatoma cells at these lower concentrations are not much higher than the normal liver rates previously quoted. However, the rates obtained were for cells, although hepatic in origin, are hepatoblastoma. Cancer cells are inherently highly active therefore may be able to achieve higher reaction rates.

Furthermore, it is interesting to note that these cells seem able to function at these higher ammonia concentrations without appearing to be poisoned by them. If the human body is unable to tolerate these higher concentrations, it is probably because these levels of ammonia are toxic to other organs in the body.

2.2.2 Lignocaine Uptake

The role of the liver in metabolic conversions of foreign compounds is particularly important in its susceptibility to chemical injury (SCHIFF, 7th edn). Drug biotransformation is one important function that is impaired in the presence of liver disease (SCHIFF, 7TH EDN). In general, the degradation of drugs that primarily undergo oxidative transformation is significantly impaired in the presence of liver disease. Since most pharmacological agents in use are biotransformed by oxidative processes of the P450 system, this observation assumes considerable clinical relevance. In patients with acute or chronic liver disease, drugs that depress the central nervous system may precipitate encephalopathy (SCHIFF, 7th edn).

Lidocaine (lignocaine) was synthesised for the first time in 1943. Since then it has been used as a local anaesthetic agent (STENSON *et al*., 1971). The study by Stenson *et al* (1971) was the first to demonstrate that the liver accounts for the majority of metabolism or removal of lidocaine in man. (NATION *et al*., 1979; STENSON *et al*., 1971). Lignocaine is metabolised primarily by the liver cytochrome P450 system through oxidative N-dealkylation, the major initial metabolite in humans being MEGX (OELLERICH *et al*., 2001). MEGX, the primary metabolite of lignocaine has been employed by many studies as an index for liver function (TREDGER *et al*., 1997; OKAMOTO 1998; EGUCHI *et al*., 1999; BURDELSKI *et al*., 1987; LOREC 1994; OELLERICH *et al*., 1987). Within the context of developing an artificial liver, the cytochrome P450 dependent mixed function oxidases (MFO) capability of cultured hepatocytes is one of the most labile of functions in terms of lignocaine metabolism (ANDERSON *et al*., 1998).

Preliminary data on the survival rates of patients indicate that MEGX formation may provide valuable prognostic information. Comparative studies with various established liver function tests, such as indocyanine green clearance and galactose elimination capacity, demonstrated that a single MEGX serum concentration obtained 15 minutes after an intravenous administration of lidocaine, had a distinctly higher predictive value for the assessment of dysfunction than any of the other liver function tests (OELLERICH *et al*., 1987).

It is reported that the MEGX concentration measured 15 minutes after the introduction of lignocaine reflects the initial formation rate of this metabolite very well (CHEN *et al.,* 1992, TREDGER & SHERWOOD, 1997). At the time of experimentation, the metabolite MEGX was not available from any commercial source. Since the decrease in lignocaine concentration would correspond to MEGX formation, the disappearance of lignocaine as a function of time was measured instead. Lignocaine concentrations were determined by adapting the HPLC method described by CHEN & co-workers (1992). The method is reported to be both reproducible and sensitive to both lignocaine and MEGX (CHEN *et al.,* 1992).

2.2.2.1 Materials and Methods

Various concentrations (500, 800, 1000, 1500, 2000, 2500 ng/ ml) of lignocaine were prepared by serial dilution with tissue culture medium. HepG2 and HuH-7 cell lines were grown in 50ml culture flasks to approximately 85% confluency. Cells were incubated in 3ml of the lignocaine/medium cocktail. Samples were aliquoted at time intervals of 15, 30, 45 and 60 minutes. Experiments were conducted in triplicate. Lignocaine concentration in the medium was determined by HPLC (Appendix A).

At the end of the experiments, the medium was removed and cells trypsinised, counted and viability assessed.

Chromatographic System

HPLC system employed a UV detector set at 205nm, manual injector equipped with a 100-µL sampling loop, a µBondapak C18 (300 x 3.9 mm I.D.) column (Millipore). Chromatography was performed at room temperature using a mobile phase of acetonitrile- potassium dihydrogen phosphate buffer (pH 4.0) (14:86) at a flow-rate of 1.3 ml/min.

2.2.2.2. Results

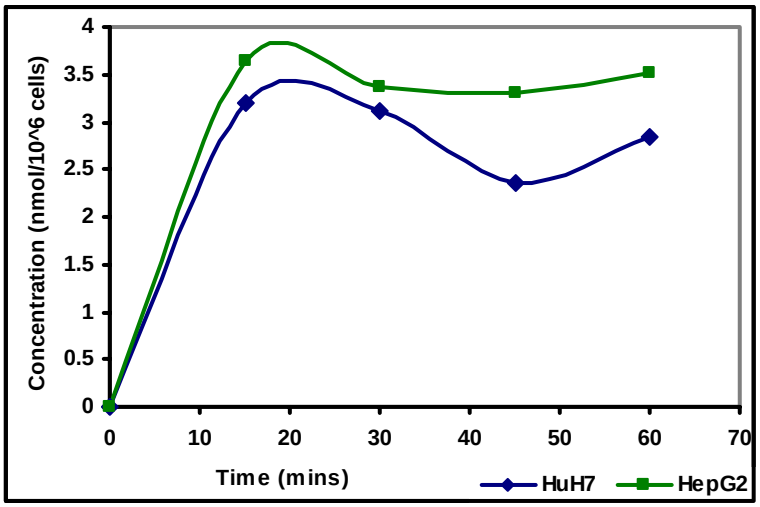

Figure 2.2.2.2.1: Lignocaine concentration in cells versus time of incubation

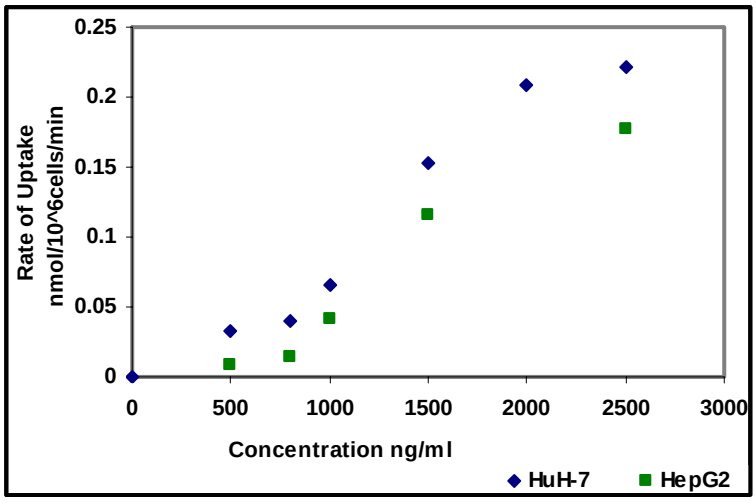

Figure 2.2.2.2.2 Comparison of the rate of uptake of lignocaine as a function of initial lignocaine concentration for transformed cell lines HuH-7 and HepG2

The ability of HuH-7 and HepG2 to remove lignocaine from the cell culture medium in order to test the P450 activity of these cells was investigated. Lignocaine concentration in the medium was measured as a function of time for a period of 60 minutes. Figure 2.2.2.2.1 illustrates the cellular uptake of lignocaine measured as a function of time. It is evident that both cell lines are almost equally capable of removing lignocaine from solution with HepG2 performing marginally better.

Initial uptake rates ($r_{max,0}$) of lignocaine were calculated from the slopes of the cumulative uptake curves for various initial concentrations of lignocaine in complete culture medium during the first 15min of incubation. This decrease in concentration reflects the initial formation rate of MEGX, the lignocaine metabolite very well (CHEN *et al.,* 1992, TREDGER & SHERWOOD, 1997). Results were expressed as nanomoles per 10^6cells per min as demonstrated in Figure 2.2.2.2.2.

For the computation of the kinetic constants, values for lignocaine, R_{max} and concentration [L] were fitted directly to the Hill equation:

$$r = \frac{R_{\max}[L]^h}{K_m{}^h + [L]^h}$$

Equation 2.2.2.2.1

Where: r is the uptake rate (nmol/10^6cells/min),

R_{max} is the maximal uptake rate (nmol/10^6cells/min),

K_m is the lignocaine concentration at half the maximal rate,

L is the lignocaine concentration,

h is the Hill Coefficient.

An iterative non-linear least squares fitting procedure was employed to determine the parameters of the mathematical curve describing the data set for each cell line. As is evident from Figure 2.2.2.2.3 and Figure 2.2.2.2.4, the model explains the measured data adequately.

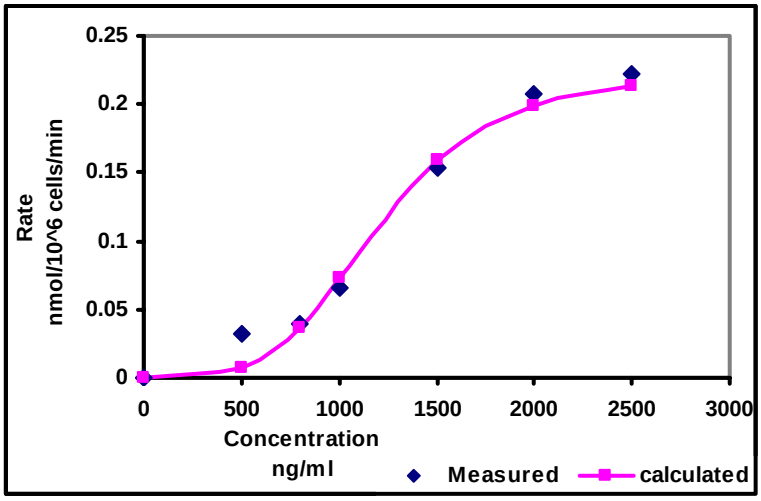

Figure 2.2.2.2.3 Rate of lignocaine uptake by the HuH-7 cell line fitted to the Hill Kinetic Model.

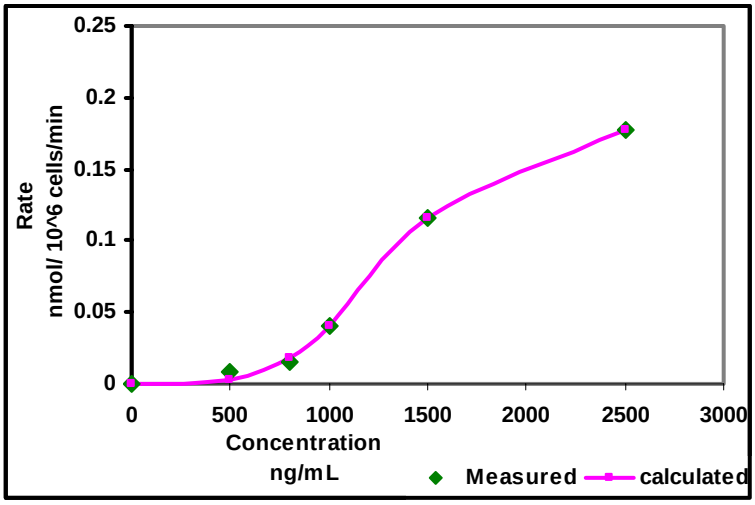

Figure 2.2.2.2.4 Rate of lignocaine uptake by the HepG2 cell line fitted to the Hill Kinetic Model.

The maximal rate was estimated at, R_{max}= 0.22 nmol/10^6cells/min, with h=3.98 and K_m= 4.17 nmol/ml for HuH-7 and R_{max}= 0.178 nmol/10^6cells/min, with h= 4.33 and K_m= 4.69 nmol/ml for HepG2 (Refer to Figures 2.2.2.2.3 and 2.2.2.2.4 for graphical representation).

The clearance of lignocaine by normal hepatocytes is quoted in the literature (HARDMAN, 2001; KATZUNG, 2001) as 9.2 ml/min/kg for a 70kg adult male with a steady state infusion rate of 1-4 mg/min to maintain a therapeutic plasma concentration of 2-6 μg/ml.

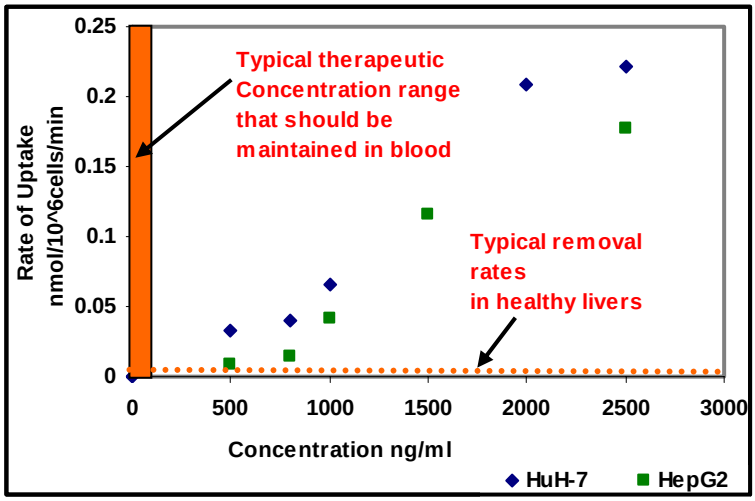

Figure 2.2.2.2.5: Hepatic removal rate of lignocaine in a healthy liver superimposed on the rate/concentration curve obtained for HuH-7 and HepG2 also indicating the therapeutic concentration of lignocaine maintained in blood.

How do the rates of normal hepatocytes in the body compare with the maximum rates measured for HuH-7 and HepG2? A summary is given below:

$r_{Liver\ cells} = 4.53 \times 10^{-12}$ mol/10^6cells/min
$r_{maxHuH7} = 0.22 \times 10^{-9}$ mol/10^6cells/min
$r_{maxHepG2} = 0.178 \times 10^{-9}$ mol/10^6cells/min

The average rate at which normal liver cells remove lignocaine from blood is significantly slower than the maximum rate at which the two hepatoma cell lines can remove lignocaine from solution. There are many other competing reactions that occur in the liver which utilise the P450 enzyme pathway including lignocaine metabolism. We only introduced lignocaine into the experimental system, therefore saturating this pathway. It makes sense then that the rate obtained could be higher than the average rate of normal cells quoted in the literature for the liver. In any event, at these low therapeutic concentrations, it is difficult from our results to predict the rates at which these hepatoma cells would perform (it is possible to compute the rate from the Hill model for therapeutic concentrations and compare them with normal liver cell rates).

As the concentration of lignocaine is increased, the rate of reaction increased accordingly until saturation and maximum rate occur at a concentration of around 2500 ng/ ml. It is quite surprising that the cells performed so well at such high concentrations. Obviously, the high concentrations of lignocaine are not toxic to the liver cells themselves. In the human body however the high concentrations most probably result in secondary organ toxicities.

