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From the Editor’s Desk

It is a matter of great pleasure for us to bring out the XVI volume of PHARMBIT, biannual scientific journal, an official publication of the Pharmaceutical Society, Department of Pharmaceutical Sciences, Birla Institute of Technology, Mesra, Ranchi.

The encouraging response and wide acceptance of our updated last issue (Vol. XV) of PHARMBIT by Pharmaceutical fraternity of national and international organizations, motivated us to further improve the content of the journal to maintain international standards. Now, the journal has been made available on-line and it can be accessed through our institute website (www.bitmesra.ac.in).

We are thankful to the scientists and faculty members of the institutions of India and abroad for contributing their review and research papers in large numbers within three months of release of the last issue of PHARMBIT. But due to page constraint and peer review by reviewers, only four review papers and seven research papers have been published in this issue.

The timely publication of the journal has been made possible only due to the support of dedicated and devoted group of personnel in the form of contributors, editorial board members, editorial advisory board members, reviewers, well wishers and members of our society.

We would further request contributors to strictly follow the instructions for authors as given to avoid undue delay as well as unnecessary rejection of their paper by refree.

As an editor of this journal, I am thankful to all authors, reviewers, editorial board members, advertisers, well wishers, Head, department of Pharmaceutical Sciences, Vice Chancellor, BIT, Mesra and Vice Chancellor Emeritus, BIT, Mesra for their support and encouragement in bringing out this edition of PHARMBIT. Lastly we invite suggestions from readers and well wishers to further improve the PHARMBIT.

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Role of Antioxidants in the Inhibition of Free-Radical Mediated NF-κB Activation: Adjunct Therapy in the Treatment and Prevention of Dreaded Disorders

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The transcription factor NF-κB, since its discovery in 1986, developed widespread interest on account of its unusual regulation, activation through variety of stimuli, its control over diverse genes and biological responses, and its apparent involvement in variety of human diseases like AIDS, Arthritis, Asthma, Atherosclerosis, Cachexia, Cancer, Diabetes, Euthyroid sick syndrome, Inflammatory bowel disease, and Stroke etc. Since oxidative stress, that excessively generates the free radicals, is one of the prominent factors activating NF-κB, the role of antioxidants in regulating NF-κB activity comes into the picture. The key role that NF-κB plays in inflammatory disorders as well as in promoting innate immune responses, clearly illustrate the “good and evil” aspects of NF-κB whereby NF-κB is required for immunological mechanisms but detrimental when it is dysregulated. Consequently, NF-κB, in recent years, has consistently become the target for the discovery of several anti-inflammatory and anticancer drugs.

REACTIVE OXYGEN SPECIES (ROS) AND PATHOGENESIS:

Oxidative stress has increasingly been recognized to play a pivotal role in influencing pathophysiology of critical illness, and development of organ failure, in particular. The role of Reactive Oxygen Species (ROS) and Reactive Nitrogen-Oxygen Species (RNOS) in modulating cell signaling, proliferation, apoptosis, and cell protection have now been clearly identified. Apart from this, ROS and RNOS are capable of attacking proteins, polysaccharides, nucleic acids, and polyunsaturated fatty acids; the ultimate outcome being the cellular damage and tissue dysfunction [1]. Analogous to one classically observed in septic shock [2], in critical illness ROS are generated from mitochondrial dysfunction, from NADPH oxidase enzyme of stimulated neutrophils and macrophages, and also from xanthine oxidase resulting from activated conversion from xanthine dehydrogenase during ischemia/reperfusion injury [1]. Moreover, ROS/RNOS may trigger release of cytokines from immune cells, activate inflammatory cascades, and increase the expression of adhesion molecules, probably mediated through inducing the expression of nuclear factor κB [3]. Thus, inflammation and tissue injury result in the accumulation of granulocytes in organs that leads to increased generation of ROS, which further perpetuates or amplifies the inflammatory response and subsequent tissue injury [4]. These pathways and cycles are central to the underlying pathophysiology in critically ill patients with a systemic inflammatory response and multiorgan dysfunction.

In humans, there is a complex endogenous defence system, designed to protect tissues from ROS/RNOS-induced cell injury. Special enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (including their cofactors-selenium, zinc, manganese, and iron), sulfahydryl group donors (glutathione), and vitamins (vitamins E, C, and β-carotene) form a network of functionally overlapping
defence mechanisms. In critically ill patients, there exists reduced storehouse of antioxidants, reduced plasma or intracellular concentrations of free electron scavengers or cofactors, and decreased activities of enzymatic systems involved in detoxification of ROS [5].

The circulating antioxidant levels decrease rapidly after shock, trauma, or surgery and are retained below normal levels for several days or even weeks [6]. More severe the trauma, systemic inflammatory response syndrome (SIRS), or sepsis, the larger the depletion of antioxidants [7, 8]. These observations are not mere epiphenomenon, as low endogenous stores of antioxidants are associated with an increase in free radical generation, an augmentation of the systemic inflammatory response, subsequent cell injury, increased morbidity, and even higher mortality in the critically ill patients [6, 9]. It is therefore hypothesized that the exogenous supply of defined trace elements-like zinc [10] and antioxidant vitamins-like Vitamin C, Vitamin E or β-Carotene [11] would be helpful to regain the balance between oxidants and antioxidants in the conditions of critical illness.

NF-κB AND ITS SIGNALING PATHWAY:

Amongst the inducible transcription factors that control inflammatory gene expression, Nuclear Factor Kappa B (NF-κB) plays a central and evolutionarily conserved role in coordinating the expression of various soluble proinflammatory mediators (e.g., cytokines and chemokines) and leukocyte adhesion molecules [9].

In non-stimulated cells, NF-κB is sequestered within the cytosol by an inhibitory protein known as, IκB (Inhibitor of NF-κB) that masks the nuclear localization signal present within the NF-κB protein sequence (Figure 1A). Treatment of cells with proinflammatory cytokines, such as TNF-α and IL-1 or with bacterial products such as lipopolysaccharide (LPS), leads to the activation of a specific IκB kinase (IKK) complex (Figure 1B) [9]. Recent findings do suggest that activation of IKK mainly involves ROS [12].

Figure 1: Location of NF-kB in Healthy and inflammatory conditions,
[A] Non-stimulated (healthy) state of the cell, and
[B] Stimulated (inflammatory) state of the cell
Activated IKK complex phosphorylates IκB, thereby making it available for ubiquitination and degradation by the proteasome. In the process, NF-κB is liberated that allows its translocation to the nucleus where it acts as transcription factor [13]. As a matter of fact, translocated NF-κB binds to specific elements (κB-sites) within the promoters of responsive genes to activate their transcription [9]. A wide range of genes involved in inflammation contain functional κB-sites within their promoters and are induced by NF-κB [14].

The IKK complex, as a critical activator of NF-κB function, has been the focus of intense research over last not more than two decades. The complex is now known to be composed of a core of three subunits; two of which namely, IKKα and IKKβ contain functional kinase domains which are capable of phosphorylating IκB at specific N-terminal serine residues to initiate its ubiquitination. In contrast, the third core subunit of the IKK complex, called NEMO (NF-κB essential modulator), which is also known as IKKγ or IKKAP, is a non-catalytic component that functions as a key regulator of IKK activity [13].

It may thus be summarized that in absence of inflammatory activity, the transcription factor NF-κB is primarily retained in the cytoplasm by an inhibitory-protein, IκB. Proinflammatory stimuli, while activating a specific protein kinase, results in the phosphorylative-degradation of IκB that leads to the release and translocation of NF-κB into the nucleus.

NFκB ASSOCIATED DISORDERS:

In the nucleus, NF-κB binds to target DNA elements, vis à vis regulating the transcription of genes involved in immune and inflammatory responses, cell growth control, and apoptosis. Genes encoding cytokines, cytokine receptors, cell adhesion molecules, chemo-attractant proteins, and growth regulators are positively regulated by NF-κB. Genes regulated by NF-κB, that are presented in Figure 2, include those encoding IL-2, IL-6, IL-8, the IL-2 receptor, the IL-12 p40 subunit, VCAM-1, ICAM-1, TNF-α, IFN-γ, and c-Myc [15, 16].

Consistent with the regulation of genes involved in the immune and inflammatory response, mice devoid of several of the NF-κB subunits show defects in clearing bacterial infection along with defects in B- and T-cell functions [16]. Surprisingly, the knockout of the p65/RelA subunit dies at day 16 of development from extensive liver apoptosis, thus revealing a role for NF-κB in controlling cell death [17]. The ability of NF-κB to get activated by inflammatory cytokines such as TNF-α and to regulate genes involved in inflammatory function, raised the question of whether NF-κB dysregulation would be associated with inflammatory diseases.

![Figure 2: NF-κB regulated genes.](image)
Chung et al. [18] proposed age-related upregulation of NF-κB, whereas Chen [19] demonstrated the crucial role that NF-κB and NF-κB-regulated cytokines can play in diabetogenesis. Hiscott et al. [20] and Collister & Albensi [21] pointed out the involvement of NF-κB signal pathway in AIDS and alzheimer’s disease, respectively. While Lee et al. [22] focused on the role of NF-κB in carcinogenesis and the therapeutic potential of targeting NF-κB in cancer therapy, Li & Gao [23] reviewed therapeutic strategies against atherosclerosis and cancer, including inactivation of NF-κB signal pathway. Okamoto [24] indicated that NF-κB is constitutively activated in some rheumatic conditions such as rheumatoid arthritis.

**Figure 3:** Diseases and conditions that may be alleviated by specific NF-κB inhibiting drugs.

Evidences generated from the involvement of many distinct in vivo models demonstrate that development of the drug selectively targeting NF-κB activity may yield pharmacologically relevant and therapeutically valuable treatments for a wide range of diseases and conditions in which inflammation plays a critical role. Some major diseases realized to be effectively treated by inhibitors of NF-κB in animal models may be gathered from Figure 3.

**ANTIOXIDANTS AND INHIBITION OF NF-κB:**

A variety of drugs and antioxidants can inhibit NFκB. Figure 4 summarizes various intermediate steps involved in proinflammatory signal transduction pathways and their targeting by inhibitors of NF-κB.