2.2.3 Uptake of the Radionuclide ^{99m}Tc-DISIDA

2.2.3.1 Materials and Methods

Bilirubin represents a class of compounds that are bound to plasma proteins. Plasma bound toxins have been implicated in hepatic coma and should therefore be removed from circulation by the artificial liver device (MATSUMURA *et al.,* 1987). An accurate measure of bilirubin conjugation by hepatocytes is difficult, due to the fact that bilirubin conjugates in the presence of light. Random drift bilirubin values can result from variations in exposure to light, temperature, water and other environmental factors (BRUNI & CHANG, 1995). Therefore, kinetics of a suitable radioactive bilirubin analogue was studied instead.

Bilirubin, the main bile pigment in most mammals, is the principal end product of haemoglobin degradation and subsequently heam catabolism (CUI *et al*., 2001). When red blood cells have out lived their life span of approximately 120 days, and become too fragile to exist longer in the circulatory system, their cell membranes rupture, and the released haemoglobin is phagocytised by tissue macrophages throughout the body. Haemoglobin is split into globin and heme. The heme ring is opened to give a free iron and a straight chain of four pyrrole nuclei, which will eventually form bilirubin. Bilirubin is a dicarboxylic acid characterised by minimal solubility at physiological pH. It is neither hydrophilic nor lipophilic, necessitating biotransformation by hepatocytes into a more polar conjugate for excretion (via secretion into bile)(ZUCKER, 1999; CUI *et al*., 2001).

The radioactive bilirubin analogue selected for study was based on a radioisotope technetium-99m, which is widely used in medicine [15]. It has a physical half-life of 6 hours (HARDMAN, 2001; HENKIN, 1996; HIBBARD, 1982). The chemical versatility of technetium-99m allows it to be incorporated into a range of biologically active substances to ensure that it concentrates in the tissue or cell of interest.

The criteria for an ideal radiopharmaceutical would be:

- No reaction with hepatocytes occurs;
- It has a short biological half life, and
- Low radiation dose (YALLOW, 1981).

A large number of ^{99m}Tc agents were developed for hepatobiliary scintigraphy. Amongst the most successful agents used were derivatives of *N*-substituted iminodiacetic acid or commonly known as Tc-HIDA. Tc-HIDA was found to clear the blood rapidly. The generic name for the ^{99m}Tc agent used in this study is disofenin (DISIDA) (HENKIN, 1996; HIBBARD, 1982).

DISIDA was chosen for this study as it presents certain structural and behavioural similarities to bilirubin. Bilirubin and DISIDA share a number of analogies with regard to hepatocellular uptake:

- Transported in plasma bound to albumin,
- Accelerated dissociation of their albumin complexes at the hepatocyte surface,
- Uptake kinetics display features of carrier mediated transport.

It is against this background that studies on ^{99m}Tc-DISIDA were carried out as opposed to bilirubin. Okuda's research demonstrates that ^{99m}Tc-DISIDA appears to enter the liver principally over the bilirubin transport system (OKUDA, 1986).

Preparation of ^{99m}Tc-DISIDA

^{99m}Tc-DISIDA (donated by Syncor International) was serially diluted to the required dose with cell culture medium (10% DMEM for HuH7 and 5% DMEM HepG2). The hepatobiliary agent was then ready for use in the *in-vitro* cellular uptake studies.

Hepatocytes were grown in 50 ml Nunc flasks to 100% confluency. Culture medium from the flasks containing cells was replaced with the radioactive medium. After 1 minute of incubation, the radioactive medium was removed and cells were washed with 3 ml phosphate buffered saline (PBS), to remove any radioactivity not trapped within the cells. Trypsin (1 ml) was added to the flask to remove the cells from the flask surface. Cell culture medium (3 ml) was added to the flasks to inhibit the action of trypsin. 100 ml of cell suspension were sampled and sent for gamma counting. Cells were counted using a haemocytometer. Cell viability was assessed by Trypan Blue exclusion. Each data point was the result of three separate experiments.

2.2.3.2. Results

Cells were incubated in radioactive medium for the various time periods, after which the cellular uptake was determined. Figure 2.2.3.2.1 suggests that both the HuH-7 and HepG2 cell lines exhibit similar uptakes characteristics for ^{99m}Tc-DISIDA.

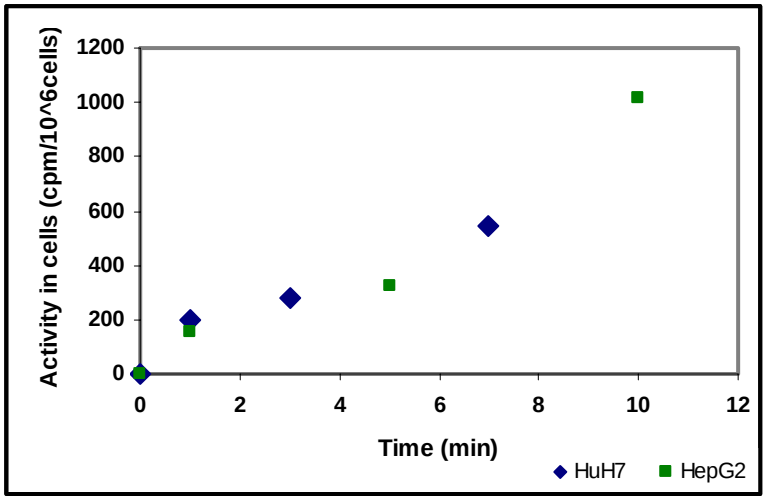

Figure 2.2.3.2.1: Graph of uptake of ^{99m}Tc-DISIDA (cpm/10^6cells) by HuH-7 and HepG2 as a function of time

Initial rates ($r_{max,o}$) of ^{99m}Tc-DISIDA were calculated from the slopes of the cumulative uptake curves during the first 60s of incubation as this was the steepest linear portion of the concentration/ time curve (indicating the maximum rate). Results were expressed as curies per 10^6cells per min as a function of the concentration of activity the cells were incubated in.

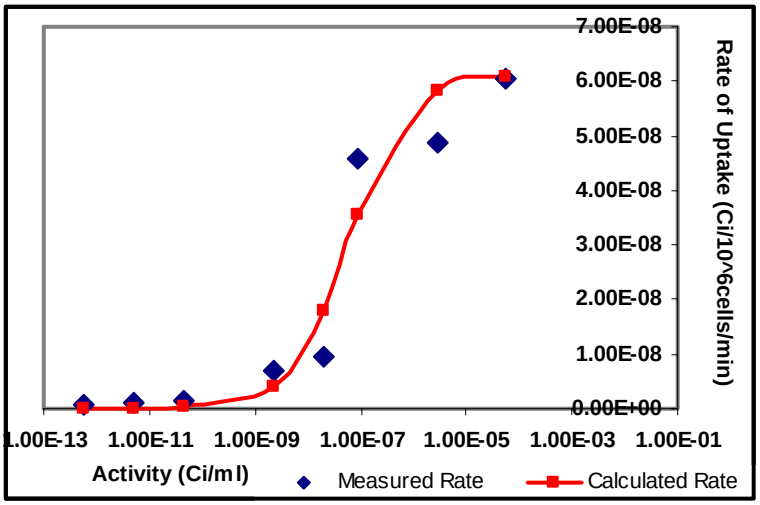

Figure 2.2.3.2.2: Rate of uptake of ^{99m}Tc-DISIDA (Ci/ 10^6cells/min) by HuH-7 cells fitted to the Hill Kinetic Model

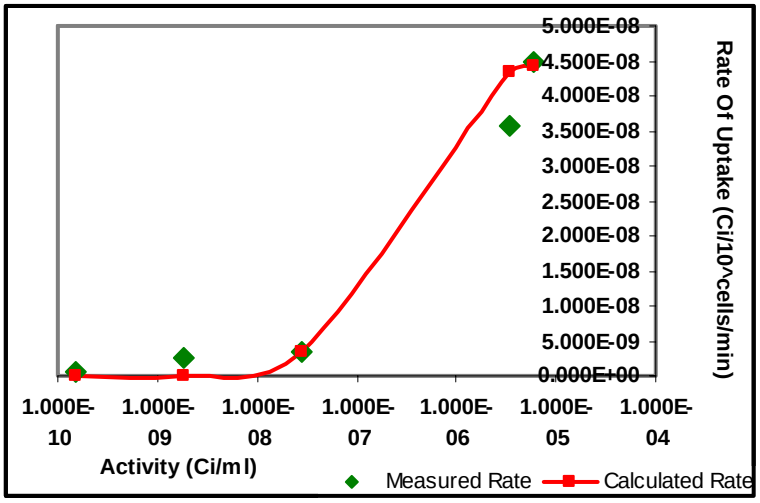

Figure 2.2.3.2.3: Rate of uptake (Ci/ 10^6cells/min) of ^{99m}Tc-DISIDA by HepG2 Cells fitted to the Hill Kinetic Model

For the computation of kinetic constants, values for ^{99m}Tc-DISIDA, $r_{max,o}$ and concentration of activity were fitted directly to the Hill equation (Refer to Equation 2.2.2.2.1), as was done for lignocaine. The following values were obtained by iteration:

$R_{max, HuH7}$ =	6.10 x 10^{-8} Ci/10^6cells/min	**$R_{max, HepG2}$ =**	4.50 x 10^{-8} Ci/10^6cells/min
K_m =	6.00 x 10^{-8} Ci/ml	**K_m =**	6.55 x 10^{-7} Ci/ml
h=	0.8	**h =**	1.30

Table 2.2.3.2.1: Values obtained for the Hill model variables for HuH-7 and HepG2

Both hepatoma cells lines are able to remove the radioactive isotope from solution implying the same capability with respect to bilirubin removal. The mechanism of ^{99m}Tc-DISIDA hepatocellular uptake was not investigated in this study only the capability to remove it from the complete culture medium was studied. The maximum rates achievable by both cell lines are fairly similar with the maximum achievable rate of HuH-7 appearing slightly higher. This once again illustrates very similar kinetic behaviour demonstrated by the respective R_{max} values, being of the same order of magnitude.

^{99m}Tc-DISIDA has a physical half-life ($t_{½phys}$) of 6.04hr (i.e. decay rate) and a biological half-life ($t_{½biol}$) of 18.8 min (for a normal liver cells) (HENKIN, 1996). In order to compare measured reaction rates to the half life and hence to the reaction rates quoted in literature, we assumed that the uptake of ^{99m}Tc-DISIDA exhibits first order kinetics, where:

$$rate = {}^{dC_A}\!/_{dt}\, xn = -nkC_A \qquad \textbf{Equation 2.2.3.2.1}$$

then the rate constant *k* is:

$$n\,k = {}^{Ln2}\!/_{t_{1/2}} \qquad \textbf{Equation 2.2.2.2.2}$$

with *n* being the number of cells.

Assuming maximal removal rates calculated for HuH-7 ($6.03\text{x}10^{-8}$ Ci/10^6cells/min) and HepG2 ($4.48\text{x}10^{-8}$ Ci/10^6cells/min) at approximately $1\text{x}\ 10^{-5}$ Ci/ml, the rate constant (*k*) for both cells lines was computed and compared with the rate constant (*k*) for normal liver cells.

	Normal Liver Cells (whole liver)	Normal Liver Cells (10^6 cells)	HuH-7 (10^6 cells)	HepG2 (10^6 cells)
Rate Constant (*k*)	0.0369 min^{-1}	1.609x10^{-8} min^{-1}	0.0017 min^{-1}	0.00255 min^{-1}
Half Life ($t_{1/2}$)	18.8 min	~ 4x10^7 min	399 min	271 min

Table 2.2.3.2.2: Comparison of rate constants (*k*) and half-lives ($t_{1/2}$) for normal liver cells and transformed liver cells (HuH-7 and HepG2).

From the table above, it is evident that the immortalised cells lines once again outperform normal liver cells.

^{99m}Tc-DISIDA is usually administered to patients as a bolus injection of 5mCi. Assuming a plasma volume of 3*l* one can compute the plasma concentration to be 1.67x10^{-6}Ci/ml.

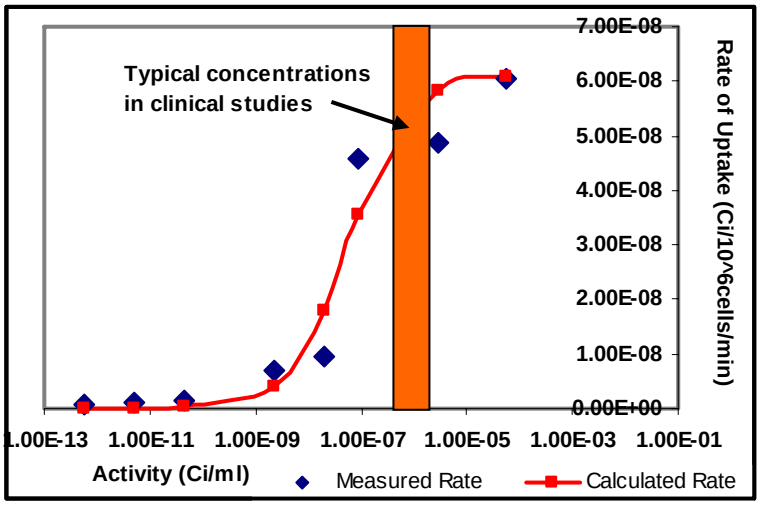

Figure 2.2.3.2.4: Concentration range of ^{99m}Tc-DISIDA administered to patients superimposed on the HuH-7 rate curve.

Results indicate that for this liver function test as for the previous two discussed, the transformed hepatocytes, HuH-7 and HepG2 have the ability to remove ^{99m}Tc-DISIDA at a much faster rate than that which occurs *in vivo* [See Table 2.2.3.2.2]. This could have positive implications for the design of our hybrid artificial liver reactor (HALR) in terms of the mass of cells required. Since this reaction was studied to test the ability of bilirubin removal from the blood by studying its analogue ^{99m}Tc-DISIDA,we can assume that both the hepatoma cell lines are highly capable of removing toxins utilising the bilirubin pathway (anionic carrier mediated removal). It is however interesting to note that these toxins are not life threatening as such. Jaundice is only life threatening in babies, as their blood brain barrier is not completely impermeable to bilirubin.

Once more the data demonstrate that both cells lines have similar reaction kinetics over a wide range of concentrations or activities that the cells were incubated in, once more emphasising that the type of cell incorporated into our reactor is not particularly significant in the reactor design process.