Oxidative stress, on account of its involvement in critical illness, is gaining increased attention. Many
factors of concern which contribute to oxidative stress are changing lifestyle in developing countries, pollution, eating habits, climatic changes (which are either not avoidable or cannot be changed), etc.

Diverse agents that cause oxidative stress can activate NF-κB [25]; and numerous stimuli (cytokines, phorbol esters, LPS, and CD3 engagement) that activate NF-κB increase the levels of intracellular ROS [26]. This generation of ROS, however, is cell- specific and also stimulus-specific. Stimulation of NF-κB with proinflammatory cytokines (IL-1 and TNF-α) leads to the generation of substantial intracellular ROS in lymphoid and monocytic cell lines, no such increase have been located in epithelial cell lines derived from ovaries, colon, breast or cervix [26]. Although evidence for the role of ROS in proinflammatory NF-κB activation remains circumstantial, more convincing studies demonstrate that a variety of antioxidants like vitamin C [27], vitamin B6 [28], vitamin E & its derivatives [29-31] & flavonoids [32-37], and those present in apple juice extract [38, 39], garlic juice [40, 41], Ginkgo biloba extract [42], tomato peel polysaccharide [43], etc. possess potential to inhibit the activation of NF-κB.

Until recently, it was thought that low-density lipoprotein cholesterol oxidation and its biological
effects could be prevented by using antioxidant supplements. The American Heart Association (AHA) [44] may, however, be found quoting, “more recent clinical trials have failed to demonstrate a beneficial effect of antioxidant supplements however; and some studies even suggest that antioxidant supplement use could have harmful effects”. AHA, therefore does not recommend using antioxidant vitamin supplements to prevent cardiovascular diseases until more complete data are available (i.e. until their effect is proved in clinical trials that directly test their impact on CVD endpoints). Beneficial effects must be demonstrated in well designed (randomized, placebo-controlled) clinical trials, before recommending widespread use of antioxidants to prevent cardiovascular disorders. At this moment, according to AHA, the scientific evidence supports a diet high in food sources of antioxidants, and other heart – protecting nutrients, such as fruits, vegetables, whole grains and nuts instead of antioxidants supplements to reduce the risk of CVD.

Such reports, occasionally appearing in a section of literature, seem to have warned against the harmful effects that an excessive consumption of supplementary antioxidants can produce. Such a finding or two, do recommend closely guarded and very precise monitoring of supplementary antioxidant intake. The nature, quantum and duration of therapy should be such as to ensure its just effectiveness to completely inhibit unwarranted biochemical processes, yet is not excessive enough to adversely interfere with the normal functioning of body cells that the original activities are restored as early as the therapy is discontinued or altered.

Some pertinent questions, more of basic nature that still remain to be addressed for effective management of the patient from the viewpoint of “antioxidant therapy” appear to be as under:

i) Does the efficiency of antioxidant remain free radical specific? (If so, the choice of antioxidant, among others, would be governed by the type/nature or the class of generated free radical).

ii) Does the type of free radicals generated from oxidative stress be illness specific? (If so, the choice of antioxidant would also be influenced by the type of disease)

iii) a. Would quantum of free radicals being generated in the body vary with the severity of illness?
   b. Although appears least likely, has the severity of the illness associated with stress-related disorders to do any thing with the nature of free radicals being generated during the course of illness and thus antioxidant therapy?

iv) a. Could in vivo /in situ activity of antioxidants be predicted on the basis of some “Universal Parameter” under varying conditions of the formulations and type of illness? or
   b. Would such a parameter be governed by the mechanism the antioxidants acts?

V) Would the combination of antioxidants, one acting as free-radical chain breaker (poisoning agent) and the other, working on the mechanism of free radical scavenger (reducing agent), serve more effective antioxidant therapy? and if so, the adverse effect, such a combination therapy, resulting from the suppression of normal generation of free radicals, is likely to have on normal metabolic processes of healthy human being.
REFERENCES:


An Insight overview of Lysosomal Enzymes and their Biological significance.

Samyukta B., Annu Singh and Pratyooosh Shukla
Department of Biotechnology, Birla Institute of Technology, Mesra, Ranchi, India.

ABSTRACT
Role of different Lysosomal enzymes has been discussed in the present review. Lysosome is a digestive structure found within virtually all types of animal cells and is bounded by a membrane and contains several dozen different species of digestive enzymes, each of which can sever particular chemical bonds found in natural materials. Most lysosomal enzymes function best in an acid environment and this acidification is accomplished by a proton pump, built into the membrane surrounding the lysosome, which affects the transport of hydrogen ions into the lysosomes. In the present review the overview of current discoveries and potential of different lysosomal enzymes which are one of the most versatile enzymes and potential have been discussed.

INTRODUCTION

DISCOVERY: Lysosomes were first discovered by a Belgian biochemist, Christian de Duve (1955 AD)\(^1\) in the liver cells and were earlier named pericanalicular dense bodies. On investigating carbohydrate metabolism of liver cells it was observed that the cells released an enzyme called acid phosphatase in larger amounts when they received proportionally greater damage in the centrifuge. To explain this phenomenon, de Duve suggested that the digestive enzyme was encased in some sort of membrane-bound organelle within the cell, which he dubbed the lysosome. After estimating the probable size of the lysosome, he was able to identify the organelle in images produced with an electron microscope.

Lysosomes are electron microscopic, vesicular structures of the cytoplasm which are involved in intra-cellular digestive activities, so called lysosomes. They are membrane bound organelles contain hydrolytic enzymes, have acidic contents (pH 4.5-5.5), have electron-dense heterogeneous contents, digest ingested material and aged or damaged organelles.

Lysosomal enzymes are those enzymes (acid hydrolases)\(^2\) which are responsible for breaking down complex chemicals within a cell which have expended their useful life. The breakdown products are then eliminated from the cell or reused.

They catalyse the reaction: \[ AB + H_2O \rightarrow AH + BOH \] \(^3\)

LYSOSOMES:

Occurrence: These are absent in the prokaryotes but are present in all the eukaryotic animal cells (except mammalian RBC’s), some fungi (yeasts, neurospora), some protists (euglena) and meristematic cells (root tip cells of maize).

Shape: These are generally spherical in shape but are irregular in plant root tip cells.

Size: Size range is 0.2 to 0.8micrometer while average size is 500nm\(^4\)

Ultrastructure: Under electron microscope, a lysosome is formed of two parts:
Fig 1. Electron microscope picture of a lysosome.

(source  http://biology.unm.edu/ccouncil-Biology_124-Images-lysosomes_jpeg)

1. **Limiting membrane:** It is outer, single layered, lipoproteinous and trilaminar unit-membrane. It keeps a limit on glyco-proteinous digestive enzymes.

2. **Matrix:** It is inner, finely granular and highly heterogeneous ground substance inside the membrane.

   On the basis of nature of matrix lysosomes are of four types, and shows polymorphism.

   (a) Primary lysosomes
   (b) Secondary lysosomes
   (c) Autophagosomes or Autolysosomes
   (d) Tertiary lysosome or Telolysosome

**Chemical composition:** Matrix of primary lysosome is formed of hydrolases (enzymes involved in hydrolysis or digestion of polymeric compounds) which operate in acidic medium (pH=5.0), so called acidic hydrolases. The acidic medium is maintained by proton pumps which actively transport the protons into the interior of lysosomes. These enzymes are synthesised on RER, transported to cisternae of Golgi body where these are packed into the lysosomes.

**Functions:** Primary function of lysosomes is intra-cellular digestion, as it contains enzymes to digest all the types of macro-molecules. The functions are estated below:

1. **Heterophagy:** In heterophagy, the cell takes up particles or molecules by the process of endocytosis, engulfing them in membrane-bounded vesicles or vacuoles that are formed at the cell surface. The endocytosed material enters lysosomes via intermediate membrane-bounded compartments known as endosomes. In higher animals, heterophagy is most prominently used by leukocytes and macrophages. These specialized cells endocytose invasive microorganisms and use endocytosis in clearing debris and disposing of dead or senescent cells.

2. **Autophagy:** In autophagy, cells segregate regions of their own cytoplasm within compartments that come to be bounded by single membranes and to receive lysosomal enzymes. Autophagic lysosomes take part in the remodeling of cells as part of the processes of development and during stressful circumstances. They also participate, along with nonlysosomal enzymes and heterophagic lysosomes, in normal turnover of the body's constituents—the balanced synthesis and destruction through which most molecules of most cells are replaced by new molecules.
3. Autolysis: It involves self digestion of cell by lysosomal enzymes when after the cell death, lysosomal enzymes are released. So the lysosomes are also called suicidal bags.

4. Extracellular digestion: The lysosome releases its enzymes out of the cell by exocytosis to digest food outside. e.g., acrosome release hyaluronidase (lytic enzyme) to digest egg envelope locally for sperm entry. Similarly, osteoclasts release the enzymes to digest the bone.

5. Lysosomes also engulf the carcinogens.

6. Lysosomes initiate cell division by digesting repressor molecules.

7. Lysosomes of osteoclasts digest the cartilage material during the formation of replacing or cartilaginous bones (osteogenesis).

**Lysosomal Enzymes**

Lysosomal enzymes are hydrolases that catalyse the reaction:

\[
AB + H_2O \rightarrow AH + BOH
\]

**TABLE 1: Different Lysosomal Enzymes**

<table>
<thead>
<tr>
<th>CATEGORY</th>
<th>EXAMPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteases</td>
<td>cathepsin and collagenase</td>
</tr>
<tr>
<td>Nucleases</td>
<td>DNAse and RNAse</td>
</tr>
<tr>
<td>Glycosidases</td>
<td>beta-galactosidase, beta-glucuronidase</td>
</tr>
<tr>
<td>Phosphatases</td>
<td>ATPase, acid phosphatase</td>
</tr>
<tr>
<td>Sulphatases</td>
<td>for sulphate linked organic compounds</td>
</tr>
<tr>
<td>Esterases</td>
<td>phospholipase, acid lipase</td>
</tr>
</tbody>
</table>

Today more than 60 lysosomal enzymes are known. The name and their action on biological molecules are stated below:

**TABLE 2**

| Peptidases | hydrolyse proteins |
| DNAases and RNAases | hydrolyse DNA and RNA |
| Lipases | hydrolyse lipids |
| Phosphatases | hydrolyse phosphates |
| Glucosidases | hydrolyse glycogen |
| Carboxylases | hydrolyse carboxyl groups |
| Sulphatases | hydrolyse sulphates |
| Esterases | hydrolyse esters |

Acid phosphatase and beta-glucuronidase have been used as histochemical markers for lysosomes.