2.3 DISCUSSION AND CONCLUSION

The aim of this study was to determine the sensitivity of the reactor design to the cell line incorporated. The reaction rates measured for the transformed cell lines are of the same order of magnitude for each liver function tested. This implies that the reactor design is not significantly sensitive to which of the two cell lines is incorporated in the design of the HALR. Refer to Table 2.3.1 below for a summary of the results obtained.

Transformed Cell Line	R_{max} Urea Metabolism	R_{max} for Lignocaine	R_{max} for ^{99m}Tc-DISIDA
HuH-7	2.91×10^{-5} mmol/10^6cells/min	0.222 nmol/10^6cells/min	6.03×10^{-8} Ci/10^6cells/min
HepG2	3.61×10^{-5} mmol/10^6cells/min	0.178 nmol/10^6cells/min	4.48×10^{-8} Ci/10^6cells/min

Table 2.3.1: Comparison between the maximum rates achievable for HuH-7 and HepG2 cell lines for selected liver biochemistries.

The HuH-7 cell line appears to be a slightly better choice with respect to lignocaine and DISIDA uptake, however marginally. The HepG2 cell line seems to metabolise urea at a slightly higher rate. The maximum rates achievable for ammonia metabolism and lignocaine uptake were compared to the rates at which normal liver cells perform these functions *in-vivo* to get an idea of the rate limiting reaction between the liver functions investigated. For both of these reactions, the maximum rates observed were higher than the average rates found in the literature for normal liver cells. This can be explained by referring back to the graphs, which illustrate the range of concentrations the reaction rates were measured for. It is quite interesting to discover that as one increases the concentrations of the various substrates to levels higher than those regarded as toxic in the human body, the cells seem to perform better.

For the third liver function test, ^{99m}Tc-DISIDA uptake was studied as a bilirubin analogue due to the difficulties encountered with accurate bilirubin determination. The half-life of ^{99m}Tc-DISIDA uptake for the two cell lines was compared to the *in-vivo* half-life of ^{99m}Tc-DISIDA uptake. Results demonstrate that just as for the previous two liver function tests, the HuH-7 and HepG2 cell lines have similar reaction rates and significantly higher rate constants than that calculated for normal liver cells normalised to 10^6cells.

The cells have demonstrated that much higher rates than those quoted in literature are achievable. These findings could be explained by assuming the higher concentrations (related to the higher reaction rates measured) are not toxic to the cells themselves. This would imply that during liver failure or injury, the concentrations would be expected to elevate, and therefore the cells are driven to perform at a much higher rate.

It is also possible that the liver has a tremendous amount of redundancy built into it. Therefore, at any one time, not all the hepatocytes are engaged in performing all the various reactions. Those involved in the various biochemical conversions at the prevalent substrate concentrations are not performing at their highest possible rate either. All of these findings make for interesting implications with respect to the design of the HALR. This would also explain the survival and normalcy of function in patients who have had a partial hepatectomy.

If one had to choose a reaction to design for, based on reaction rates and the number of cells required, either ammonia metabolism or lignocaine uptake could be selected. This is as a result of the ability of the transformed cell lines to function at significantly higher rates than those quoted for normal cells functioning in-vivo. Hyperbilirubinemia is not life threatening to an adult. High concentrations of bilirubin could be removed by other devices connected in series with an artificial

reactor, or by some other means (not within the scope of this study) and is therefore not the first choice reaction to design the reactor for.

The next step in the design of an artificial liver reactor is to investigate what effect microencapsulating these cells would have on the mass transfer characteristics of the reactor and the design implications of those findings.

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3. CHAPTER TWO

Determination of the Design Implications of Transport Phenomena Across Membranes of Microencapsulated Hepatocytes For Use in a Hybrid Artificial Liver Reactor

This chapter was written in the form of a paper for publication. Parts paper was presented at the PSE2004 Conference held in China in January 2004.

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DETERMINATION OF THE DESIGN IMPLICATIONS OF TRANSPORT PHENOMENA ACROSS MEMBRANES OF MICROENCAPSULATED HEPATOCYTES FOR USE IN A HYBRID ARTIFICIAL LIVER REACTOR

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Abstract

The aim of this study was to determine the membrane effects on mass transfer across microencapsulated hepatocytes and subsequently the implications of microencapsulation for the reactor design. Cell viability within the capsular environment was ascertained via α-fetoprotein production. Mass transfer data for substrates (urea, lignocaine) that would potentially cross the microcapsule membrane in a HALR scenario were measured. The kinetics of external mass transfer were compared to the intrinsic cell kinetics previously measured to determine the rate-limiting step. Results indicate that the capsular membrane does not significantly impede mass transfer and that intrinsic cell kinetics is the rate-limiting step. This implies that the reaction rate will depend only on the quantity of cells incorporated in the reactor and not on membrane area, structure or size.

3.1 INTRODUCTION

Process synthesis methodology aims to advance technology through the application of market related concepts to process development. The paradigm shift that is taking place within the scientific arena has resulted in the move from an isolated sequential approach in the development of new processes and technologies to a more iterative, time, energy and financially efficient process.

In the traditional sequential approach to developing a new process, laboratory measurements are often obtained in the absence of the designer. The process is then developed from the laboratory data in isolation from the laboratory personal. Instead, we have employed the iterative approach described by GLASSER, HILDEBRANDT & McGREGOR (1998). In this approach, the process is designed with whatever information, albeit limited, is available, and measurements are then obtained depending on which variables need optimising.

The development and modelling of artificial organs, such as the liver, is one such area where engineering methodology, and in particular, process synthesis has seen wide applicability. Our focus is on developing and modelling bioartificial liver support. Despite recent advances in modern medical therapy, patients with fulminant hepatic failure (FHF) have a mortality rate approaching 90% (DIXIT & GITNICK, 1996). While technology has made human organ transplantation a common occurrence, the chronic shortage of donor organs impels the need for the development of an extracorporeal liver assist device, either as a bridge to transplantation or to provide reprieve for a damaged liver to recover.

Laboratory experimentation included determining the liver functionality expressed by two hepatoma cell lines, namely, HuH-7 and HepG2, aimed at assessing specific liver functionality, as well as the sensitivity of the reactor design to the cell line chosen. Research has demonstrated that both cell lines chosen exhibit a stable phenotypical expression of some differentiated liver functions in long term cell culture. Both these cells have been reported to produce a number of plasma proteins, liver specific enzymes and bile acids (NAKABAYASHI, 1984; MIYASHITA, 2000; NAGAKI *et al*, 2000). Our own experimentation has demonstrated the capability of both cell lines for ureagenesis, P450 activity (drug metabolism) and bilirubin type functionality (Chapter 1).

The aim of this study is to determine the membrane effects on mass transfer across microencapsulated hepatocytes and subsequently the implications of encapsulation for the reactor design.

3.1.1 Microencapsulation of cells

Bioartificial liver support research harnesses the natural capabilities of liver cells by encapsulating them in a semipermeable membrane and incorporating them in bioreactors of various configurations (DIXIT & GITNICK, 1996). This encapsulation can be achieved in a number of ways, and in our research, the cells are protected from immune rejection by microencapsulating them in sodium alginate beads. Microencapsulation is based on the premise that cells, once sequestered within a semipermeable membrane, are isolated from the immune system and therefore cannot be recognised and destroyed by the host's immune defence system. This is achieved by designing the membrane, permeability so that the large antibodies are prevented from crossing the membrane thus circumventing the need for immunosuppression, while ensuring cell viability *via* bi-directional diffusion of nutrients, oxygen, metabolites and allowing their products unimpeded diffusion out. The capsular environment provides cells with cell-cell interaction (synergistic interactions between

adjacent cells), which exist *in-vivo*, nutrient-product and pH gradients (Reddy, 2001; REID & JEFFERSON, 1984). Furthermore, the microencapsulation prevents the hepatoma cells away from infecting the patient.

Microencapsulation of cells provides the advantage of obtaining high cell densities in the reactor as compared to cells in hollow Fibre devices (HFDs) or cells in free suspension. Hepatocyte isolation within a microcapsule allows the cells to organise themselves into three-dimensional structures. Wu *et al.* (1996) hypothesised that this organisation contributed to enhanced liver specific activity. The high cell loading leads to high volumetric productivities. It also enables the use of higher dilution rates, as there is no culture washout.

CHANG first reported artificial cells, prepared from microencapsulation of biologically active materials, in 1964. The development of artificial cells led to the microencapsulation of biological materials becoming an area of study for industrial purposes as well as biomedical research (WONG & CHANG, 1991). Artificial cells were first prepared as ultrathin polymer membranes of cellular dimensions, microencapsulating the proteins and enzymes extracted from biological cells (DIXIT & GITNICK, 1996).

3.1.2 Kinetic Determination of Rate Limiting Step

The reactor incorporating these encapsulated cells is modelled on data obtained in the laboratory, and reaction rates are fundamental to the design process. The overall reaction rate could be limited by intrinsic cell kinetics, mass transfer within the bead, diffusion across the membrane, or external mass transfer. The design process thus only requires laboratory tests that provide this information. This approach is advantageous as a better process is developed more rapidly as less time and money are spent on irrelevant experiments.

Laboratory experiments performed included cell encapsulation to ascertain cell viability within the capsular environment, demonstrated by α-fetoprotein (AFP) production, as well as mass transfer measurements for the semipermeable membrane. Mass transfer data produced were compared with the intrinsic cell kinetics previously determined (Chapter 1) to establish the rate-limiting step.

If intrinsic cells kinetics limit the reaction rate, then the rate will depend only on the quantity of cells in the capsular beads and not on the membrane area, structure nor particle size. If experiments indicate that the internal mass transfer is rate limiting, then the overall reaction rate is strongly dependent on bead radius. Membrane structure and area will only influence the reaction rate if diffusion across the membrane is rate limiting. If external mass transfer is determined to be rate limiting then it would imply a badly designed reactor. In terms of process synthesis, this would be the best-case scenario, as the flow in the reactor could be redesigned to better facilitate mass transfer.

3.2 Materials and Method

Hepatocyte Cell Culture

Hepatocytes were cultured in Dulbecco's Modified Eagle's Medium DMEM (1.0 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1250 μL penicillin/ streptomycin) supplemented with 5-10% fetal calf serum. *(All chemicals purchased from Sterilab Services, Kempton Park, South Africa).* Spent medium was replaced every four days. Cell cultures were incubated at 37°C in a 95% air and 5% CO_2 atmosphere.1.0ml of trypsin *(Sterilab Services, Kempton Park, South Africa)* solution was added to the flask and cells observed under an inverted light microscope until cell layer was dispersed. Cells that were difficult to detach were placed at 37°C to facilitate dispersal. 3ml of complete tissue culture medium were added and mixed by gently pipetting. Cells were now ready for use.

Droplet Generation

The droplet generator used for microencapsulation consists of 2 co-axially arranged jets: the central jet consists of a 26G stainless steel needle (Perfektum; Popper & Sons, Inc.,New Hyde Park, NY) through which the sample is extruded, within a 16G surrounding air jet through which air is passed. To prevent sample occlusion at the end of the air jet, the tip of the sample jet is fabricated such, that the sample jet extends 0.5mm beyond the end of the air jet (See Figure 3.2.1 & 3.2.2 below). The droplet generator is so designed that the tip of the needle is subjected to a constant laminar airflow, which acts as an airknife. The syringe filled with cell suspension is mounted on a syringe pump, which is activated to incrementally force drops of the solution to the tip of the needle, where the droplet is cut-off by the air stream and falls into the gelling solution where the absorption of cross-linking ions causes immediate gellation. The distance between the tip of the needle and gelling solution is set to allow the alginate cell suspension to assume the most favourable shape to reduce mass transfer resistance – a sphere (maximum surface area to volume ratio).

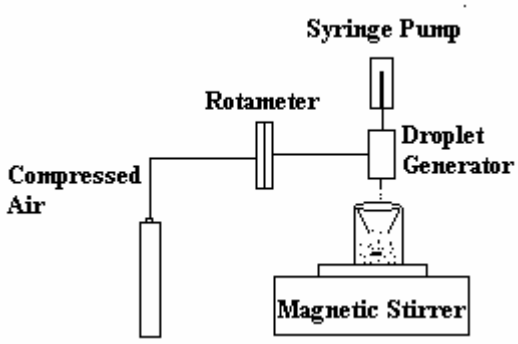

Figure 3.2.1: Apparatus set-up for microencapsulation

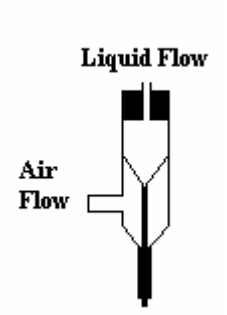

Figure 3.2.2: Droplet needle assembly

Microencapsulation

The method used for encapsulation was based that described by CHANG (1992). The isolated hepatocytes were resuspended in medium, or in the case of mass transfer experiments, saline was used. The cell suspension or saline was then resuspended in 4% sodium alginate (4.0% sodium alginate, 0.9% saline, 20mM fructose). The final working concentration of cells and sodium alginate was adjusted to obtain a working concentration of 20 x 10^6 cells/ml and 2.0% respectively. In the case of mass transfer experiments, a final working concentration of 2% alginate was produced.

A syringe (Becton Dickinson & Co; Rutherford, N.J.) was filled with the 2% alginate suspension, the sample then extruded with a syringe infusion pump (Harvard apparatus; Millis, Mass) set at 0.1ml/min through the 26-gauge needle of the droplet generator. The droplets were sheared off by the air jet flowing through the16 gauge needle at 2.5 l/min.

The droplets formed were allowed to fall dropwise into a beaker containing 200ml of an ice cold, gently stirred 100mM $CaCl_2$ solution (100mM $CaCl_2$, 10mM HEPES, 20mM fructose, pH 7.4), where they were allowed to gel for 15 minutes on contact with the $CaCl_2$ solution.

The alginate matrix on the surface of the microcapsule was stabilised with poly-L-lysine by immersion in 80ml of a 50 mg% solution of poly-L-lysine (50 mg% poly-L-lysine, 0.9% NaCl, 10mM HEPES, 20mM fructose, pH 7.4) for 20 minutes.

The microcapsules were then drained, washed with buffered saline and immersed in 200ml of 0.1% sodium alginate (0.1% sodium alginate, 0.9% NaCl, 10mM HEPES, 20mM fructose, pH 7.4) for 5 minutes to apply an external layer of alginate.

The microcapsules were collected and immersed in 200ml of 50mM sodium citrate (50mM sodium citrate, 0.8% NaCl, 20mM fructose, pH 7.4) for 6 minutes to solubilise the intracapsular calcium alginate.

The resulting microcapsules were then washed with saline and ready for use.

Determination of Encapsulated Hepatocyte Viability

HuH-7 cells were cultured in the manner described above and viability prior to encapsulation was determined via trypan blue exclusion. All apparatus to be used in the microencapsulation procedure was sterilised by autoclaving at 200^0C for an hour. Cells (1 x 10^7cells/ml) were microencapsulated as per method described above and incubated in complete culture medium at 37^0C in a 95% air and 5% CO_2 atmosphere. Daily samples of the medium were analysed for AFP.

Mass Transfer Across the Capsular Membrane

Empty microcapsules were prepared with saline suspended in 4% sodium alginate to produce a working concentration of 2% as per method described above. The microcapsules were then added to solutions of lignocaine (7nmol/ml in saline) and urea (20mmol/l in saline) respectively which were being gently stirred on a magnetic stirrer. Aliquots of the supernatant liquor were sampled at particular time intervals while attempting not to remove any microcapsules. These samples were added to eppendorf vials equipped with 0.45 μm filters and centrifuged (10°C, 3000rpm, 5minutes) to remove any capsular debris and analysed via HPLC for lignocaine and UV-VIS Spectrometer at 340nm using the Merck Ecoline 25 ® Urea Kit (1.14855.0001) for urea.

3.3 RESULTS

Encapsulated Hepatocyte Viability

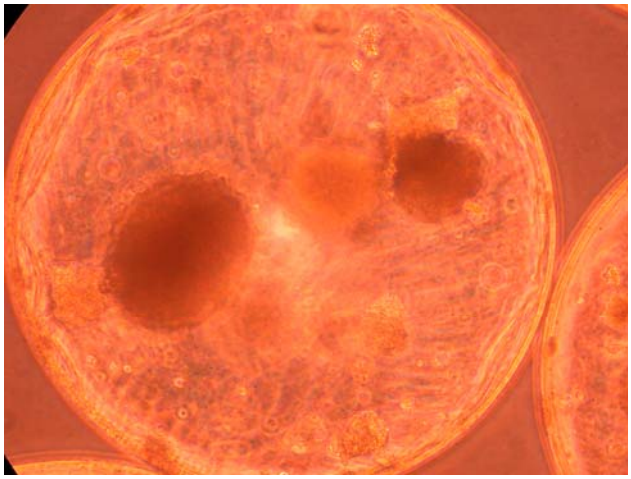

Figure 3.3.1: Photograph showing HuH-7 cells in an alginate-polylysine-alginate (APA) microcapsule on Day 7.

Time	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Concentration ng/ml	1.8	277.56	289.68	293.64	297.36	297.6	308.16

Table 3.3.1: AFP production by encapsulated HuH-7 cells (2.5 x 10^7cells initially on Day 0) over a period of six days.

Results demonstrate that encapsulating the hepatoma cells does not affect their viability as is indicated by the production of AFP for up to six consecutive days. Capsules containing cells were ruptured daily and viability assessed by trypan blue exclusion. Cell viability according to trypan blue exclusion had decreased by day six by approximately 10%.

Thus, in designing an artificial liver, one could expect cells to remain viable for at least six days. The implications of these findings for clinical application are particularly promising. Firstly, if one had to be guided by kidney dialysis, microcapsules containing cells do not need to be replaced during a therapeutic perfusion session. Secondly, cartridges or reactors can be prepared in advance.

Mass Transfer Across the Capsular Membrane

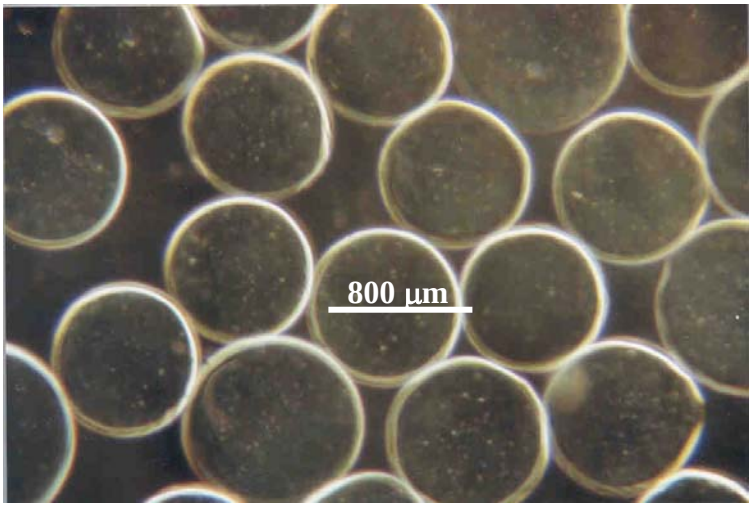

Figure 3.3.2: Empty microcapsules suspended in phosphate buffered saline (PBS) for use in mass transfer experiments

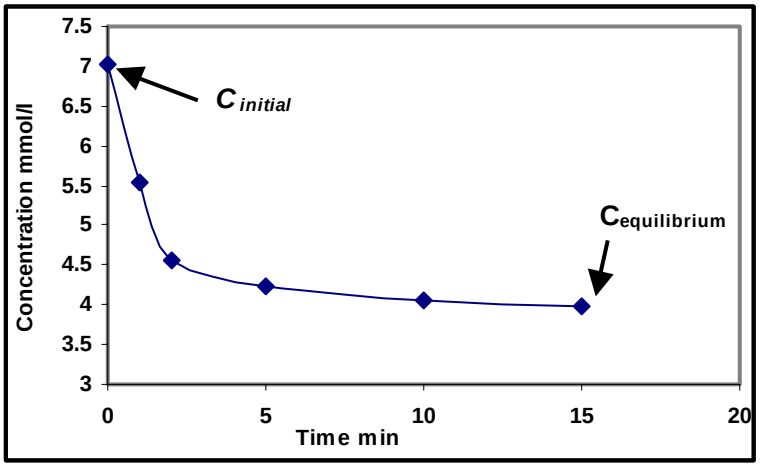

Figure 3.3.3: Lignocaine concentrations in bulk solution containing empty microcapsules measured as a function of time

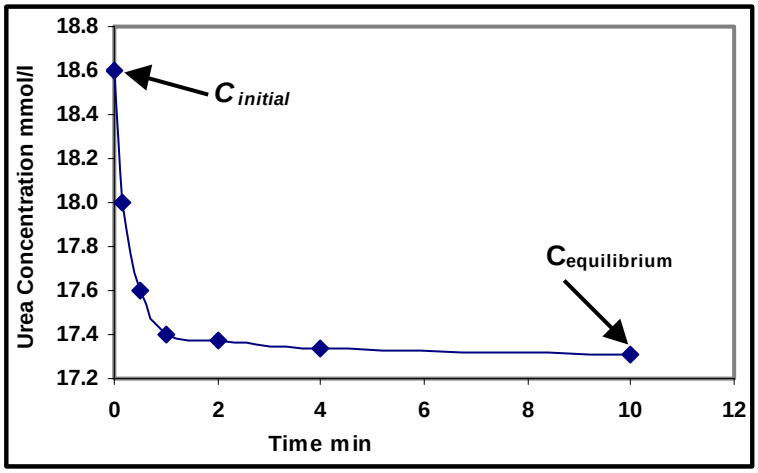

Figure 3.3.4: Urea concentrations in bulk solution containing empty microcapsules measured as a function of time

If we assume that the diffusion of lignocaine and urea across the capsular membrane follows first order kinetics i.e. the mass transfer coefficient is approximately constant and the concentration within the microcapsule tends to zero, then the half-life of diffusion is calculated using:

$$dC/dT = -k(C - C_{equilibrium}) \qquad \textbf{Equation 3.3.1}$$

Integrating both sides yields a straight-line equation, where k is the slope of the line:

$$Ln(C_{(t)} - C_{equilibrium}) = kt + Ln(C_{(t=0)} - C_{equilibrium}) \qquad \textbf{Equation 3.3.2}$$

$$t_{1/2} = Ln2/k \qquad \textbf{Equation 3.3.3}$$

Where:

dC/dT	change in concentration as a function of time,
k	mass transfer co-effecient,
C	substrate concentration in the bulk solution;
$C_{equilibrium}$	the concentration reached at equilibrium
$t_{½}$	half-life or time at which C= $C_0/2$

In order to establish whether intrinsic cell kinetics or external mass transfer is the rate-limiting step, the half-lives ($t_{½}$) of both reactions will have to be calculated and compared. Using equations 3.2 and 3.3 we were able to establish the transport half lives for diffusion of urea and lignocaine across the capsular membrane.

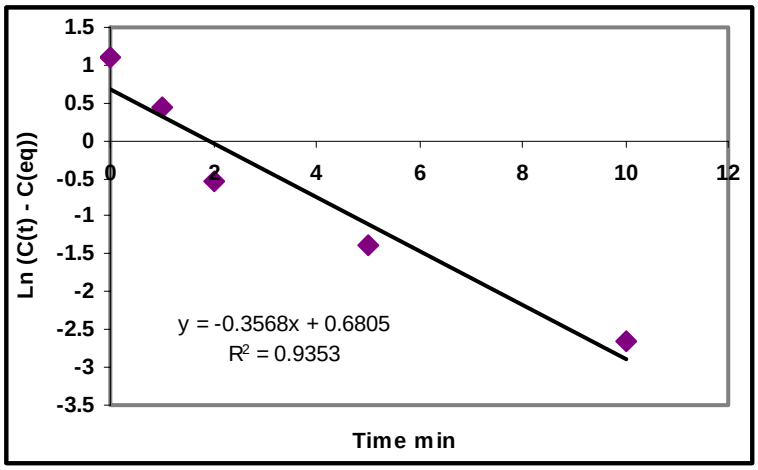

Figure 3.3.5: Determination of t ½ for lignocaine from the linearisation of the rate equation

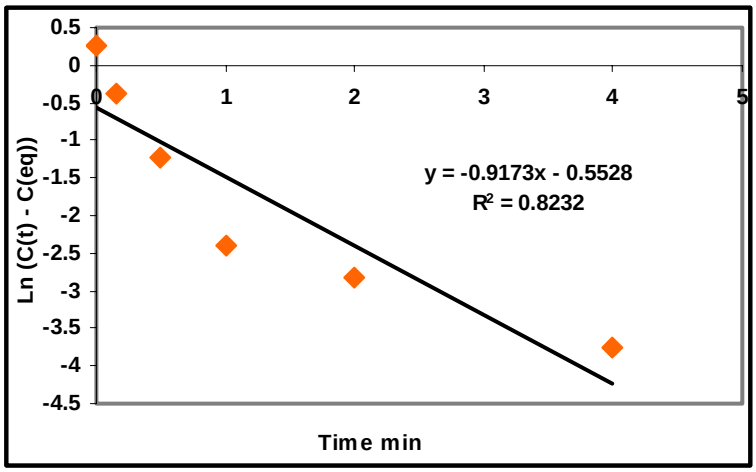

Figure 3.3.6: Determination of t ½ for urea from the linearisation of the rate equation

Clearly, neither of the two systems appears to be well approximated by the first order system. However, as we are only interested in order of magnitude results, we have fitted the data to a straight line, as illustrated by the graphs Figure 3.3.5 and 3.3.6, and computed the following half lives for mass transfer across the microcapsule membrane:

$t_{½\ \text{lignocaine}} = 1.94$ minutes

$t_{½\ \text{urea}} = 0.76$ minutes

A similar calculation can be done to calculate the intrinsic cell kinetics of encapsulated cells for the exact same experimental conditions as the mass transfer experiments described above. These include using the same initial concentrations of both substrates as was used in the experiment above, fabricating the same number of beads except that this time they contain hepatocytes and maintaining the cell loading (cells per bead) derived from the concentration of cells (20 x 10^6cells/ ml) suggested by CHANG's patent (1992).

As our interest is in establishing which of intrinsic cell kinetics or mass transfer across the capsular membrane is the rate limiting reaction - therefore the reaction the designer would have to design the reactor for, an order of magnitude calculation will suffice to reach this conclusion. For this reason, we did not measure the functionality of encapsulated cells and made the assumption that the rate determined for unencapsulated cells would be similar to that of encapsulated cells. Other researchers have conducted these studies and have concluded that microencapsulating hepatocytes does not alter the functionality of the cells (CHANG, 1992, 2001; CANAPLE, 2001; HAMAZAKI, 2002).

Using an initial bulk concentration of 7nmol/ml of lignocaine (the concentration used in the mass transfer experiments), and 1800 microcapsules (the number of capsules formed using 5ml of 2% cell/ alginate suspension), with a cell concentration of 20 x 10^6cells/ml and a maximal rate of uptake by hepatoma cells of lignocaine (Chapter 1), R_{max} = 0.1778 nmol/ 10^6cells/min[2], $t_{½\text{lignocaine}}$ for the cells can be computed:

$$t_{\frac{1}{2}} = {}^{0.5C_{(t=0)}}\Big/_{R_{\max}} \qquad \textbf{Equation 3.3.4}$$

$t_{½\ \text{lignocaine (encap. cells)}}$ = 3.9min

Similarly for urea, using $C_{(t=0)}$ = 18 mmol/l (the concentration used in the mass transfer experiments), and $R_{max} = 2.91 \times 10^{-5}$ mmol/ 10^6cells/min (Chapter 1), the half-life of ammonia metabolism is calculated using Equation 3.3.4, yielding:

$t_{½\ \text{urea (encap. cells)}}$ = 30.92 min

[2] The maximal rate of HepG2 was used as it was the lower maximum rate reached between HuH7 and HepG2.

The half-life computed for mass transfer and intrinsic cell kinetis for urea differed by a factor of ten. For lignocaine, the values calculated were of the same order of magnitude. The values computed for the half-life of the intrinsic cell kinetics was based on the maximum rate achievable by these cells. This implies that the scenario modelled would be the worst case, yet the half-life of mass transfer across the membrane is faster, indicating that mass transfer is not rate limiting even when compared to the half-life of cell kinetics at the highest reaction rates. For a real case scenario, the reaction rates achievable would depend on the maximum concentration of the substrate in blood and further more the half-life would also depend on the cell loading of the microcapsules. As the reactor will most probably not be operating at maximum rate, the real situation would be one where mass transfer has a far shorter half-life than intrinsic cell kinetics (a much higher differential then obtained in these calculations).