**Cell Digestion and the Secretory Pathway**

Lysosomes break down cellular waste products, fats, carbohydrates, proteins, and other macromolecules into simple compounds, which are then returned to the cytoplasm as new cell-building...
To accomplish the tasks associated with digestion, the lysosomes use some 40 different types of hydrolytic enzymes, all of which are manufactured in the rough endoplasmic reticulum from where they are transferred in a transport vesicle to the cis face of the Golgi apparatus. Inside the Golgi complex, the enzymes undergo additional processing and are transformed from an inactive to an active state. Lysosomes may then bud from the trans face of the Golgi apparatus, though they may also form via other mechanisms. Several different varieties of macromolecules may be digested by lysosomes and arrive at the organelles by different pathways.

Table 3. Digestive function of lysosomes

<table>
<thead>
<tr>
<th>Macromolecule</th>
<th>Degradation product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleic acids</td>
<td>Mononucleosides, phosphate</td>
</tr>
<tr>
<td>Protein</td>
<td>Amino acids, dipeptides</td>
</tr>
<tr>
<td>Glycoprotein</td>
<td>Mono-, disaccharides</td>
</tr>
<tr>
<td>Polysaccharide</td>
<td>Ceramide</td>
</tr>
<tr>
<td>Glycolipids</td>
<td>Choline, phosphate, P-diester</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>Fatty acids, glycerol</td>
</tr>
<tr>
<td>Neutral lipids</td>
<td></td>
</tr>
</tbody>
</table>

Thus lysosomes play a critical role in the metabolic function of our bodies. One of their primary roles is to pick up substances such as carbohydrates and lipids and break them down into smaller molecules so that they can be used again in the metabolic process. This process is possible because lysosomes contain enzymes, which are proteins that help the body’s chemistry work better and faster. Working continuously, enzymes break down carbohydrates and lipids and assist in the transfer of their byproducts throughout the rest of the cell for the production of energy or excretion.
Thus, Lysosomal enzymes (acid hydrolases) are responsible for breaking down complex chemicals within a cell. The breakdown products are then eliminated from the cell or reused. A deficiency of any one of these enzymes will lead to a “storage disease” which is usually associated with developmental regression.

**Lysosomal diseases**

Genetic defects in lysosomal enzymes and related proteins are known to be associated with a large number of rare disorders in humans and animals (such as Tay-Sachs disease and Niemann-Pick disease type C). Defective lysosomal function leads to storage of particular classes of molecules that cannot be degraded and, in long-lived cells such as neurons, to complex pathogenic cascades with widespread impact on endosomal-lysosomal function, membrane trafficking, and signal transduction. Such disorders are most often fatal.

The causes of the deficiencies may include total absence of the gene, absence of the appropriate mRNA, impaired conversion of a proenzyme to active enzyme, rapid degradation of a precursor or of the enzyme itself, incorrect transport of the enzyme precursor to its proper destination, presence of mutations that inactivate the enzyme, or absence of protective proteins.

**Table 4 : Lysosomal Diseases and Respective Defective Enzymes**

<table>
<thead>
<tr>
<th>NAME</th>
<th>DEFECTIVE ENZYME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Niemann-Pick disease</td>
<td>Sphingomyelinase</td>
</tr>
<tr>
<td>Faber disease</td>
<td>Ceramidase</td>
</tr>
<tr>
<td>Gaucher disease</td>
<td>Beta-Glucocerebrosidase</td>
</tr>
<tr>
<td>Lactosyl ceramidosis</td>
<td>Beta-Galactosyl hydrolase</td>
</tr>
<tr>
<td>Tay-Sachs disease</td>
<td>Beta-Hexosaminidase A</td>
</tr>
<tr>
<td>Fabry disease</td>
<td>Alpha-Galactosidase</td>
</tr>
<tr>
<td>Sandhoff disease</td>
<td>Beta-Hexosaminidases A and B</td>
</tr>
<tr>
<td>Globoid cell leukodystrophy</td>
<td>Galactocerebrosidase</td>
</tr>
<tr>
<td>Metachromatic leukodystrophy</td>
<td>Arylsulfatase A</td>
</tr>
<tr>
<td>Pompe disease</td>
<td>Alpha-Glucosidase</td>
</tr>
<tr>
<td>Huler Syndrome</td>
<td>Alpha-L-Iduronidase</td>
</tr>
<tr>
<td>Hunter Syndrome</td>
<td>Iduronate-2-sulfate sulfatase</td>
</tr>
<tr>
<td>Maroteaux-Lamy Syndrome</td>
<td>Arylsulfatase B</td>
</tr>
<tr>
<td>Sly Syndrome</td>
<td>Beta-Glucuronidase</td>
</tr>
<tr>
<td>Aspartylglucosaminuria</td>
<td>Aspartylglucosaminidase</td>
</tr>
</tbody>
</table>

**Important Lysosomal Enzymes : Some of the Enzymes are described below :**

1) **Alpha-galactosidase**°: It is a glycoside hydrolase enzyme encoded by the GLA gene. This gene encodes a homodimeric glycoprotein that hydrolyses the terminal alpha-galactosyl moieties from glycolipids and glycoproteins. This enzyme predominantly hydrolyzes ceramide trihexoside, and it can catalyze the hydrolysis of melibiose into galactose and glucose. A variety of mutations in this gene affect the synthesis, processing, and stability of this enzyme, which causes Fabry disease, a rare lysosomal storage disorder that results from a failure to catabolize alpha-D-galactosyl glycolipid moieties.
2) β-galactosidase: It is a hydrolase enzyme that catalyzes the hydrolysis of β-galactosides into monosaccharides. The active site of β-galactosidase catalyzes the hydrolysis of its disaccharide substrate via “shallow” and “deep” binding. Monovalent potassium ions (K⁺) as well as divalent magnesium ions (Mg²⁺) are required for the enzyme’s optimal activity. The beta-linkage of the substrate is cleaved by a terminal carboxyl group on the side chain of a glutamic acid. β-galactosidase is an essential enzyme in the human body. Deficiencies in the protein can result in galactosialidosis or Morquio B syndrome.

3) Glucocerebrosidase: (glucosylceramidase, β-glucosidase, or D-glucosyl-N-acylsphingosine glucohydrolase) It is needed to cleave, by hydrolysis, the beta-glucosidic linkage of the chemical glucocerebroside, an intermediate in glycolipid metabolism. It is localized in the lysosome and has a molecular weight of 59700 Daltons. Mutations in the gene cause Gaucher disease, a lysosomal storage disease characterized by an accumulation of glucocerebrosides.

4) Hexosaminidase A (alpha polypeptide): Hexosaminidase A is the alpha subunit of the lysosomal enzyme beta-hexosaminidase that, together with the cofactor GM2 activator protein, catalyzes the degradation of the ganglioside GM2, and other molecules containing terminal N-acetyl hexosamines. Beta-hexosaminidase is composed of two subunits, alpha and beta, which are encoded by separate genes. Both beta-hexosaminidase alpha and beta subunits are members of family 20 of glycosyl hydrolases. Mutations in the alpha or beta subunit genes lead to an accumulation of GM2 ganglioside in neurons and neurodegenerative disorders termed the GM2 gangliosidoses. Alpha subunit gene mutations lead to Tay-Sachs disease (GM2-gangliosidosis type 1).

5) Sphingomyelinase phosphodiesterase: (Sphingomyelinase) It is a hydrolase enzyme that is involved in sphingolipid metabolism reactions. SMase is found in the lysosomes of both prokaryotic and eukaryotic cells and is responsible for breaking sphingomyelin (SM) down into phosphocholine and ceramide. Without SMase, SM builds up within the cell, eventually causing cell death and major organ failure; the disease associated with this disorder is Niemann-Pick disease.

6) UDP-N-acetylglucosamine-lysosomal-enzymeN-acetylglucosaminephosphotransferase: It catalyzes the chemical reaction as stated below:

\[
\text{UDP-N-acetyl-D-glucosamine} + \text{lysosomal-enzyme D-mannose} \rightarrow \text{UMP} + \text{lysosomal-enzyme N-acetyl-D-glucosaminyl-phospho-D-mannose}
\]

Thus, the two substrates of this enzyme are UDP-N-acetyl-D-glucosamine and lysosomal-enzyme D-mannose, whereas its two products are UMP and lysosomal-enzyme N-acetyl-D-glucosaminyl-phospho-D-mannose.

This enzyme belongs to the family of transferases, specifically those transferring phosphorus-containing groups transferases for other substituted phosphate groups.

7) Glucuronidases: These are glycoside hydrolases that cleave glucuronic acid glycosides (glucuronides). Human glucuronidase is a lysosomal glycosidase, and hydrolyzes glucuronide moieties from proteins. Glucuronidase exhibits both endo-glycosidase and exo-glycosidase activities, meaning that it can cleave monosaccharides from the middle of a chain or from the end. In eukaryotes, glucuronidase is located in the lysosome and plays an important role in recycling cellular components. A deficiency is associated with Sly syndrome.

CONCLUSION

Lysosomes play a critical role in the metabolic function of our bodies. One of their primary roles is to pick up substances such as carbohydrates and lipids and break them down into smaller molecules so that they can be used again in the metabolic process. This process is possible because lysosomes
contain enzymes, which are proteins that help the body’s chemistry work better and faster. Working continuously, these enzymes break down carbohydrates and lipids and assist the transfer of their byproducts throughout the rest of the cell for the production of energy. Lysosomes contain as many as 40 or 50 different enzymes, each responsible for a highly specialized function. At present there are around 60 lysosomal enzymes are known but majority of them are not well studied separately. Therefore, a deficiency in one particular enzyme or activator protein causes symptoms that may be somewhat different from the symptoms caused by the deficiency of another type of enzyme.