3.4 DISCUSSION AND CONCLUSION

This drop method employed to form the microcapsules results in alginate-polylysine-alginate (APA) microcapsules containing cells. The formation of non-spherical microcapsules was eliminated by adjusting the height of the needle assembly from the surface of the $CaCl_2$ solution to greater than 20cm. We used a clearance height of approximately 30cm. As the rate of the air inflow increases, the size of the microcapsules decreases. An air infusion rate of 2l/min produced microcapsules of a diameter of 800μm $\pm$100μm.

Capsule diameter is a very important property. The capsule must be sufficiently large to contain the cells. Larger capsules are preferable as they are easier to handle during washing and settling or in reactor operations such as fluidisation. The probability of finding cells in the microcapsule is therefore related to Poisson's Law. To ensure at least one cell per capsule, the mean cell number per capsule must be higher than four.

Capsules that are too large suffer the problem of limitations to mass transfer created by an internal dead volume. Therefore, we chose to keep our bead diameter as small as possible.

There have been many modifications made to the original encapsulation by researchers (DORIAN, R.E., 1995; GOOSEN, M.F.A., 1987; SOON-SHIONG, 1998; CHANDY, 1999) to increase hepatocyte viability and functionality for transplanted microcapsules, as well as to better control the permeability properties and uniformity of the membrane (GOOSEN, 1985; TSANG, 1987). DIXIT's study (1992) showed that Matrigel could significantly enhance hepatocyte function when used for microencapsulation. MIURA and co-workers (1986) demonstrated that hepatocytes within calcium alginate showed liver specific characteristics, and these activities were almost comparable with those of monolayer-cultured cells.

The concept of an artificial liver based on microencapsulated hepatocytes has been validated by this and others' previous studies. Functions of the encapsulated hepatocytes were the focus of many a study (CHANG, 1992, 2001; CANAPLE, 2001, HAMAZAKI, 2002). Our own studies indicate that cells can be kept alive and functional for up to six days. It most probably is longer but laboratory experiments were only conducted for a period of 6 days. Intraperitoneal implanted encapsulated hepatocytes prolonged the survival time of galactosamine-induced FHF in rats (WONG; 1986); as well as lowered the bilirubin level in Gunn rats (BRUNI, 1989, 1991; DIXIT, 1990). Their viability was maintained for long periods *in vitro* and *in vivo* (WONG, 1988; CAI, 1989). These hepatocytes also release a hepatotrophic factor that is retained within the artificial cells (LANDRY, 1985). BENOIST, *et al*. (2001) demonstrated the survival and functionality of porcine hepatocytes transplanted in large quantities in the peritoneal cavity of allogeneic animals following semiautomatic encapsulation.

Very little is known of the structure of hepatocytes in the microcapsules. A proper three-dimensional cytoarchitecture is important for hepatocytes to maintain differentiated functions. It is important that the correlation between the structure of the encapsulated hepatocytes and their viability be investigated. ITO and co-workers (1992) have shown morphologically distinguishable forms of hepatocyte structures in microcapsules. The tendency to form spheroids in microcapsules increased when high molecular weight poly-L-lysine was used to form the microcapsular membrane. Protein-producing ability of hepatocytes is exerted more in the spheroid culture than in the monolayer culture. Once spheroids were formed in microcapsules, the viability and protein-producing ability were maintained for at least 30 days (ITO, 1992).

Our own data demonstrate that encapsulated hepatocytes are able to maintain their viability for up to six days. Mass transfer data indicate that for the capsules we fabricated the presence of the capsular membrane does not hinder mass transfer to and away from the cells. Work by BRUNI & CHANG (1995) has demonstrated that both free and microencapsulated hepatocytes were equally effective in lowering the serum bilirubin thus validating our results that the capsular membrane does not affect the efficacy of encapsulated cells. Mass transfer experiments were conducted for urea and lignocaine only due to the availability of ^{99m}Tc-DISIDA (source - generous donation). Urea would be a representative of small molecules and lignocaine of larger molecules that would have to cross the membrane. Results are summarised in the table below:

Substrate of Interest	**$t_{½}$ Membrane Mass Transfer**	**$t_{½}$ Cellular Uptake**
Lignocaine	1.94 minutes	3.9 minutes
Urea	0.76 minutes	30.92 minutes

Table 3.4.1 Comparison of the half-life of mass transfer across the microcapsule membrane with the half-life of cellular uptake for the two substances of interest.

The intrinsic cell kinetics for ammonia metabolism to urea is a factor of two slower than the diffusion rate across the membrane. Since the reaction rates used for comparison were based on the maximum rates (r_{max}) achievable, this reflects the best-case scenario. In clinical application, the reaction rates will most probably be much lower than the r_{max} depending on the concentration in blood that requires removal, thus implying that the half-life of intrinsic cell kinetics will be much slower than the diffusional rates.

Thus, we can conclude that mass transfer across the capsular membrane is not rate limiting. Hence, the membrane does not require optimisation for improved mass transfer across it. Intrinsic cells kinetics rather than mass transfer limits us. Hence as we do not believe that we can affect intrinsic cell reaction rates we have the best-case scenario as far as reactor design is concerned. The reactor design would thus depend mainly on the number of cells we can incorporate into into a given volume as the reaction rate is limiting. This simplifies the choice of reactor type and design of reactor.

From these simple relatively inexpensive laboratory experiments we can illustrate the following:

- Membrane structure does not significantly impede the flow of substrates or products across the capsular membrane compared to the cell reaction rate.
- The rate-limiting step was determined to be the **intrinsic cell kinetics,** which cannot be enhanced.
- Optimisable parameters are microcapsule size and the number of microcapsules incorporated in the final reactor design, in order to match cellular uptake rate to membrane transfer rate.
- The cell loading i.e. cells per bead could be increased to increase the achievable reaction rates.

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4. CHAPTER THREE

A FEASIBILITY STUDY FOR THE DESIGN OF A HYBRID ARTIFICIAL LIVER REACTOR

This chapter was written in the form of a paper for publication. Parts of this paper were presented at the PSE2004 Conference held in China in January 2004.

A FEASIBILITY STUDY FOR THE DESIGN OF A HYBRID ARTIFICIAL LIVER REACTOR (HALR)

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Abstract

Loss of more than 60% of liver function can become very detrimental to a patient's health and consequently life. Liver failure results in the loss of maintenance of a normal metabolic balance. This causes a build-up of toxins in the body, which may subsequently lead to secondary organ failure, as the liver is no longer producing important nutrients. To date, there is not a single therapeutic device capable of prolonging a patient's life until the diseased or injured liver recovers or is able to bridge a patient to liver transplantation. Various hybrid artificial liver systems have been researched, tested and proposed as possible therapeutic modalities. They include the use of hollow fibre devices in their various configurations, the use of non-woven fabric encased in a plastic cartridge and the use of encapsulated hepatocytes in fluidised bed reactors. We propose the use of a packed bed reactor incorporating microencapsulated hepatocytes perfused by heparin anticoagulated arterial blood. This configuration circumvents the need for immunosuppression, plasmapheresis, as well as *in vitro* ventilation while at the same time overcomes the limitations of low mass transfer properties and cell volume design constraints. Furthermore it provides a uniform microenvironment as well as ease of scale up. Previous work (Chapter 1 and 2) determined that intrinsic cell kinetics were rate limiting and that microencapsulating hepatocytes does not significantly impede mass transfer when compared to intrinsic cellular kinetics. The aim of this study was together with data obtained in the laboratory to design and assess the feasibility of a reactor for use as a bioartartificial liver reactor.

4.1 INTRODUCTION

If one were to imagine the body as a chemical processing plant, then the liver would be equated with the reactor responsible for almost all the metabolic activities of the body. There is hardly any chemical produced, secreted or regulated that the liver is not directly or indirectly responsible for, making the design of an artificial liver a daunting task for any researcher. It is currently impossible to replicate the multitude of functions of a single liver cell, even given all the knowledge and technological advances of the 21st century.

The first step in designing the reactor is ascertaining the actual or effective reaction rate. This is primarily determined by the intrinsic cell kinetics of the cells to be incorporated. The preferred method chosen to incorporate cells into the reactor is via sequestration within a semipermeable membrane. The next step is determining what the rate-limiting step in the process is. Intrinsic cell kinetics, mass transfer within the bead, diffusion across the membrane, or external mass transfer could limit the overall reaction rate. Intrinsic cells kinetics depends only on the quantity of cells in the capsular beads and not on the membrane area, structure or particle size. Internal mass transfer is strongly dependent on bead radius. Membrane structure and area will only influence the reaction rate if diffusion across the membrane is rate limiting. If for instance external mass transfer is determined to be rate limiting, as is the case with most hollow Fibre devices (HFDs) and plate dialyser models, then it would imply a reactor that could in principle be improved. If this were the case, it would allow the designer to improve the reactor or redesign it such that flow in the reactor could better facilitate mass transfer. Once the situation is achieved where the intrinsic cell kinetics is the rate controlling mechanism, no further improvements in this regard are possible.

The most critical issue in terms of reactor design is mass transfer resistance, i.e. mass transfer to the membrane surface (controlled by design) and mass transfer across the membrane. Each hepatocyte in the intact liver functions under conditions of perfusion and close blood contact. Most bioreactor designs currently under investigation do not duplicate these conditions, which are essential for optimum substrate and metabolic exchange between the hepatocytes and media. Consequently, non-physiological gradients will occur which impair the metabolic activity of the cultured cells. Therefore, optimal mass transfer is an important aspect that should be addressed when developing a HALR.

In order to determine which of the aforementioned processes was rate limiting, an initial study of hepatocyte intrinsic cell kinetics was undertaken. The functionality of two cell lines, HuH-7 and HepG2, was assessed for ureagenesis, P450 activity and bilirubin type uptake activity (Chapter 1). Laboratory results indicated the capability of both cell lines for all three functions and comparable intrinsic cell kinetics for both cell lines were exhibited. Laboratory data also indicate that both the transformed hepatoma cell lines were capable of significantly higher maximum rates of reaction than those quoted in the various literature sources for normal liver cells.

Further work (Chapter 2) demonstrated that mass transfer across the microcapsule, was not significantly hindered by the membrane and therefore not the rate limiting reaction.

The second critical issue involves the reactor volume constraints, i.e the number of cells that can be incorporated in to a given volume. This is important as this determines the intrinsic reaction ability of the reactor to process blood. Clearly the size of this device is critical- as the less blood required to prime the device the less strain is placed on the already struggling patient.

4.1.1 Review of Some of the Bioartificial Liver (BAL) Devices

4.1.1.1. Hollow Fibre Devices

The most researched reactor configuration is the hollow fibre device (HFD). Hollow fibre devices are reactors, which allow the isolation of cellular material from the bulk medium by means of hollow membranes (Refer to figure 1.1.1.1). The reactor configuration is conceptually equivalent to that of the shell and tube heat exchanger. Cells are cultured in either the lumen of the hollow fibre device , analogous to the tube of the heat exchanger, or in the extra capillary space (ECS), analogous to the shell, and the medium pumped through the cell deficient compartment.

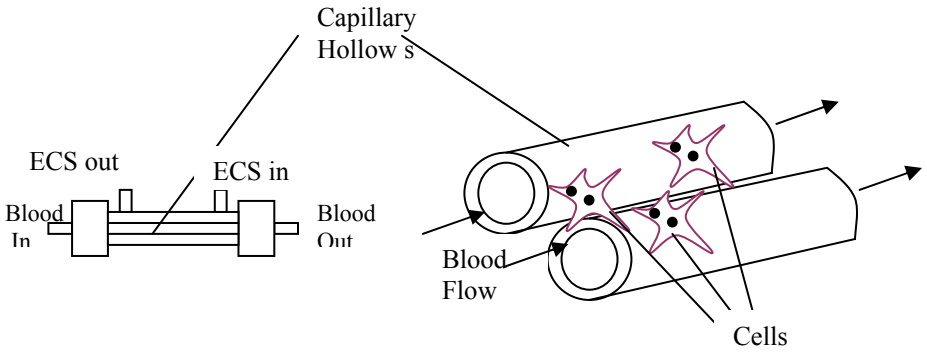


Figure 4.1.1.1.1: Schematic of a capillary hollow fibre device. Hepatoma cells are cultured on the exterior surface of the capillary hollow s, which are bundled together within an enclosing plastic shell. Nutrient medium or blood is circulated within the capillaries (Adapted from DIXIT,1996).

Research with this particular device began with the idea of WOLF & MUNKELD (1976), which was subsequently carried forward by SUSSMAN *et al*. (1992, 1996). The first HFD consisted of a combination of perfused hollow fibres with malignant rat hepatocytes. Cerra and co-workers modified the device by introducing the cells into the lumen of the hollow fibres i.e. intracapillary space (ICS). Gerlach in Germany created a three dimensional arrangement of three different classes of hollow Fibres that supported nutrition, oxygenation and blood transfer to 2.5 x 10^9 porcine hepatocytes (SHEIL, 2002). This device is labour intensive and expensive, which makes prolonged successful support unlikely. Demetriou and collaborators managed to treat some patients with his plasmapheresis circuit, which included the separation of porcine hepatocytes for use in the extracorporeal bioreactor (JAUREGUI, 1999).

Sussman's Extracorporeal Liver Assist Device (ELAD)

Sussman's extracorporeal liver assist device (ELAD®) is being developed by a private company in the United States called VitaGen, Incorporated. The ELAD® is based on a hollow fibre device with C3A cells (cell line originating from a well-differentiated hepatoblastoma, HepG2) inoculated in the ECS. During treatment, the ELAD® will be connected to a continuous haemodialysis machine which separates the cells from plasma which is then perfused through the ICS of the ELAD® (SUSSMAN, 1992&1996). VitaGen is involved in FDA approved clinical trials to evaluate the safety and efficiency of this device in patients with FHF while bridging them to liver transplantation (VitaGen, 2002).

All HFDs are restricted because of their inherently low mass transfer properties across a limited capillary membrane surface. HFDs are also subject to design constraints, which limit the volume of cells that can be incorporated in the device. In addition to these, even though most BAL systems utilise a filtering membrane to isolate bioreactor cells from the patient's blood, micro-breakage of the filter may occur.

Unequal distribution of cells in the network is possible, which could result in a decrease in nutrient diffusion to the cells. Establishing hepatocyte cultures in a HFD is not easy, due to the specific geometry of the device and the nature of hepatocytes as anchorage dependent cells. Hepatocytes introduced into the HFD tend to drop by gravity onto the bottom of the device shell. Researchers have used various attachment processes to anchor cells in a HFD (KARLIK, 1999) but prolongation of the attachment process negatively influences the metabolic function of hepatocytes and consequently the bioreactor performance.