One major significant activity of these enzymes is that they are needed to break down molecules called glycosaminoglycans - long chains of sugar carbohydrates in each of our cells that help build bone, cartilage, tendons, corneas, skin and connective tissue. Furthermore these glycosaminoglycans (formerly called mucopolysaccharides) are also found in the fluid which lubricates our joints for better locomotory movements. Lysosomal enzymes (acid hydrolases) are responsible for breaking down complex chemicals within a cell, thus playing a very crucial role in all animal cells including human. A deficiency of any one of these enzymes will lead to a ‘storage disease’ viz. Niemann-Pick disease, Farber disease, Tay-Sachs disease etc. which is to be addressed correctly and precisely. Deciphering the novel developments about such enzymes will help us to know, how the therapeutic potential of these enzymes can be harnessed and it will lead novel discoveries for better understanding of lysosomal enzyme related disorders and ‘storage diseases’.

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Nutraceuticals and Diabetes- An update

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The term “nutraceutical” was coined from nutrition and pharmaceutical in 1989 by Dr. Stephen Defelice. According to him, nutraceutical can be defined as a food or part of a food that provides medical or health benefits, including the prevention of a disease. However, nutraceuticals differ from dietary supplements as they not only supplement the diet but also help in preventing disease and it is represented for use as a conventional food or as the sole item of a meal or diet. The healthcare practitioners are investigating alternative treatments and nutritional compounds to help in controlling the symptoms of the insidious disease. In this regard, the use of nutraceuticals is of great help as it is an attempt to accomplish desirable therapeutic outcomes with reduced side effects as compared with other therapeutic agents.

Diabetes is a collection of diseases distinguished by high levels of blood glucose caused by defective insulin production, often responsible for severe health problems and early death. Scientists classify diabetes into several varieties, the most common being type I and type II. Type I diabetes, known previously as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes, accounts for 5% to 10% of all diagnosed cases of diabetes. Type I diabetes occurs when the body’s immune system destroys pancreatic beta cells, manufacturers of the vital hormone insulin, a blood glucose regulator. Patients with this variety of diabetes are typically children and young adults, take regular doses of exogenous insulin, delivered by injection or pump, to control their blood sugar. Risk factors for type I diabetes may be autoimmune, genetic or environmental and currently there is no cure for the disease. Type II diabetes, formerly called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, accounts for 90% to 95% of all diagnosed cases of diabetes. Type II diabetes normally develops when cells lose the ability to use insulin correctly, a disorder known as insulin resistance. Risk factors for type II diabetes include old age, obesity, family history of diabetes, history of gestational diabetes, impaired glucose metabolism and physical inactivity. There is considerable need for safe agents that can reduce risk for diabetes. Although certain drugs like metformin, acarbose have shown diabetes preventive activity in large randomized studies, nutraceuticals have potential in this regard as well. In general, diets lacking adequate dietary fiber may put individuals at risk of developing type II diabetes. According to an eight-year study investigating the association between dietary fiber intake, glycemic index and the risk of developing type II diabetes among 90,000 women, researchers at the Harvard school of public health found that fiber intake was significantly associated with an increased risk of developing type II diabetes, while fiber intake decreased the risk. A good source of dietary fiber including the soluble fiber beta-glucan, may help diabetics control blood glucose and cardiovascular problems. These natural agents which slow carbohydrate absorption may mimic the protective effect of acarbose; most notably glucomannan, chlorogenic acid likely responsible for reduction in diabetes risk associated with heavy coffee intake and legume derived alpha amylase inhibitors. Metformin’s efficacy reflects activation of AMP-activated kinase, there is preliminary evidence that certain compounds in Barley malt have similar activity, without the side effects associated with metformin. Beta-glucan from barley has also been shown clinically to reduce serum lipid levels.
In addition to producing low G1 functional foods and ingredients marketed for blood glucose control, suppliers and manufacturers have flooded the market with a number of low or no calorie sugar replacements intended for stand alone use as dietary supplements or inclusion in functional foods. A few of these ingredients include luo han kuo (LHK), an Asian botanical with naturally sweet fruits; stevia, another naturally sweet plant sourced product that can be used in dietary supplements. LHK may have benefits for diabetics beyond its capabilities as a sweetener, as it may help diabetics control serum lipids. In supraphysiological concentrations, biotin directly activates soluble guanylate cyclase this implies that at some sufficient intake, biotin should exert effects on beta cells, the liver and skeletal muscle that favor good glucose tolerance and maintenance of effective beta cell function.

The mineral magnesium may also be important for diabetes. A Taiwanese study found an inverse association between plasma magnesium concentration and prevalence of diabetes in a national population based cross-sectional nutrition survey, and researchers from Harvard found an inverse association between consumption of magnesium and the risk of developing type II diabetes, as lower fasting insulin concentrations accompanied higher magnesium intake.

Chromium is proven efficacious for blood glucose control in clinical trial, a mineral shown to enhance the body’s utilization of insulin. The combination of chromium picolinate and biotin may have beneficial effects on blood glucose, coadministration of chromium picolinate and biotin as Diachrome and prescription treatment regimens to type II diabetics produced a 0.7% to 1.9% reduction in HbA1c.

Calcium or vitamin D may help preserve insulin sensitivity by preventing secondary hyperparathyroidism. Vanadium may control insulin sensitivity in diabetics mainly type II. It is effective as its metabolite vanadyl sulfate which enhances insulin mediated stimulation of glucose uptake.

Antioxidant micronutrients also appear critical in helping diabetics maintain good health. Coenzyme Q 10 (coQ 10) may positively affect cardiovascular health in diabetics. Supplementation may improve blood pressure and long term glycemic control in type II diabetics. Taking the potent antioxidant alpha-lipoic acid (ALA) may inhibit diabetic neuropathy, a peripheral nerve disorder.

Beyond micronutrients there are many botanical extracts that may benefit diabetics. Consumption of cinnamon may help control glucose levels as well as other symptoms of diabetes. Cinnamon’s blood sugar balancing capabilities may be a function of the compound’s double linked Type-A polymers.

Fenugreek is another botanical compound with applications in blood sugar control. Fenugreek galactomannan dose dependently lowered blood sugar levels in non - diabetics subjects according to results of a small human trial conducted at glycemic index testing in Toronto. In subjects with severe diabetes, fenugreek reportedly improved the reduction of fasting blood sugar when combined with conventional medical treatment.

Aloe Vera may have notable antidiabetic activity. Oral aloe supplementation has beneficial effects on blood sugar, cholesterol and triglycerides. Another route to glucose homeostasis among diabetics may be consumption of gymnema. In type II diabetic patients gymnema in supplementation, diabetic patients showed a significant reduction in blood glucose, glycosylated hemoglobin and glycosylated plasma proteins and also able to decrease their intake of conventional antihyperglycemia drugs.
Bitter melon may help diabetics maintain optimal blood glucose levels. Supplementation with an aqueous homogenous suspension of bitter melon pulp led to significant reduction in type II diabetic patient\(^\text{17}\).

Pinitol, a sugar alcohol found in various legumes, plants, and fruits also appear to affect blood sugar. Another botanical based ingredient touted for blood sugar control is extract of French maritime pine bark as pyenogenol, administered in conjunction with standard anti-diabetic treatment; the extract lowered blood glucose and improved endothelial function in type II diabetic patients. Supplementation with it may also help diabetics in maintaining good eye health\(^\text{18}\).

According to numerous clinical trials, bioactive substances present in maitake mushroom may ameliorate certain symptoms of diabetes.

Nutraceuticals featuring meaningful doses of combinations of these agents would likely have substantial diabetes preventive efficacy and presumably could be marketed legally as aids to good glucose tolerance and insulin sensitivity. Clearly many nutraceuticals have hypoglycemic, anti-hyperglycemic, insulin sensitizing and anti-hypertensive activities. So, supplementation with vitamins, minerals, botanicals and other natural compounds may comprise gentle treatment programs for patients with diabetes and could possibly replace or compliment use of prescription drugs among some diabetes patients.

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INTRODUCTION

A Gene Knockout is a genetic technique in which an organism is engineered to carry genes that have been made inoperative. Eliminating a gene (Gene Knockout) completely from a diploid organism requires knocking out both copies of the gene in the cells. Knocking out the activity of a gene provides valuable clues about what that gene normally does. The effect of the absence of a gene can be very informative about the normal function of the gene. There are a variety of methods for producing gene knockouts in different model organisms. Knockout organisms or simply knockouts are used in learning about a gene that has been sequenced, but which has an unknown or incompletely known function. Researchers draw inferences from the difference between the knockout organism and normal individuals. The term also refers to the process of creating such an organism, as in “knocking out” a gene.

Knockout is accomplished through a combination of techniques, beginning in the test tube with a plasmid, a bacterial artificial chromosome or other DNA construct, and proceeding to cell culture. Individual cells are genetically transformed with the DNA construct. The goal of knockout gene method is to create a transgenic animal that has the altered gene. If so, embryonic stem cells are genetically transformed and inserted into early embryos. Resulting animals with the genetic change in their germline cells can then often pass the gene knockout to future generations.

The construct is engineered to recombine with the target gene, which is accomplished by incorporating sequences from the gene itself into the construct. Recombination then occurs in the region of that sequence within the gene, resulting in the insertion of a foreign sequence to disrupt the gene. With its sequence interrupted, the altered gene in most cases will be translated into a nonfunctional protein, if it is translated at all. A conditional knockout allows gene deletion in a tissue or time specific manner.

Because the desired type of DNA recombination is a rare event in the case of most cells and most constructs, the foreign sequence chosen for insertion usually includes a reporter. This enables easy selection of cells or individuals in which knockout was successful. Sometimes the DNA construct inserts into a chromosome without the desired homologous recombination with the target gene. To avoid isolation of such cells, the DNA construct often contains a second region of DNA that allows such cells to be identified and discarded.

In diploid organisms, which contain two alleles for most genes, and may as well contain several related genes that collaborate in the same role, additional rounds of transformation and selection are performed until every targeted gene is knocked out. Selective breeding may be required to produce homozygous knockout animals. Knock-in is similar to knock-out, but instead it replaces a gene with another instead of deleting it.

Knockout Mouse

A knockout mouse is a laboratory mouse in which researchers have inactivated, or “knocked out,” an existing gene by replacing it or disrupting it with an artificial piece of DNA. The loss of gene activity
often causes changes in a mouse’s phenotype, which includes appearance, behavior and other observable physical and biochemical characteristics. Humans share many genes with mice. Consequently, observing the characteristics of knockout mice gives researchers information that can be used to better understand how a similar gene may cause or contribute to disease in humans.