It is very likely that non-physiological gradients will occur which impairs the metabolic activity of the cultured cells. In this respect, semipermeable hollow-membranes seem to be more a burden than a solution to the problem. The membranes can foul and act as a diffusional barrier.

4.1.1.2. Flendrig's Bioartificial Device

Flendrig's approach to the BAL has been to devise a bioreactor that does not include hollow fibre membranes for blood or plasma perfusion. The device comprises a three-dimensional non-woven polyester fabric for high-density hepatocyte culture as small aggregates and an integral oxygenator for on-site oxygenation of the cells. A scaled up 400 ml version of the bioreactor can hold up to $20x10^9$ porcine hepatocytes (FLENDRIG, *et al.*, 1997,1998,1999[5],1999[6]). This configuration is purported to mimic the *in vivo* environment of liver cells. If this device is to attain clinical

application, then concerns such as the potential risk of porcine endogenous retrovirus (PERV) infection and the possibility of emergence of virulent agents must be addressed for purposes of medical safety as PERVs have been shown to be capable of infecting human cells *in vitro* (SHEIL, 2002).

The non-woven fabric (NWF) module approach has also been adopted by a Japanese research group. SAKAI & NARUSI (1998) have developed a bioartificial liver module which is filled with hepatocytes immobilised on non-woven polyester fabric. Apparently, the cell density in the module is comparable to a packed bed type bioreactor. The module is a spin filter-type perfusion bioreactor immobilising 2% number of human liver cells within a 150 ml volume.

The reactor configuration we chose incorporates a higher number of cells per volume of reactor. To compare, Flendrig's reactor configuration immobilises $20x10^9$ porcine hepatocytes in a 400 ml reactor volume (quoted above) whereas our reactor incorporates $4.58x10^{11}$ hepatocytes in a 409ml reactor volume.

4.1.1.3. Plate Dialysers

Multiple membranous plates of primary hepatocyte monolayer cultures are placed in a configuration such that blood flows over the opposite surface of the membrane on which hepatocyte monolayers are cultured. One Japanese group has reported inconclusive *in vitro* data using this system (DIXIT & GITNICK, 1996). Our reactor configuration is able to contain more cells. Further, this configuration would impede mass transfer to the membrane and perhaps across it.

4.1.1.4. Hepatocyte Haemoperfusion

DIXIT & GITNICK (1996) chose to pursue the path of incorporating microencapsulated hepatocytes in an extracorporeal chamber. The plastic chamber is composed of biocompatible polycarbonate, closed at both ends, except for blood

access via two external ports at either end. Results report improved survival time of animals with FHF and no significant adverse effects on the animal's heamodynamics.

4.1.2 Approach to the Reactor Design

Having reviewed all the literature on the various reactor configurations, it was obvious that the best possible route to pursue would be a reactor configuration that faciliated good mass transfer and allowed the most number of cells to be incorporated. This left two choices, the first being the membrane hollow fibre configuration actively being researched by many other research groups and the second being either the fluidised or packed bed reactor containing microencapsulated hepatocytes.

The HFD approach was not investigated for all the inherent disadvantages mentioned in section 4.1.1.1. Furthermore, if one pictures the arrangement of cells with in the capillaries of a HFD and compares it to the spheres within a fluidised/ packed bed reactor, then it is obvious that the spheres contain more cells when packed into the reactor.

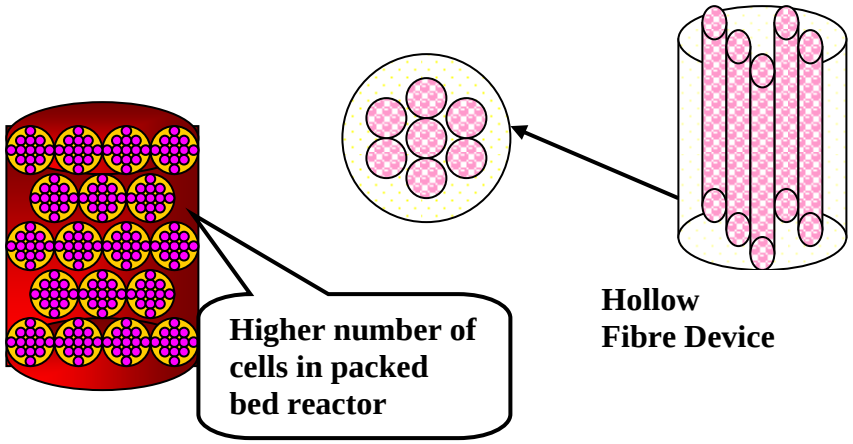


Figure 4.1.2.1: Schematic comparison between the cell loading in a packed bed reactor containing microencapsulated hepatocytes and a capillary HFD.

Furthermore, microencapsulation, based on CHANG'S (1992) method was chosen as it prevents immunological responses from the patient's blood. A spherical microcapsule membrane has the greatest surface area to volume ratio then any other shape, thus maximum nutrient/product exchange across the microcapsule membrane may be achieved. It is also postulated that the capsular environment allows better cell-to-cell communication thereby enabling cells to retain their functionality for extended periods (DIXIT & GITNICK, 1996). Spherical microcapsules offer better three-dimensional stability with respect to mechanical stresses.

The next step was to determine which reactor configuration i.e. fluidised bed or packed bed reactor, was the best route to pursue. Both fluidised and packed bed reactors allow direct perfusion of blood over microencapsulated hepatocytes.

Fluidised bed reactors have the properties of a liquid. One of the properties is excellent heat transfer. The same temperature is quickly established through out the fluidised system because the general agitation of the particles disperses local regions of hot and cold. A fluidised bed is therefore very suitable for biological systems requiring close temperature control (DAVIDSON, 1985). There is also a high rate of heat transfer to the beads in the bed. Fluidised bed reactors are also characterised by the following features:

- Low pressure drop across the bed (low ΔP);
- No dead volumes;
- Low shear;
- Low cell number per volume of beads (OTHMER, 1956).

Packed bed reactors don't have freeboard and therefore can accommodate a higher number of cells per volume of reactor. They are characterised by:

- Higher-pressure drop across the bed (high ΔP);
- High velocities could result in high shear forces;
- High ΔP could result in high shear forces.

To summarise:

PROPERTY	FLUIDISED BED	PACKED BED
External Mass Transfer	Good	Less Good
Pressure Drop	Low	High
Shear on Bead	Low	High
Dead Volumes	Low	Likely
Cell Density	Low	High

Table 4.1.2.1: Comparison of the physical properties between Fluidised and Packed Bed Reactors

Both these reactor configurations will be investigated.

4.2 REACTOR DESIGN

We have adopted a Process Synthesis approach to the theoretical design of our bioartificial reactor by employing chemical engineering principles to the design process. In order to apply the Process Synthesis methodology, one needs to be able to specify the important parameters of the reactor so that in the end one is able to do a more rigorous Process Simulation. Using this approach to Process Synthesis, we have advocated, we will use simplified calculations to test the feasibility of the reactor design.

4.2.1 Introduction

Ureagenesis functionality is very important as accumulation of ammonia can lead to coma then death. The hepatocytes tested in our laboratory appeared to perform this function more than adequately and the maximum reaction rates for the two hepatoma cell lines measured were significantly higher than those quoted for normal liver cells in the literature. Therefore, in terms of the reactor design, we can ignore this reaction.

Bilirubin conjugation was measured in an indirect manner by studying the uptake kinetics of an analogue demonstrated by other studies (OKUDA, 1986) to use the same anionic pathway. From those results, kinetic data for bilirubin conjugation were inferred. A radioactive bilirubin analogue, ^{99m}Tc-DISIDA was studied instead as this would circumvent the accuracy problems associated with measuring bilirubin concentration under laboratory conditions. Bilirubin is life threatening in an infant, as the blood brain barrier has not developed sufficiently to prevent the bilirubin from crossing over. In an adult, an increase in bilirubin concentrations is not life threatening as such and can be treated by various other means such as exchange transfusion and exposure to UV light. Therefore, this reaction can also be ignored for the reactor design.

The P450 activity of the two cell lines mentioned previously was determined by measuring the uptake kinetics of lignocaine by these cells. The functions of the P450 system, which if decompensated is life threatening. Therefore this reaction would be a good choice as the primary basis for the design of the reactor.

Laboratory work described previously in Chapter Two demonstrated that encapsulation of the hepatoma cells in beads does not result in cell reactions being mass transfer limited with respect to both lignocaine and urea. Thus we were able to conclude that the rate limiting step, at least with respect to these two reactions, is the cell reactions themselves. This implies therefore that the measured rates of reaction

obtained for the non-encapsulated cells can be employed for design purposes. Furthermore, we were also able to demonstrate that encapsulated cells could be kept alive for at least six days with only a small drop in activity. This implies that the essential metabolites could be supplied to the cells at the required rates. Microencapsulation therefore represents a powerful method for isolating the hepatoma cells from the whole blood.

4.2.2 Reactor Specific Rates

The variation in reaction rates has a direct impact on the reactor design. In order to obtain the required removal rates in the reactor, a certain number of cells has to be incorporated to effect this removal rate. The important question is what that **number of cells** is?

4.2.2.1 Estimate of the Number of Cells

The measured removal rates as a function of composition for all the reactions studied were given by an S-shaped curve, with a rapid increase in reaction rate at low concentrations rising to a maximum value at high concentrations of the metabolite (or substance requiring removal). The high concentrations of these metabolites do not appear to impede the functioning of the cells, so that in all likelihood the toxicity levels will be set by the sensitivity of other organs to these materials. Thus, the hepatoma cells would have no difficulty in functioning at these high concentrations.

The data tabled below were obtained from various literature sources for normal liver cells. In table 4.2.2.1 reaction rates from the literature (column 1) were normalised for 10^6cells and expressed in the units (column 2) that data measured in our laboratory were expressed, allowing easier comparison between normal liver cells and the transformed hepatoma cell lines.

	[1]Production/ Removal Rates (In units quoted in literature)	Production/ Removal Rates (Converted to uniform units)	Concentration in Blood (To be removed)
Ammonia	2.08×10^{-9} mol/10^6cells/min	2.08×10^{-9} mol/10^6cells/min	100×10^{-6} mol/ l
Lignocaine	[2]3×10^{-3}g/min	4.53×10^{-12} mol/10^6cells/min	1.73×10^{-5} mol/ l
Bilirubin	[2]250 – 350 mg/day	[3]3.088×10^{-13} mol/10^6cells/min	17.1×10^{-6} mol/ l

[1] Production rate for urea from ammonia (YOON *et al*., 1999; ZUCKER *et al*., 1995), removal rates for lignocaine (HARDMAN, 2001; KATZUNG, 2001) and bilirubin (HAWKER, 1993).

[2] Assume this is the clearance rate for entire mass of hepatocytes =1200g or 2.292×10^{12}cells.

[3] Used maximum rate of 350mg/day (HAWKER, 1993).

Table 4.2.2.1: Removal rates for the three reactions and the upper bound concentrations of the substrates in blood obtained from various literature sources.

Without information of the toxicity levels, we are not in a position to accomplish a full design. The best we can do under the circumstances is to note that at the normal concentration conditions at which the body operates, the hepatoma cells appear to exhibit reaction rates that are in excess of those for the normal liver. Under these circumstances, the obvious option would be to take into account the anecdotal mass of cells quoted in the literature by various researchers and clinicians to be compatible with preserving life and designing the reactor for this mass of cells.

Assuming that 20% of hepatocytes mass is required to sustain life, the mass of cells to be designed for is 240g (LIU, 2002). To mimic as closely as possible the *in-vivo* conditions of the liver, we have attempted to design our reactor such that flows and pressure drops through the reactor are as close as possible to those of a normal functioning liver. To this end, the flow rate (Q) used is 300ml/min (normal arterial delivery to the liver). The pressure drop across the hepatocytes in the liver is 9mmHg (1200 Pa). Pressure drops across dialysis units are in the range 100-200 mmHg. For the purposes of this design, an upper limit of 100 mmHg is set.

4.2.3. Design Specifications

The first question one must ask is: **What specifications must the reactor meet?**
The constraints on the reactor design include limiting the amount of priming blood from the patient to 500ml. This is set at a maximum. For a fluidised bed reactor configuration, the reactor volume should lie somewhere between the packed bed volume and the expanded bed volumes (microencapsulated cells + priming blood). For a packed bed reactor design, the volume of the reactor is just the volume of the encapsulated cells incorporated, assuming a bed voidage of e=0.4. Secondly, the design should ensure that the pressure drop across the bed is as small as possible.

How do we design the reactor to ensure that it meets these specifications?
Since we are not diffusion limited but rather limited by the intrinsic cell kinetics either reactor configuration would be feasible. However external mass transfer in the reactor would be better in a fluidised bed reactor than a packed bed reactor.

For the design of a fluidised bed reactor, the parameters that are available for the designer to choose and optimise include the geometry or dimensions of the reactor, so the length of the bed (L), the cross sectional area of the bed (A) and the voidage or bed expansion during fluidisation (e). e is the fraction of the volume of the bed not occupied by solid material and is termed the fractional voidage. It is dimensionless. Therefore, the fractional volume of a bed occupied by solid material is (1-e) (COULSON & RICHARDSON, 1960).

For the design of a packed bed reactor, the voidage is determined by the shape of the particles in the reactor. Since our beads are spherical in shape, they would probably assume the loosest form of packing with e = 0.4. The flow rate is the choice of the designer. Other parameters available for optimisation include the reactor geometry, L and A, and the pressure drop (ΔP) across the bed.

4.2.4 Is a Fluidised Bed Reactor Design Feasible for Clinical Application?

L and A are dependent on the aspect ratio (L/D) of the reactor vessel. A longer, thinner geometry is preferred to a shorter fatter type for better flow properties. The voidage is dependent on the pressure drop across the bed. Initially spheres in a bed would assume the loosest possible mode of packing (COULSON & RICHARDSON) therefore e_{min} =0.4 (bed initially assumes packed bed configuration).

The range of allowable reactor volumes can be calculated from the volume of beads and the volume of blood required constrained by the maximum volume of priming blood (500ml).

V_{beads} = 246 ml

Assuming e = 0.4

V_{min} = 410 ml for the reactor, implying that V_{blood} = 164 ml

If the maximum blood volume allowable is 500ml, the V_{max} for the reactor would be the sum of the bead and blood volume, i.e. 746 ml. Therefore the allowable range of reactor volumes is given by $410 < V_{reactor} \leq 746$ ml. The maximum voidage can be calculated using the maximum allowable volume of the reactor, V_{max} as follows:

$$e = \frac{V_m - V_b}{V_m} = 0.67 . \qquad \textbf{Equation 4.2.4.1}$$

where V_b refers to the volume of the beads or spheres containing hepatocytes.