Examples of research in which knockout mice have been useful include studying and modeling different kinds of cancer, obesity, heart disease, diabetes, arthritis, substance abuse, anxiety, aging and Parkinson disease. Knockout mice also offer a biological context in which drugs and other therapies can be developed and tested.

Many of these mouse models are named after the gene that has been inactivated. For example, the p53 knockout mouse is named after the p53 gene, which codes for a protein that normally suppresses the growth of tumors by arresting cell division. Humans born with mutations that inactivate the p53 gene suffer from Li-Fraumeni syndrome, a condition that dramatically increases the risk of developing bone cancers, breast cancer and blood cancers at an early age.

How are knockout mice made?

Researchers begin by harvesting embryonic stem (ES) cells from early-stage mouse embryos four days after fertilization. ES cells are used because they are able to differentiate into nearly any type of adult cell, which means that if a gene is knocked out in an ES cell, the effects can be observed in any tissue in an adult mouse. In addition, ES cells grown in the lab can be used to make knockout mice as long as 10 years after they were harvested.

To produce knockout mice, researchers use one of two methods to insert artificial DNA into the chromosomes contained in the nuclei of ES cells. Both methods are carried out in vitro, which are in cultured cells grown in laboratory conditions.

1. Gene Targeting or Homologous Recombination
2. Gene Trapping

In the gene targeting or homologous recombination researchers specifically manipulate a gene in the nucleus of an ES cell. Typically, this is done by introducing an artificial piece of DNA that shares identical, or homologous, sequence to the gene. This homologous sequence flanks the existing gene’s DNA sequence both upstream and downstream of the gene’s location on the chromosome. The cell’s own nuclear machinery automatically recognizes the identical stretches of sequence and swaps out the existing gene or portion of a gene with the artificial piece of DNA. Because the artificial DNA is inactive, bearing only a genetic tag, or “reporter gene,” designed for use in tracking, the swap eliminates, or “knocks out,” the function of the existing gene.

In the gene trapping, researchers again manipulate a gene in an ES cell. However, instead of directly targeting a gene of interest, a random process is used. A piece of artificial DNA containing a reporter gene is designed to insert randomly into any gene. The inserted piece of artificial DNA prevents the cell’s RNA “splicing” machinery from working properly, thus preventing the existing gene from producing its designated protein and knocking out its function. As in the first strategy, researchers can track the activity of the artificial reporter gene to figure out the existing gene’s normal pattern of activity in mouse tissues.
For both gene targeting and gene trapping, the vehicle used to ferry the artificial DNA into ES cells often consists of a modified viral vector or a linear fragment of bacterial DNA. After the artificial DNA is inserted, the genetically altered ES cells are grown in a lab dish for several days and injected into early-stage mouse embryos. The embryos are implanted into the uterus of a female mouse and allowed to develop into mouse pups.

The resulting mouse pups have some tissues in which a gene has been knocked out - those derived from the altered ES cells. However, they also have some normal tissues derived from the non-altered embryos into which the altered ES cells were injected. Consequently, they are not complete knockout mice. It is necessary to crossbreed such mice to produce lines of mice in which both copies of the gene (one on each chromosome) are knocked out in all tissues. Researchers refer to such mice as homozygous knockouts.

The advantage of gene trapping is that researchers do not need to know the DNA sequences of specific genes in order to knock them out. In addition, in gene trapping, a single vector can be used in a high throughput capacity to generate a suite of mice in which a variety of genes have been knocked out.

The disadvantage of gene trapping is that it is not as efficient or specific as gene targeting because not every successful insertion of artificial DNA into a gene leads to a loss of function. Researchers often must spend considerable time conducting tests to identify ES cells in which gene(s) actually have been knocked out. In addition, since gene trapping is a random process, certain genes may never get hit due to statistics or because the gene is not active in ES cells, which means they will not produce the marker indicating that the gene has been knocked out.

**Drawbacks of Knockout**

While knockout mice technology represents a valuable research tool, some important limitations exist. About 15 percent of gene knockouts are developmentally lethal, which means that the genetically altered embryos cannot grow into adult mice. The lack of adult mice limits studies to embryonic development and often makes it more difficult to determine a gene's function in relation to human health. In some instances, the gene may serve a different function in adults than in developing embryos.

Knocking out a gene also may fail to produce an observable change in a mouse or may even...
produce different characteristics from those observed in humans in which the same gene is inactivated. For example, mutations in the p53 gene are associated with more than half of human cancers and often lead to tumors in a particular set of tissues. However, when the p53 gene is knocked out in mice, the animals develop tumors in a different array of tissues.

Despite these drawbacks, knockout mice offer one of the most powerful means now available for studying gene function in a living animal. Such studies will accelerate efforts to translate newfound knowledge of the human and mouse genomes into better strategies for diagnosing, treating and preventing human disease.

The 2007 Nobel Prize in physiology or medicine is awarded to Drs Mario R. Capecchi, Martin J. Evans and Oliver Smithies for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells. Their work has made it possible to modify specific genes in the germline of mammals and to raise offspring that carry and express the modified gene. The toolbox of experimental genetic methods developed by Capecchi, Evans and Smithies, commonly called the Knockout Technology, has permitted scientists to determine the role of specific genes in development, physiology, and pathology. It has revolutionized life science and plays a key role in the development of medical therapy.

**BIBLIOGRAPHY**


Bioequivalence Evaluation of a Pravastatin 80 mg Generic Formulation: Matching Pravastatin Variable Kinetics

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Abstract
The objective was to demonstrate bioequivalence of a new formulation of pravastatin 80 mg developed by Ranbaxy Laboratories Limited with that of Pravachol 80 mg of Bristol-Myers Squibb Company in fasting and non-fasting conditions in healthy male volunteers. The studies were conducted as single-dose, two-treatment, two-sequence, four-period with replicate design. Results revealed high intersubject variability for all pharmacokinetic parameters and high intrasubject variability for $C_{\text{max}}$ despite a very uniform study population. Cross comparison of both studies revealed a food effect as reported in literature. The mean and 90% confidence intervals for the ratios of test and reference products (least-squares means) derived from the analysis of log transformed pharmacokinetic parameters $\text{AUC}_{0-t}$, $\text{AUC}_{0-\infty}$ and $C_{\text{max}}$ were 100.49 (93.94-107.48), 104.81 (95.53-115.0) and 100.49 (94.01-107.43) in the fasting state and 92.51 (87.03-98.33), 92.84 (87.25-98.78) and 90.12 (82.32-98.66) in the fed state, respectively. In conclusion, the new 80 mg pravastatin formulation was found to be bioequivalent to Pravachol 80 mg.

Introduction
Statins are the most effective and the best tolerated agents for treating dyslipidemia available now. Multiple well conducted studies have demonstrated safety and efficacy of statins in reducing fatal and non-fatal CHD events, strokes and total mortality.¹ In a prospective meta-analysis of data from 90,056 participants in 14 randomized trials of statins, Baigent, et al documented that statin therapy can safely reduce the 5-year incidence of major vascular events about one fifth per mmol/L reduction in LDL cholesterol, benefiting most among those at higher risk of such events depending on level of absolute LDL cholesterol reduction and concluded that such finding reinforce the need to consider prolonged statin treatment in all patients at high risk.²

Choice of statin is a controversial issue; a matter of continued debate as relative efficacy of different statins for long-term cardiovascular prevention remains largely undetermined. The statins have been shown to differ in their efficacy in reducing the LDL cholesterol level in different RCTs.³,⁴,⁵ Prescription patterns regarding choice of statin is not determined only by high-quality RCTs.⁶ It is usually based on the clinician’s judgment of the relative importance of three factors: evidence of beneficial clinical outcomes, efficacy for lowering LDL and cost. It is often constrained by cost-oriented managed care formularies and differential co-payment levels.⁷ Not surprisingly, emergence of generic statins influences prescription patterns of physicians to a large extent. A recent metaanalysis of published randomized placebo-controlled trials suggests that pravastatin, simvastatin, and atorvastatin, when used at their standard dosages, show
no statistically significant difference on long-term cardiovascular prevention. However, statins do have certain differences that suits needs of particular patient population. Pravastatin has unique pharmacokinetic characteristics among the members of its class. It is metabolized by chemical degradation in the stomach rather than by cytochrome P450-dependent metabolism in the liver. The intact drug and its metabolites are cleared through both hepatic and renal routes. This dual route of pravastatin elimination coupled with low plasma protein binding reduce the need for dosage adjustment if the function of either the liver or kidney is impaired, and also reduce the possibility of drug interactions compared with other statins, which are largely eliminated by metabolism. It makes pravastatin a favored one in patients co prescribed with antiretroviral therapy, antidepressants,azole antifungals, macrolide antibiotics, cyclosporine, verapamil or diltiazem to reduce the risk of myopathy or rhabdomyolysis. 10, 11, 12, 13

Like other statins pravastatin also has variable pharmacokinetics. 14, 15, 16 Variable pharmacokinetics pose distinct challenges for generic drug development. Two kinds of variability are encountered during bioequivalence studies i.e. intersubject and intrasubject variability. Intersubject variability is usually taken care in the conventional crossover studies. It is the intrasubject variability from unique interaction of subject, formulation, period and sequence of treatment that determines the fate of a bioequivalence study. A well designed study and a robust matching dissolution profile dissipates it to some extent. High intrasubject variability can be attributed to the drug molecule itself and often hinders to meet the arbitrarily defined bioequivalence criteria (i.e., the 90% CI for the percentage ratio of generic/innovator formulations to fall within the bioequivalence interval of 0.80-1.25 based on log-transformed AUC and C\text{max} data) with a reasonable number of subjects, despite a good formulation as the CIs may be so wide that there is a low probability of two formulations meeting the criteria, even if the geometric mean ratio is 100%. 17 Traditional methods to overcome the difficulty with a reasonable number of subjects are use of multiple-dose, replicate, stable isotope, and/or group sequential study designs. These approaches can assess and, in some cases, reduce intrasubject variability. 17, 18 Various statistical approaches including widening the acceptable range have also been proposed. 18 Although this phenomenon is well recognized by regulatory authorities worldwide, it has not been effectively dealt with so far. Therefore, replicate design in large number of subjects remains the only practical means to demonstrate bioequivalence while dealing with highly variable molecules.

The objective of the present studies was to demonstrate bioequivalence of a new formulation of pravastatin 80 mg developed by Ranbaxy Laboratories Limited with that of Pravachol 80 mg of Bristol-Myers Squibb Company in fasting and non-fasting conditions.

Subjects and Methods

Clinical phases of both the studies were carried out at PRACS Institute, Ltd. US. Selection criteria, safety monitoring and evaluation were similar in both the studies. All subjects met study inclusion and exclusion criteria by an acceptable medical history, medication history, physical examination, sitting blood pressure, heart rate, electrocardiogram, clinical laboratory evaluations, non-reactive screens for HIV antibody, hepatitis B surface antigen and hepatitis C antibody, and drugs of abuse within twenty-eight days prior to Period I dosing. Exclusion criteria were history of allergic response(s) to pravastatin or related drugs, use of any systemic prescription medication in the 14 days prior to Period I dosing, use of any drug known to induce or inhibit hepatic drug metabolism in the 28 days prior to Period I dosing, history of drug or alcohol addiction or abuse within the past year, current use tobacco products,
blood donation of greater than 150 mL within 28 days prior to Period I and presence of any significant organ dysfunction. Volunteers were advised to refrain from using any over the counter and prescription medicines 3 days and 14 days prior to period I dosing, respectively, till study completion. Alcohol, caffeine and/or xanthine, grapefruit and/or grapefruit containing products were prohibited within 48 hours prior to each dosing and during the periods when blood samples were collected. At study check-in, the subjects were briefly evaluated to assess if they continued to meet the study inclusion/exclusion criteria and lifestyle guidelines. A blood sample was also collected for clinical laboratory tests. The study was conducted under medical supervision. Safety evaluation (general observations, a physical examination, vital sign measurement and laboratory evaluation) was repeated at the exit to elicit any abnormalities in the volunteers. All subjects gave written informed consent and were allowed to ask and answered questions concerning conduct of the study prior to enrollment. The studies were approved by the PRACS Institute, Ltd. Institutional Review Board.

Design

Both the fasting and non fasting studies were planned as randomized, single-dose, two-treatment, two-sequence, four-period with replicate design to compare the bioavailability of 80 mg of test Pravastatin Sodium Tablets (by Ranbaxy Laboratories, Ltd.) or 80 mg of reference Pravastatin Sodium Tablets (Pravachol by Bristol-Myers Squibb Company). Washout interval of 7 days was kept between the periods in each study.

Study medications were administered under supervision after an overnight fast of 10 hours in case of the fasting study and 30 minutes after initiation of a standardized, high-fat and high-calorie fat breakfast following an overnight fast in the non fasting study, along with 240 ml of water, as per randomization schedule. The standardized breakfast consisted of two eggs fried in butter, two strips of bacon, two slices of toast with butter, four ounces of hash brown potatoes and eight fluid ounces (240 mL) of whole milk (nearly 1000 calorie). No food was allowed for 4 hours after dosing. Drinking water restriction was observed for a period of one hour before and after dosing. Subjects remained upright for four hours after dosing was over. The meal plans were similar for every period in each study during confinement.

Blood collection schedule:

Fasting study: Predose (within one hour of dosing) and at 0.167, 0.33, 0.5, 0.67, 0.83, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 6, 8, 10, 12, 16, and 24 hours post dose in each period.

Non Fasting study: Predose (within one hour of dosing) and at 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.25, 3.5, 4, 4.5, 5, 6, 8, 10, 12, 16 and 24 hours post dose in each period.

After collection, samples (7 ml in K3 EDTA vacutainers) were placed on ice, centrifuged at approximately 3000 RPM and 4°C for 15 minutes, the plasma pipetted into duplicate polypropylene tubes, flash frozen in a dry ice/methanol bath and stored at approximately -80°C ± 15°C (or colder) until analysis.

Analytical procedure:

An assay to measure the concentration of pravastatin after separating the 3 alpha hydroxy pravastatin (isomeric metabolite) in human plasma (containing EDTA as anticoagulant) over a range of 0.500 to 500 ng/mL using a high performance liquid chromatographic method combined with mass spectrometry...
Pravastatin was extracted from a 500 µL aliquot of human plasma (containing EDTA as anticoagulant) sample using 30 mg Strata-X™ solid phase extraction cartridges (Phenomenex, Torrance, United States).

The LC system consisted of a solvent delivery module (Series 200 LC Pump of Perkin Elmer, Toronto, Canada), an auto sampler (Series 200 Auto sampler of Perkin Elmer, Toronto, Canada), and a LUNA™ column, 4.6x50 mm, 5 µm (Phenomenex, Torrance, United States). The mobile phase was a mixture of acetonitrile, Milli-Q water, acetic acid, and ammonium acetate and the flow rate was set to 0.85 mL/minute. The injection volume was 20 µL.

Mass spectrometric detection was carried out with a Sciex API 3000 equipped with a Turbo Ion Spray interface (MDS Sciex, Toronto, Canada). The ion source was operated in the negative mode. A transition m/z 423 to 321 amu was monitored for pravastatin with a dwell time of 500 msec. The Analyst™ software (version 1.4, MDS Sciex, Toronto, Canada) was used for data acquisition and processing. Weighted (1/C^2) calibration curves were constructed using the ratio of the peak areas of pravastatin and the internal standard (lovastatin acid).

Pravastatin was obtained from Changzhou Dahua Corp (Changzhou, China). Acetonitrile (HPLC grade), and acetic acid (HPLC grade), were obtained from Fisher (Ottawa, Canada), ammonium acetate (AnalaR), was obtained from EMD (Toronto, Canada). Human plasma (containing EDTA as anticoagulant) was obtained from Biological Specialties Corp (Colmar, United States). Water was produced with a Milli-Q water purification system (Milford, United States). High purity liquid nitrogen was supplied by BOC (Laval, Canada).

Fourteen (14) calibration curves were processed and the correlation coefficient was equal to or greater than 0.9961. The accuracy and precision of back-calculated calibration standard concentrations ranged from 95.6-104.9% and 2.5-5.4%, respectively. The intra-day accuracy and precision of quality control samples ranged from 94.8-108.0% and 1.6-8.7%, respectively. The inter-day accuracy and precision of quality control samples ranged from 96.8-104.8% and 5.6-14.5%, respectively. Similar accuracy and precision values were observed during study sample analysis.

The recovery of pravastatin was consistently approached 85%. No matrix effect, affecting quantification was observed. Pravastatin was found to be stable in human plasma for at least 867 days at -20°C and at least 45 days at -80°C. The integrity of pravastatin was not affected after undergoing 3 freeze-thaw cycles. It was determined that the extracted samples could be injected after at least 112 hours when stored at room temperature. Dilution integrity and a high level of selectivity of the assay were also demonstrated.

**Pharmacokinetic procedure:**

Non-Compartmental Analysis for deriving pharmacokinetic parameters was performed with WinNonlin version 4.0. The actual time of sample collection was used in pravastatin dataset for pharmacokinetic analysis. Area under the plasma concentration versus time curve \( (AUC_{0-\infty}) \) from time zero to the last measurable concentration, was calculated by the linear trapezoidal method. Area under the plasma concentration versus time curve, from time zero to infinity \( (AUC_{0-\infty}) \) was calculated as the sum of \( AUC_{0-t} \) and the ratio of the last measurable plasma concentration to the elimination rate constant.
C<sub>max</sub> was calculated as the maximum measured plasma concentration over the time span specified. Apparent first-order terminal elimination rate constant (K<sub>el</sub>) was calculated from a semi-log plot of the plasma concentration versus time curve. The parameter was calculated by linear least-square regression analysis using the maximum number of points in the terminal log-linear phase (e.g. three or more non-zero plasma concentrations).

Statistical Analysis

Sample size of 40 (fasting study) and 50 (non-fasting study) were chosen to prove bioequivalence with 80% power and to take care of dropouts/withdrawals. Arithmetic means, standard deviations and coefficients of variation were calculated for the pharmacokinetic parameters. Additionally, geometric means and percentage coefficient of variation for geometric means were calculated for AUC<sub>0-t</sub>, AUC<sub>0-inf</sub> and C<sub>max</sub>. Analysis of Variance (ANOVA) was performed on the log (natural)-transformed pharmacokinetic parameters C<sub>max</sub>, AUC<sub>0-t</sub> and AUC<sub>0-inf</sub>. The ANOVA model included sequence, formulation and period as fixed effects and subjects nested within sequence as random effect. The sequence effect was tested at the 10% level of significance while other main effects were tested at the 5% level of significance against the residual error (mean square error) from the ANOVA model as the error term. Each analysis of variance included calculation of least-squares means (LSM), the difference between the adjusted formulation means and the standard error associated with the difference. The intrasubject variability for the C<sub>max</sub>, AUC<sub>0-t</sub> and AUC<sub>0-inf</sub>, which reflect the residual variability after accounting for differences between subjects, periods, sequences and formulations, was derived from the analyses of the ln-transformed data.

90% confidence interval for the ratio of the test and reference product averages (least square means) was calculated for pravastatin by first calculating the 90% confidence interval for the differences in the averages (arithmetic means) of the log-transformed data and then taking the antilogs of the obtained confidence limits. Ratio of means was calculated using the LSM for log-transformed C<sub>max</sub>, AUC<sub>0-t</sub> and AUC<sub>0-inf</sub>. Ratio of means was expressed as a percentage of the LSM for the reference formulations.

The statistical analysis was done using SAS system for Windows, release 9.1 (SAS Institute Inc., USA).

Results

Subject disposition

Fasting Study: The target enrollment of 40 subjects was not met secondary to insufficient number of qualified volunteers. 36 of 38 enrolled male subjects completed the study. One subject was withdrawn secondary to abnormality in laboratory parameter (ALT and AST) prior to period 3 dose administration. Another volunteer discontinued from the study because of personal reasons. Age, weight and BMI of the subjects were 30.1 ± 12.3 years, 181.8 ± 26.9 lbs and 25.6 ± 3.2 kg/m<sup>2</sup> respectively. There were twelve adverse events reported by ten subjects, of mild to moderate severity.