The pressure drop across the fluidised bed is constant irrespective of increasing velocity. To calculate the pressure drop at minimum fluidisation, the minimum fluidisation velocity (u_{mf}) must first be calculated. The minimum velocity, which will produce fluidisation if flow conditions within the bed are streamline, is obtained by:

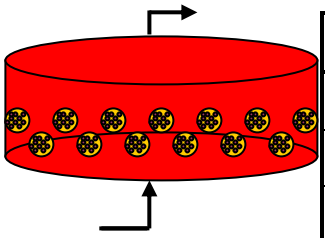
$$u_{mf} = 0.0055\left(\frac{e^3}{1-e}\right)\frac{d^2(\rho_s - \rho)}{\mu}g \qquad \textbf{Equation 4.2.4.2}$$

$$u_{mf} = 0.000191 \text{ m/s}$$

Since the flow rate through the reactor is defined as 300 ml/min, the area of the reactor can be computed from $Q = u_{mf} A$. The volume of the reactor at minimum fluidising velocity is known and using the area just calculated, the reactor length, L, can be calculated. Pressure drop is related to the bed voidage and reactor length as follows:

$$-\frac{\Delta P}{l} = (1-e)(\rho_s - \rho)g \qquad \textbf{Equation 4.2.4.3}$$

What would this reactor look like at incipient fluidisation?



Reactor Specifications	
Reactor Volume	410 ml
Bead Volume	246 ml
Number of cells	4.58×10^{11}
Reactor Area	0.026 m^2
Reactor Length	16 mm
Reactor Diameter	183 mm
Flow rate	300 ml/min
Velocity (u_{mf})	**0.191 mm/s**
Pressure Drop	0.01 Pa
L/D	**0.08**

Figure 4.2.4.1: Fluidised bed reactor configuration and specifications

From these quick and simple initial calculations, one can see that the fluidised bed is not the most feasible option. If one were to increase the voidage, hence the bed volume, the area of the reactor would increase by a greater amount than the length. A fluidised bed reactor is meant to be long and thin, i.e. L/D≅10, not short and broad as this shape adds problems associated with fluid flow and inhomogeneous concentrations through the reactor.

This result is not unexpected since the bead density is approximately equal to the blood density. This results in the extremely small minimum fluidisation velocity (u_{mf}). Velocities cannot be enhanced very much as this would result in entrainment of the microcapsules at the top of the fluidised bed. Ideally, one would want a larger density differential between the fluid in the reactor and the particles being fluidised. Even though this reactor type is characterised by better mass transfer and temperature control, it is not essential that our reactor is a fluidised bed.

A packed bed reactor does not have the exceptional mass transfer and temperature control characteristics of the fluidised bed, but since we are not diffusion limited this is not a issue for us. We are limited by the intrinsic cell kinetics. Therefore, the reactor we design should have scalability such that the number of cells incorporated may be increased at the designer's or clinician's will.

4.2.5. Is a Packed Bed Reactor a Feasible Design for a Hybrid Artificial Liver Reactor?

The feasibility of this reactor configuration will be determined by initial calculations using the design constraints and initial estimates of parameters available to the designer. The volume of the reactor is determined by the volume of beads required with a bed voidage, e=0.4, i.e. $V_{reactor}$ = 410 ml. The flow rate is set at 300 ml/min. The only parameters available for the designer to optimise are the reactor geometry, L

and A, and the pressure drop (ΔP), across the bed, which increases as the velocity of blood perfusing through the bed increases.

Since reactor volume is the product of the reactor area (A) and reactor length (L) which can both be written in terms of the diameter, and assuming an aspect ratio of L/D =10 the geometry of the reactor can be computed yielding:

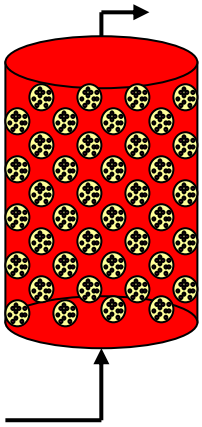
A = 1104 mm^2
L = 375 mm
D = 37.5 mm

The velocity (u) through the bed is then computed from $Q = u\,A$ yielding a velocity of 4.53x10^{-3} m/s. If we assume the flow through the packed bed is laminar as $(D_s G_o / \mu)(1-e)^{-1} < 10$ (2.38 for these conditions) where $G_o = \rho u_0$ the pressure drop across the bed may be calculated using the Blake Kozeny equation (BIRD, STEWARD & LIGHTFOOT, 1960):

$$u_o = \frac{\Delta P}{L}\frac{D_s^{\,2}}{150\mu}\frac{e^3}{(1-e)^2} \qquad \textbf{Equation 4.2.5.1}$$

ΔP = 6045 Pa (45.35 mmHg)

What does this reactor look like?



Reactor Specifications	
Reactor Volume	410 ml
Bead Volume	246 ml
Reactor Area	1100 mm^2
Reactor Length	375 mm
Flow rate	300 ml/min
Velocity	4.53 mm/s
Pressure Drop	45.34 mmHg
Residence time	0.73 min
L/D ratio	**10**

Figure 4.2.5.1: Packed bed reactor configuration and specifications

Based on the assumption that only 20% of liver mass is required to sustain life, we are able to design the reactor with the specifications above. This reactor only requires a blood volume of 164 ml. This implies that the priming volume of blood for the reactor itself is also only 164ml. Therefore the apparent scalability of this reactor is a highly desirable feature as this packed bed reactor volume can be increased by 67%. This allows for some flexibility as there is also a need to prime the lines leading to and from the device with blood.

Since lignocaine clearance was the rate limiting reaction of the three liver function tests, we examined the range of reaction rates achievable for various masses of cells.

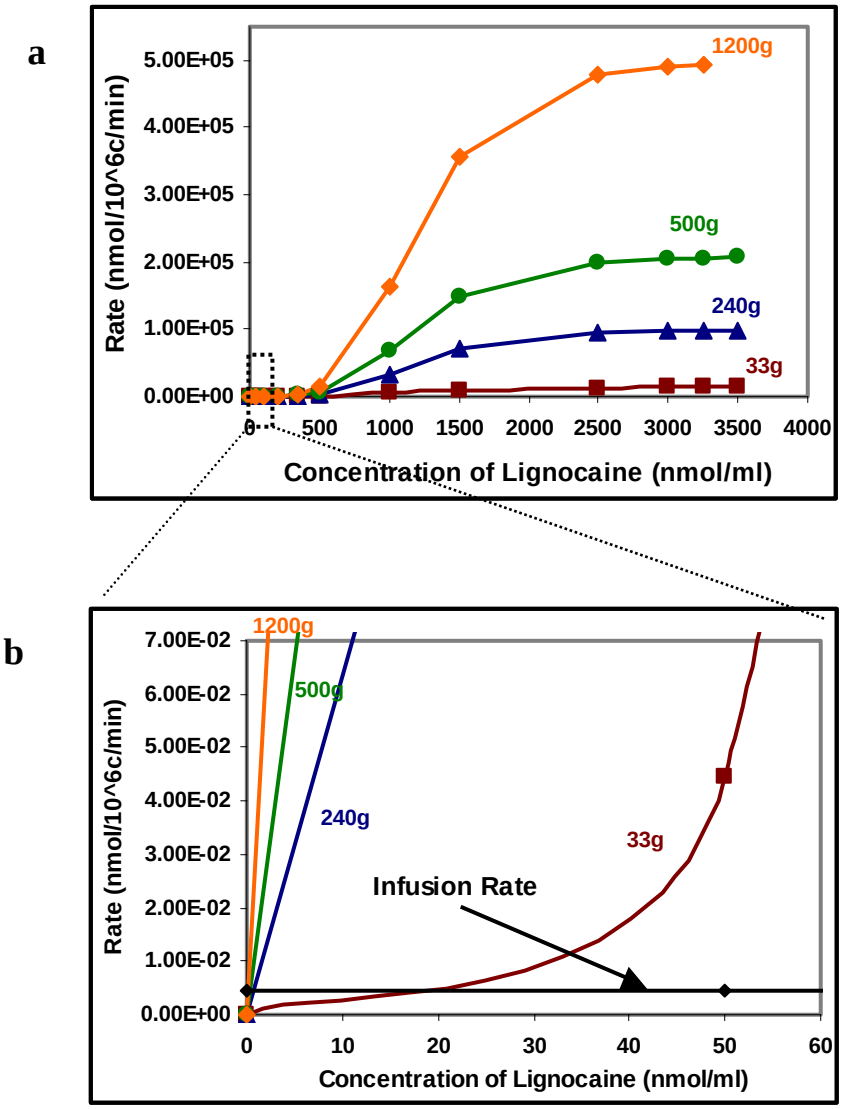


Figure 4.2.5.2: Reactor Rates for various amounts of cells (a), with the expanded region illustrating the infusion rate of lignocaine plotted on the Reactor Rate curve for various amounts of cells (b).

From the graphs plotted above, 240g seems to be a reasonable estimate of cells. As the concentration builds up the reaction rate will increase to a new steady state. An important question that would need to be answered is what the maximum tolerable steady state reaction rate is.

We have achieved our desired goals by using an L/D ratio of 10. However, if we wished to decrease the pressure drop for any reason, this value could be decreased without any significant problems, which in turn allows further flexibility in our design.

4.3. DISCUSSION AND CONCLUSION

This paper has raised some interesting questions, firstly about the reaction kinetic data available in the literature and secondly about the use of fluidised bed reactors as bioartificial liver devices.

Initially, our attempt was to calculate the number of cells required for use in our hybrid artificial liver reactor (HALR) based on a comparison of rate data obtained in the laboratory with that of normal cells obtained in the literature. We would then have used the limiting reaction based on the liver function that required the most cells to base our reactor design on. Anecdotal reports suggest that flow rates used in dialysis machines range between 300 and 600 ml/min of whole blood. We wanted to calculate the flow rates that would correspond with the reaction rates for normal liver cells and base our reactor flow rate on those rates, the aim - to mimic as close as possible the *in-vivo* environment. Incompleteness in the data from various literature sources resulted in us abandoning this route.

The approach taken assumed approximately 20% of liver hepatocyte mass was essential to keep a patient alive, and therefore the reactor design could be based on this cell mass. Process synthesis methodology was then applied to the reactor design process. It was always the intention of this research group that a fluidised bed reactor would be the best reactor type to incorporate the encapsulated hepatocytes for all the various properties of this particular reactor type. From the initial calculations, we found that this was not the most feasible option as the reactor geometry was contrary to the desired long and thin configuration (L/D ~10). Scalability of this reactor would result in non-feasible clinical applications based on the area and size of the bed that would result, as well as from the volume of priming blood that would be needed.

The main reason that a fluidised bed reactor will not work for this application is that the density of the microcapsules is very close to the density of the blood. This implies that small increases in velocity could result in entrainment of the beads, or velocities exceeding terminal velocity. As the velocity through the bed increases, the bed would expand such that the area of the reactor would have to increase to accommodate this increasing velocity, as the pressure drop during fluidisation remains constant. This would result in a worse reactor geometry.

If however we were mass transfer limited, which we believe is not the case, and there was really a need to use a fluidised bed reactor, the density problems could be solved in one of two ways. The first option would be to make the beads heavier. This could be achieved by encapsulating a dense material (e.g. metal filings) together with the cells. To avoid any unfavourable reactions of the cells to the denser material, the material could first be microencapsulated then encapsulated with the cells into a bigger bead. This would create a more favourable density differential between the beads and the blood, resulting in better reactor geometry and higher achievable superficial velocities.

The other option would be to make the beads less dense so that they float in the blood and then fluidise downwards. This could be achieved by trapping air bubbles in the alginate/ cell suspension used to form the microcapsules.

Based on simple calculations performed we were able to ascertain the non-feasibility of a fluidised bed reactor as a bioartificial liver reactor. There are other researchers however who have proposed the use a fluidised bed reactor (BARBE, DAVID & LEGALLAIS, 2002) and have gone further to determine how the motion in a fluidised bed affects the mechanical properties of the beads. The flow rates and superficial velocities would be so minute that the shear stress on the beads are insignificant.

The other option of course would be to consider an alternate reactor type such as a packed bed reactor.

We then proceeded to determine the feasibility of a packed bed reactor. Initial calculations yielded a feasible reactor design. The specifications of the reactor proposed were consistent with the clinical requirements as stated in the literature. The flow rate through the packed bed was the same as the arterial delivery to the liver, Q = 300ml/min. The pressure drop through the bed was not as small as would have been preferred but essentially not too harsh a pressure drop for the blood or the hepatocytes. Our HALR only requires a priming volume of 164 ml, implying that scalability is not a problem with this device. If however mass transfer was the rate limiting process, one would struggle to enhance mass transfer between the blood and microcapsule membrane in this type of reactor.

Extracorporeal devices perfused with whole blood require the patient to be heparinised to prevent clotting in the device. However, heparinisation of the patient often leads to haemorrhage complications (LANGER, 1982). Therefore, our research group has undertaken the development of a heparin bioreactor (JOSHUA, 2003), which can be used as part of the clinicians' armament when treating patients with extracorporeal therapy. One of the most salient benefits of the development of such a reactor is that is can be used in conjunction with our artificial liver reactor, which would be based on heparin anti-coagulated whole blood. Since heparin requires metabolism by the liver, the heparin bioreactor would remove this added metabolic burden from an already decompensated liver.

The *in vitro* studies and calculations performed during this research, suggest the feasibility of a hybrid artificial liver. However, prior to any extension of the research to clinical tests, an in depth risk analysis would need to be conducted on the use of transformed cell lines, as this may ultimately prove to be the factor that makes such reactor design using transformed cells unacceptable.

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5. CONCLUSION OF THE THESIS

Designing a hybrid artificial liver reactor required investigation into various disciplines. Given that the reactor being developed is a chimera of both synthetic and biological components, an understanding of the critical variables available to the designer within each of these areas was necessary. In order to answer those questions that would allow us to determine the parameters available to the designer, a high-level systems approach to the problem being researched was adopted. Throughout the course of this thesis, the reader may have found that certain areas could have been researched much further but were not. The main reason for this is that only those factors that contributed to the design of the reactor were explored. For example, the biological component we chose to incorporate in the reactor was a transformed liver cell line. Further research could have been conducted to determine the best cell line to use. We could have made the topic of the thesis a comparison of cell lines and their kinetics, but this was not the focus of this work. Our experiments and development was aimed at determining the critical factors in the design of the reactor. Research terminated at determining the functional integrity of the selected cell lines and selectivity of the reactor design to these cell lines.