Non Fasting study: The study was initiated in 50 male subjects of whom 46 completed it. Two volunteers discontinued citing personal reasons. Another two were withdrawn secondary to abnormality in laboratory test (ALT, AST abnormality and reactive Hepatitis C serology that was confirmed negative later) prior to period two. Age, weight and BMI of the subjects were 28.3 ± 12.0 years, 181.8 ± 26.9 lbs and 26.8 ± 3.0 kg/m<sup>2</sup> respectively. There were nine adverse events reported over the course of the
study by eight subjects of mild severity.

**Pharmacokinetic parameters (Table 1, Figure 1)**

Pharmacokinetic analysis was performed in 36 and 46 subjects in the fasting and non-fasting studies, respectively. High intersubject variability for all pharmacokinetic parameters and high intrasubject variability for $C_{\text{max}}$ was observed despite a very uniform study population. Comparison across both the studies revealed that in general, the reference product pharmacokinetics was more variable compared to the test product in fasting state study and vice versa in the non fasting state study except for $T_{\text{max}}$ and $T_{1/2}$. Approximately, 30 to 40 % reduction in mean $C_{\text{max}}$ and AUC was observed in the non fasting study compared to the non fasting study.

**Assessment of Bioequivalence (Table 2)**

The 90% confidence intervals for the ratios of test and reference products (least-squares means) derived from the analysis of log transformed pharmacokinetic parameters $\text{AUC}_{0-t}$, $\text{AUC}_{0-\infty}$ and $C_{\text{max}}$, were within the 80-125% acceptance range for pravastatin in both the studies. The ratios and CIs closely matched in the fasting study whereas in the fed state it was less than 100%.

**Discussion:**

Although pravastatin is in use for a long time, pharmacokinetics of 80 mg strength in healthy volunteers is not reported in literature. The present study will serve as a reference for future pharmacokinetic studies with this higher strength. Cross comparison of both studies revealed a food effect as reported in literature.\(^9,16\) The decreased bioavailability is speculated to be due to longer stay in the stomach in the non fasting state leading to chemical degradation. However, it does not affect the lipid lowering efficacy of pravastatin significantly. Pravastatin bioavailability is also known to be decreased by 60% if taken at bed time compared to a morning dose. Despite this, lipid lowering activity appears to be better if taken in the evening. The major degradation product of pravastatin is an isomeric metabolite 3α-hydroxy pravastatin which has one tenth to one fortieth lipid lowering activity.\(^16\) In the present study the analytical procedures were designed to measure pravastatin concentration after isomer separation.

One volunteer in each study developed biochemical abnormalities in liver function, but it did not satisfied the criteria of a major abnormality as per product information. It could be possibly because of the single high dose exposure. Clinical trial experience has showed that liver function test abnormalities observed during pravastatin therapy were usually asymptomatic, not associated with cholestasis, and did not appear to be related to treatment duration.\(^16\)

In the present studies, the intra-subject variability for $C_{\text{max}}$ was 36.3 and 30% in the fasting and non fasting studies respectively for the reference product. Although intrinsic to the molecule and not related to the formulation, intrasubject variability must be adjusted within the bioequivalence limits in addition to any difference between the two formulations. ‘Highly variable drugs’ have been defined as those for which the intrasubject variability equals or exceeds 30% for the $C_{\text{max}}$ and/or the AUC.\(^18\) In case of highly variable drugs, European regulatory agency accepts a wider range for $C_{\text{max}}$ to be prospectively defined and adequately justified.\(^19\) Canadian agency asks for point estimate of the $C_{\text{max}}$ to be within 80-125%.\(^20\) Currently, US FDA does not accept such a wider range for any of the parameters.\(^21\) All the pharmacokinetic parameters are not always equally important from a pharmacodynamic point of view.
In the present case, $C_{\text{max}}$ of pravastatin may not be critical for safety or efficacy of the product, considering its high variability that has never posed an obstacle in practice. In general, $C_{\text{max}}$ is a short lived phenomenon and inherently more variable, although an important parameter for certain drugs. Therefore, bioequivalence criteria should be flexible depending on its relevance from PK/PD perspective, inherent variable pharmacokinetics and clinical rationale.

A rough estimate of pravastatin variability was identified in pilot studies and the pivotal studies were designed as replicate studies to minimize variability while large sample sizes were chosen for adequate statistical power. The ratios and 90% CI for $C_{\text{max}}$ and AUC met the regulatory acceptance criteria of 80-125%. After successful evaluation the new pravastatin 80 mg generic formulation has been registered with US FDA.

Disclosure:

The authors marked with superscripts 1 and 2 are employees of Ranbaxy Laboratories Limited. All the expenses of the study were borne by Ranbaxy Laboratories Limited.

Table 1: Comparision of pharmacokinetic parameters and its intrasubject coefficient of variations [Mean (CV)] in the replicate design bioequivalence (Fasting and non Fasting) studies

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pravastatin 80 mg (Test)</th>
<th>Pravachol 80 mg (Reference)</th>
<th>Intrasubject CV (Test)</th>
<th>Intrasubject CV (Reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (hours)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>0.96(24.6)</td>
<td>1.43(42.9)</td>
<td>28.5</td>
<td>36.3</td>
</tr>
<tr>
<td>Non Fasting</td>
<td>1.08(44.4)</td>
<td>1.59(49.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>133.82(58.5)</td>
<td>127.38(74.1)</td>
<td>44.8</td>
<td>30.0</td>
</tr>
<tr>
<td>Non Fasting</td>
<td>77.5(73.7)</td>
<td>85.96(68.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\text{AUC}_{0-t}$ (ng.h/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>291.52(53.1)</td>
<td>289.40(59.6)</td>
<td>21.6</td>
<td>20.3</td>
</tr>
<tr>
<td>Non Fasting</td>
<td>176.41(55.2)</td>
<td>190.71(53.3)</td>
<td>28.3</td>
<td>26.4</td>
</tr>
<tr>
<td>$\text{AUC}_{0-\infty}$ (ng.h/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>296.22(52.3)</td>
<td>192.0(53.4)</td>
<td>21.4</td>
<td>26.1</td>
</tr>
<tr>
<td>Non Fasting</td>
<td>178.42(55.0)</td>
<td>293.99(58.9)</td>
<td>28.2</td>
<td>20.0</td>
</tr>
<tr>
<td>$T_{1/2}$ (hours)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting</td>
<td>2.91(59.0)</td>
<td>3.21(60.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non Fasting</td>
<td>2.41(89.2)</td>
<td>2.56(94.0)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Ratios (Test/Reference) of log transformed LSM and 90% Confidence Interval for pravastatin in fasting and non fasting studies

<table>
<thead>
<tr>
<th></th>
<th>Ln $C_{\text{max}}$</th>
<th>Ln $\text{AUC}_{0-t}$</th>
<th>Ln $\text{AUC}_{0-\infty}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasting</td>
<td>104.81(95.53-115.0)</td>
<td>100.49(93.94-107.48)</td>
<td>100.49(94.01-107.43)</td>
</tr>
<tr>
<td>Non Fasting</td>
<td>90.12(82.32-98.66)</td>
<td>92.51(87.03-98.33)</td>
<td>92.84(87.25-98.78)</td>
</tr>
</tbody>
</table>
Figure 1: Comparison of mean plasma concentrations of pravastatin (Test Vs Reference) in fasting and non fasting conditions (Data up to 12 hours has been plotted).

REFERENCES:


Preparation And Release Rate Study Of Controlled Release Matrix Tablets Of Verapamil Hydrochloride Using Kollidon®SR

Royal College of Pharmacy and Health Sciences, Berhampur, Orissa, India

Abstract

The need for controlled release formulations of Verapamil hydrochloride is well recognized. In our study, controlled release tablets of Verapamil hydrochloride were formulated by the matrix-embedding technique using Kollidon®SR as a retardant. All the tablets prepared were of good physical quality with respect to appearance, drug content uniformity, hardness, weight variation, and friability. In-vitro release study showed that Kollidon®SR prolonged the release of the drug to 12hrs or more. FTIR study of Verapamil Hydrochloride, Kollidon®SR and their mixture confirmed that there was no chemical interaction between the drugs and polymer. Release pattern from formulations followed Higuchi’s kinetics. The formulations were highly stable and possessed reproducible release kinetics across batches.

Introduction

Many problems are associated with conventional multiple dosing regimen of long duration therapy, such as, systemic accumulation of the drug leading to side effects or toxicities, flip-flop profile of the plasma drug level and patient compliance. Controlled release drug delivery systems have the potential of solving these problems. The disease symptoms such as hypertension ischemic heart disease, asthma, and rheumatoid arthritis exhibit circadian rhythms. For example blood pressure tends to be lower while asleep and tends to be elevated in the early morning. Similarly, the maximum morning occurrence of the death from heart attack is at 9am, which coincides with peaks in platelet aggregation, plasma catecholamine levels, blood pressure and in fibrinolytic activity. From these points of view, development of preparations, in which a required amount of a drug levels are reached, has been desired. Verapamil Hydrochloride (VH) is a calcium channel blocker used as a peripheral vasodilator. Verapamil Hydrochloride has short biological half-life and thus frequent administration makes it a potential candidate for the design of sustained release dosage forms.\textsuperscript{1, 2} Polymeric hydrogels are being increasingly studied for controlled- release applications because of their good biocompatibility. In addition the ability of hydrogels to release an entrapped drug in an aqueous medium, to regulate the release of such drug by control of swelling and cross-linking makes them particularly suitable for controlled release applications. Kollidon®SR is a directly compressible polymeric blend composed primarily of polyvinyl acetate (PVAc) and povidone(PVP). The amorphous nature of PVAc coupled with its unusually low glass transition temperature of 28–31°C imparts certain unique characteristics to this binary matrix\textsuperscript{3-5}. Polyvinyl acetate is a very plastic material that produces a coherent matrix; the water soluble povidone is leached out to form pores through which the active ingredient slowly diffuses outwards.