We selected the HuH-7 and HepG2 transformed hepatoma cell lines as possible candidates for incorporation into the reactor. Results demonstrated that both cell lines exhibited liver functionality with respect to three pre-selected liver function tests. Furthermore, the intrinsic cell kinetics determined for both cell lines did not differ significantly. From this, we concluded that it was probable that the reactor design was not sensitive to the type of cell line chosen. Therefore more cell lines were not tested.

Indirectly, we have also developed a quick screening technique that other researchers can use for choosing a biological component for a bioartificial liver (BAL). Firstly what we have found which was also determined by various other researchers, was that these cells are more than adequate at performing ureagenesis. The rates at which our cells remove lignocaine from solution is almost comparable to the in vivo average liver cell rates.

Metabolic reactions using the anionic carrier pathway, such as the conjugation of bilirubin were found to have the fastest reaction rates when compared to normal liver cells (with respect to all three liver function tests). So as a first pass in choosing a cell line, the bilirubin functionality should not be the first functionality test conducted. Furthermore, the accumulation of bilirubin per se is not a life threatening condition in an adult. Various alternate therapies are available to the clinician to deal with the conjugation of bilirubin such as plasma exchange therapy and exposure to UV light.

We chose to look at liver functionality for the three tests across a wider concentration range, extending beyond the C_{max} i.e. the concentration of the substrate in blood that would have to be removed. This was done in order to determine the saturation concentrations of the cells as well as the metabolic activity at these high concentrations. The results obtained were most interesting. It seems that high concentrations of substances that medical practitioners regard as toxic to patients are not toxic as such to the liver cells themselves. This is evidenced by the increasing reaction rate obtained corresponding to an increasing reactant concentration. Another hypothesis that would explain these findings is that the cells tested are tumoregenic in nature and therefore inherently highly active, therefore capable of high reaction rates.

These findings would lead one to believe that the normal liver cells in the body operate at a much lower rate than they are capable of. This would imply that there is a lot of redundancy built into the liver. At normal substrate concentrations, the liver operates at a lower reaction rate. Due to liver disease or diminished liver capacity, the concentrations of these substances could be expected to increase and therefore a new higher steady state concentration and higher reaction rate could be established in the liver. This hypothesis is validated by the fact that loss of two thirds of liver functionality does not lead to patient death.

Subsequent to establishing the nature of the biological component, the next step in the reactor development was finding a way to incorporate the cells into the reactor, while simultaneously immuno-isolating them and overcoming the drawbacks of previous BAL designs such as mass transfer limitations and cell volume constraints. This was achieved by employing the microencapsulation technique learnt in Professor Chang of McGill University's laboratory. Encapsulating the cells allows the designer the use of whole blood perfusion instead of plasma reducing the number of extracorporeal devices a patient would have to be connected too. The capsular membrane however adds a barrier to the diffusion of substrates, products and nutrients to the liver cells.

The first step in designing the reactor is determining what the rate-limiting step is. Which reaction would the designer prefer to be rate limiting? Obviously, it would be the one that the designer has the most control over, such as external mass transfer. This would be the best-case scenario as the reactor can be redesigned or the flow better designed to facilitate better mass transfer should mass transfer be rate limiting. However, a well-designed reactor would be limited by a rate beyond the designer's control. In this case, that would be the intrinsic reaction rate of the cells.

The results obtained in the second chapter revealed the rate-limiting step to be the intrinsic cell kinetics. The capsular membrane was found to not significantly hinder the transport of material across the membrane. Therefore, we were not diffusion limited. Not much can be done to enhance the intrinsic cell kinetics except to increase the number of cells in the reactor. This may be achieved by increasing either the cell loading of the beads, the bead diameter or the number of beads incorporated into the reactor. Since the bead diameter is one of the design constraints, the two other options are the only avenues available for the designer's exploitation. Another implication of these findings is that the designer does not have to design for optimising the bead membrane.

The rate-limiting step was determined by comparing the mass transfer half-life to the intrinsic cell kinetic half-life for the same set of conditions. The maximum rates obtained for the three reactions were used to do this computation. One would expect that at these high reaction rates mass transfer might be the limiting factor in the process. What we found was that even at these elevated rates of reaction, the mass transfer was not limiting. Therefore, one could infer that in reactor operation the mass transfer would be much better as the cells would most probably not be at reacting at their maximum rate in the device.

The type of substrates that would have to cross the capsular membrane would include molecules ranging from the small size represented by urea through to bigger molecules of the size of lignocaine. The membrane produced by Chang's method does not hinder the diffusion of these substances in any way as is evidenced by mass transfer data obtained in the laboratory discussed in Chapter 2. Oxygen should not have a problem diffusing through the membrane either. This was evidenced by the fact that the encapsulated cells could be kept alive for at least six days.

The reactor configuration found to be most feasible for a HALR was the packed bed reactor because the microcapsule density is very close to that of the density of blood. Thus, if one wanted to take the fluidised bed approach, the superficial velocities achievable would be too low. Since the velocity is related to the flow rate, which is in turn related to the reactor geometry or dimensions, an increase in flow rate would result in an increase in reactor diameter. The optimum shape of a fluidised bed reactor is long and thin with an L/D of approximately 10 and not short and broad. Therefore, the packed bed option was investigated. A packed bed reactor does not have the exceptional flow characteristics of a fluidised bed reactor, but since we are not mass transfer limited this was not regarded as an issue of concern.

The findings from the laboratory research are all very interesting in terms of reactor design. Traditionally, a chemical engineer will usually design a reactor based on maximum rates possible. Obviously the ultimate Chemical Engineer does not concur, as this is obviously not the case with the liver in the body (Refer to Table 5.1). It seems as though a tremendous amount of redundancy has been built into the design (of the liver) allowing for the ability to respond to all sorts of situations.

	Normal Liver Cells Reaction Rates	HuH-7 Maximum Reaction Rates	HepG2 Maximum Reaction Rates	Concentration in Blood
Ammonia Metabolism	2.08nmol/10^6cells/min	29nmol/10^6cells/min	35nmol/10^6cells/min	100 μmol/l
Lignocaine Uptake	4.53 x 10^{-12} mol/10^6cells/min	0.22 x 10^{-9} mol/10^6cells/min	0.178 x 10^{-9} mol/10^6cells/min	2-6 μg/ml (Plasma concentration)
^{99m}Tc-DISIDA Uptake	(Data unavailable in similar units for comparison)	6.10 x 10^{-8} Ci/10^6cells/min	4.50 x 10^{-8} Ci/10^6cells/min	1.67x10^{-6} Ci/ml (Plasma concentration)
Rate constant (*k*) (Based on half life)	1.609x10^{-8} min^{-1}	0.0017 min^{-1}	0.00255 min^{-1}	

Table 5.1: Comparison of reaction kinetics between normal liver cells and the transformed hepatoma cell lines for the three liver function tests.

It seems that liver cells are capable of higher rates of reaction concomitant with high substrate concentrations, i.e. higher than those quoted in column 5 of Table 5.1. What is unclear however is the range of substrate concentrations the body could actually tolerate.

What we discovered in our research is that the design of an artificial liver is not a problem in terms of achieving removal (i.e. reaction rate). The liver cells are able to operate at much higher reactions rates than occurs in the body. In order to function at such high rates, the concentrations of the substrates might be in the range regarded as toxic to the rest of the body. What is required is to test the whole system, which would imply the whole body to determine concentrations which are toxic to the body over the short term.

If you designed the liver independent of the body, you would only need a small amount of cells operating at maximum reaction rates, but since the corresponding concentrations might be toxic to the rest of the body a larger mass of cells would be required. Therefore, even though a liver contains 1200g of cells our HALR can operate efficiently at a higher rate based on 240g of cells but whether these higher concentrations allow life to be sustained remains an open question.

In conclusion, our research has shown that a packed bed configuration is a feasible reactor type capable of including the number of cells required to effect the reaction rate needed for the adequate removal of substances required for artificial liver support.

APPENDIX A – HPLC METHOD FOR DETERMINING LIGNOCAINE

SPECIMEN:

A 5ml sample.

EQUIPMENT AND MATERIALS:

EQUIPMENT:

1. A HPLC system with UV detector and integrating facilities.
2. Vortex mixer.
3. Centrifuge.
4. Waterbath at 40ºC.
5. Assorted pipettes.

MATERIALS:

1. Water µBondapak C_{18} (10µm) HPLC column 300 x 3.9mm ID – Water Cat.No.
2. Drug Free Serum – BioRad Cat.No.
3. Acetonitrile HPLC grade
4. Potassium dihydrogen phosphate-
5. Dichloromethane HPLC grade
6. Trimethoprim
7. Lignocaine hydrochloride
8. Sodium hydroxide
9. Sterilin 15ml PTFE tubes
10. Whatman Phase Sep filter papers
11. Milli-Q water

REAGENTS:

1. **0.05M Potassium dihydrogen phosphate pH 4.0:**

   Weigh out 6.805g of potassium dihydrogen phosphate and dissolve in ± 700ml water in a beaker.

   Adjust pH to 4.0 with phosphoric acid.

   Transfer to a 1 litre volumetric flask and make up to volume with water.

2. **Mobile phase:**

   Mix 860ml of the above 0.05M buffer with 140ml acetonitrile.

3. **0.05M Potassium dihydrogen phosphate pH 2.8:**

   Weigh out 6.805g of potassium dihydrogen phosphate and dissolve in ± 700ml water in a beaker.

   Adjust pH to 2.8 with phosphoric acid.

   Transfer to a 1 litre volumetric flask and make up to volume with water.

4. **0.25M Sodium hydroxyde:**

   Weigh out 1.0g of sodium hydroxide pellets and dissolve in 100ml water.

5. **Trimethoprim Internal Standard:**

   Weigh out 15mg of trimethoprim and dissolve in 1 litre of water.

CALIBRATION:

1. **Lignocaine Stock Standard:**

   Dilute the lignocaine solution.

QUALITY CONTROL:

1. Reconstitute 1 bottle each of carotene serum control level 1 and 2 with 2.0ml Milli-Q water.

   Allow to stand for 10 – 15 minutes, then mix gently.

   Store in freezer when not in use.

PROCEDURE – STEPWISE:

SETTING UP OF INSTRUMENTS:

HPLC:

1. Install correct column for lignocaine and check that sample loop is correct - 20ųl
2. Place reagent lines into bottle, seal top with sheet of parafilm.
3. Switch on helium cylinder and ESS and bubble helium through mobile phase for a few minutes, then set ESS to position 1 for intermittent sparging.
4. Switch on pump and prime mobile phase using syringe supplied.

COMPUTER AND SOFTWARE:

1. Switch ON Computer.
2. At the C:\ type in WIN
3. Maximise Program Manager.
4. Select Borwin V icon and double click.
5. Double click on next Borwin icon – Borwin loads and Main Menu appears.

   FILE EDIT USER VIEW CONTROL RUN PEAKS PROCESS HELP
6. From Borwin Main Menu top row select “Control”.
7. Control drop down menu select “Dev. Control on System 2” – Click ON.
8. Click ON “HPLC PUMP”.
9. When pump control screen appears set flow rate at 0.5 ml/min.
10. From Borwin Main Menu top row select “RUN”.
11. Then click ON “Working List”.
12. then click ON “Working on System 2”.

13. When work list screen appears, select the 🗁 (open file icon) top left hand corner and click ON.
14. From the list that appears, click ON “ligno”.
15. The worklist from the previous run loads. Edit list with new parameters and then click on 🗁 (close file icon) to save new list.
16. Click ON “Start Acquisition” icon.

DETECTOR:

1. Switch on detector and allow to go through set-up program.
2. Select λ wavelength button – enter 205nm – press “ENTER”.
3. Select AUFS – set to “ENTER”.
4. Press AITO ZERO to zero detector.

METHOD:

1. Into a 5ml plastic test tube, pipette 0.5ml serum.
2. Add 50µl internal standard mixture.
3. Add 40µl of 0.25M sodium hydroxide.
4. Vortex for a few seconds.
5. Add 4.0ml dichloromethane.
6. Vortex for 1 minute.
7. Filter through a phase Sep filter paper.
8. Place into a waterbath at ± 40ºC – and evaporate to dryness.
9. Reconstitute with 300µl 0.05M potassium dihydrogen phosphate pH 2.8.
10. Vortex for a few seconds.
11. Draw 100µl into the injection syringe and load onto rheodyne injector.
12. Switch the rheodyne injector into the INJECT position.

INTEGRATION:

1. On main screen click On 🗁 (open file icon) – a list of all files appears.
2. Highlight appropriate file name – a sample chromatogram appears in right hand corner – press.
3. When chromatogram has loaded, click ON "PEAKS".
4. Click On "Peak parameters" on drop down menu – select 🗁 (open file icon) and select "CAROT".
5. Click ON "Find peaks" on drop down menu – chromatogram will be integrated.
6. Baseline and peak starts and ends may need adjustment.
7. Select "PRINT" icon to print report – Please Note Chromatogram will be printed as seen on screen!
8. Close chromatogram.

CALCULATION:

1. Note down the area values on worksheet.
2. Calculate the analyte area/internal std area ratios for all standards and samples.
3. Calculate results as follows:
   Ratio of unknown ÷ ratio of standard x concentration of standard
4. Alternatively results can be calculated by using computer program HPLC.

Please Note: Values may be given in nmol/L on insert but results are reported in µmol/L.

Divide result by 1000.

PROCEDURE NOTES:

Reference Ranges:

0.19 – 1.58 µmol/L.

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APPENDIX B – METHOD FOR CELL ENCAPSULATION

1) Cells (hepatocytes) washed with buffered saline (medical saline
2) Cells added to **4%** stock solution of sodium alginate.
   (working concentration of **2% alginate solution containing 1x10^6 cells/ml. 5ml solution).**

3) Extrude solution with a syringe infusion pump through droplet generator
   (Air flow rate = 2-3 l/min Infusion Rate = 0.28 –0.39 ml/min).

4) Gelation upon contact with **300ml 100mM** receiving solution.
   (Height between jet and $CaCl_2$ >= **20cm**)

5) Microcapsules are cured in **$CaCl_2$** solution for **15 minutes**.

6) Microcapsules are washed at least thrice in saline.

7) 5ml microcapsules + **80 ml of 50% Poly-L-lysine** for **20 mins.**

8) Microcapsules strained and washed with saline at least thrice.

9) Immersed into **200ml of 0.2% Sodium Alginate** for **5 min**.

10) Washed and immersed in **200 ml** of 50mM Sodium Citrate for **6 min**.

11) Washed and stored in culture medium for future use.