The objective of our study was to design and compare the release characteristics of controlled release formulations of Verapamil hydrochloride by matrix-embedding techniques. Kollidon®SR was used as a retardant to achieve controlled release. The effect of varying proportions of retardant material in matrix tablet on release kinetics was studied.
MATERIALS AND METHODS

Materials

A gift sample of Verapamil hydrochloride was received from Aristo Pharmaceuticals Ltd., (Mumbai, India). Kollidon®SR was received from BASF (Germany). Double distilled water was used throughout the study and all the other chemicals used were of analytical grade.

Characterization of Materials

The drug was characterized by various official tests of identification and was analyzed in aqueous medium by ultraviolet-visible spectrophotometric method (Shimadzu, SLV-1700) at 278 nm. The IR spectrum was obtained (FT-IR, PERKIN ELMER, SWITZERLAND) and compared with that of the standard. The effect of various formulation excipients on the stability of the drug also was studied. The chemical interaction between the drugs and polymer was studied by using FT-IR.

Fabrication of Matrix Tablets

Controlled release matrix-embedded tablets were prepared separately by direct compression process using different proportions of Kollidon® SR. The composition of various formulations is given in Table 1. Verapamil hydrochloride and Kollidon® SR were mixed in a polybag, and the mixture was passed through mesh No. 40 and mixed with 1% aerosil (Aerosil-200, Degussa Corp, Dusseldorf, Germany) and 1% of Talc. Tablets were compressed of 250 mg weight on a 10-station Mini Press-I rotary tablet compression machine (Karnavati engineering Ltd, India) with 6-mm oval-shaped punches. Four different formulas, having different concentrations of Kollidon®SR (10%, 20%, 40%, and 60%), were developed to evaluate the drug release and to study the effect of polymer concentration on drug release.

Physical Characterization of Designed Tablets

Crushing strength: Ten tablets were tested using Monsanto hardness tester.

Friability: Twenty tablets were weighed (W₀) and rotated for four minutes at 25 rpm in a Roche friabilator. The tablets were then reweighed (W) and the percentage friability (%f) was calculated using equation. (1)

\[ \%f = 100 \times (1 - \frac{W_0}{W}) \]  

Drug Content uniformity test: The drug content of the prepared tablets of each batch was determined. From each batch 20 tablets were taken, weighed, and finely grounded. An aliquot amount of this powder, equivalent to 10mg of drug, was accurately weighed, suitably dissolved, and diluted and analysed by UV method at 278 nm.

Weight variation test: The Weight variation was determined on 20 tablets using an electronic balance.

Dissolution Study: Drug release was assessed by dissolution test under the following conditions: n = 6(in triplicate), USP type II dissolution apparatus (Lab India, DISSO 2000) at 50 rpm in 900 mL of phosphate buffer at pH 6.8 maintained at 37 ± 0.5°C. Five milliliters of the sample was withdrawn at regular intervals and replaced with the same volume of prewarmed (37 ± 0.5°C) fresh dissolution medium. The samples withdrawn were filtered through Whatman filter paper (No.1, Whatman, Maidstone, UK) and drug content in each sample was analyzed after suitable dilution using UV spectrophotometer method at 278nm. The effect of hardness and pH of the dissolution medium on release characteristics of the best formulations were studied and compared with release profile.
Quantification of water uptake and Erosion Determinations

Tablets were placed in flat dissolution vessels. At time intervals of 60, 120, 180, 240, 300 and 360 minutes the tablets were withdrawn from the medium and weighed after excess of water at the surface had been removed with filter paper. The wetted samples were then dried in an oven at 40°C up to constant weight. The increases in weight of the tablets reflect the weight of the liquid uptake. It was estimated according equation,

\[ Q = 100\frac{(W_i - W_w)}{W_i}, \]

where \( Q \) is the percentage of liquid uptake, \( W_w \) and \( W_i \) are the masses of the hydrated samples before drying and the initial starting dry weight, respectively.

The degree of erosion (expressed as % erosion of the polymer content, \( E \)) was determined using the equation,

\[ E = 100\frac{(W_i - W_f)}{W_i}, \]

where \( W_f \) is the final mass of the same dried and partially eroded sample.

RESULTS AND DISCUSSION
Characterization of Materials

The supplied drug passed various tests of identification and analysis as per I.P. The formulation additives (in concentrations used) did not affect the stability and ultraviolet absorbance of the drug. There is no chemical interaction between the drugs and polymer as per FT-IR study (fig.1).

Physical Characterization of Designed Tablets

Physical appearance, tablet hardness, friability, weight variation, and drug content uniformity of different formulations were found to be satisfactory as can be observed from Table 2. Tablet hardness varied between 5.3 to 5.6 kg/cm\(^2\). The friability was less than 0.8\%(w/w). The matrix tablet showed low weight variation and a high degree of content uniformity indicating that the granulation method is acceptable for manufacturing of good quality Verapamil matrix tablets.

Dissolution Rate Study

In-vitro dissolution studies were conducted on three tablets of each of the formulations VK-1, VK-2, VK-3, VK-4. The mean cumulative percent of Verapamil HCl released at different time intervals is shown in Table 3. We observed that the initial rate of release for VK-1 was high and around 91% of the drug was released within 4 hrs. In formulation VK-2 around 94% of the drug was released within 8 hrs. The formulations containing 10%, 20% of Kollidon®SR was unable to sustain the drug release for 12 hours, whereas 40% of Kollidon®SR sustained the release for 12 hours. The 60% of Kollidon®SR was unable to release 90% of drug within 12 hours. So the formulation VK-3 contains the optimum quantity polymer to sustain the drug release for 12 hours. Therefore, VK-3 formulation was kept for further study like FT-IR and stability.

The plot of cumulative percent drug released versus square root of time (Figure 2) showed a linear relationship, suggesting that the release mechanism followed Higuchi's square root kinetics. Since the tablets were nondisintegrating the hardness in all formulation was kept above 5.0 kg/cm\(^2\).
Liquid Uptake and Erosion by the Matrix Tablets

Since the rate of swelling and erosion is related and may affect the mechanism and kinetics of drug release, the penetration of the dissolution medium and the erosion of the hydrated tablets were determined. The percentage increase in weight of the hydrated tablets containing Kollidon®SR at various time intervals up to 6 h are shown in (Fig. 2). Simultaneously with the penetration liquid study, the degree of polymer erosion was measured (Fig. 3). The degree of swelling is dependent on the concentration and type of polymer presented in the matrix. The order of swelling observed in these polymers could indicate the rates at which the preparations are able to absorb water and swell. After this wetting period, the systems were only in gel form with a higher volume of water.

Effect of Storage on Release Profile and Batch Reproducibility

No significant difference was observed in the release profile of different batches, indicating that the manufacturing process used was reliable and reproducible. There were no changes in the tablet characteristics on storage.

CONCLUSION

The matrix embedding technique using Kollidon®SR as polymer has successfully extended the release of Verapamil HCl in the tablet formulations. The manufacturing method used is simple and easily adaptable in conventional tablet manufacturing units. Thus, we conclude that the clinical benefits and economics (raw material cost per dosage regimen and cost of production) of designed controlled release oral formulation of Verapamil HCl render them better alternatives to conventional formulations.

REFERENCES

3. Technical Information, Kollidon®SR; BASF Aktiengesellschaft, Germany. 1999 p. 1–10

Table – 1 Components of designed formulations

<table>
<thead>
<tr>
<th>Formulations</th>
<th>VK1</th>
<th>VK2</th>
<th>VK3</th>
<th>VK4</th>
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<tbody>
<tr>
<td>Components</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil HCl</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Kollidon® SR-</td>
<td>20</td>
<td>50</td>
<td>100</td>
<td>150</td>
</tr>
<tr>
<td>MCC (mg)</td>
<td>145</td>
<td>115</td>
<td>65</td>
<td>15</td>
</tr>
<tr>
<td>Aerosil (mg)</td>
<td>2.5</td>
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<tr>
<td>Talc</td>
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<tr>
<td>Total Weight</td>
<td>250</td>
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<td>250</td>
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</tbody>
</table>

Table-2 Physical properties of compressed tablets

<table>
<thead>
<tr>
<th>Formulations*</th>
<th>VK1</th>
<th>VK2</th>
<th>VK3</th>
<th>VK4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drug Content(mg/tab)</td>
<td>80.2±0.5</td>
<td>80.4±0.9</td>
<td>80.1±0.7</td>
<td>81.0±0.5</td>
</tr>
<tr>
<td>Weight variation</td>
<td>±5</td>
<td>±4.5</td>
<td>±5</td>
<td>±4</td>
</tr>
<tr>
<td>Hardness(kg/cm²)</td>
<td>5.4±0.3</td>
<td>5.2±0.4</td>
<td>5.1±0.5</td>
<td>5.3±0.4</td>
</tr>
<tr>
<td>Friability(%)</td>
<td>&lt;0.9</td>
<td>&lt;0.7</td>
<td>&lt;0.7</td>
<td>&lt;0.9</td>
</tr>
</tbody>
</table>

*Mean of triplicate with S.D.

Fig-1

Verapamil HCl + Kollidon SR

Table – 3 Mean cumulative percent of Verapamil HCl released from VK-1, VK-2, VK-3, VK-4 Formulations
<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>Mean Cumulative percent ± S.D of Verapamil released from the formulation*</th>
</tr>
</thead>
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<tr>
<td></td>
<td>VK1</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>29.60 ± 0.92</td>
</tr>
<tr>
<td>1</td>
<td>49.21 ± 0.80</td>
</tr>
<tr>
<td>2</td>
<td>68.53 ± 2.75</td>
</tr>
<tr>
<td>3</td>
<td>81.58 ± 1.23</td>
</tr>
<tr>
<td>4</td>
<td>91.70 ± 1.25</td>
</tr>
<tr>
<td>5</td>
<td>--</td>
</tr>
<tr>
<td>6</td>
<td>--</td>
</tr>
<tr>
<td>7</td>
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<td>11</td>
<td>--</td>
</tr>
<tr>
<td>12</td>
<td>--</td>
</tr>
</tbody>
</table>

*Mean of triplicate with s.d.

**Fig-2**

**Fig-3**
In vitro cytotoxic activity against fibroblast cells of rats (L-929 cell line) and antimicrobial activity of the fresh unripe tender fruit extract from the plant Azadirachta indica

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ABSTRACT
Fresh unripe tender fruits of Azadirachta indica were collected from the nearby forest area in the month of June 2006. Fresh unripe tender fruits were collected (5 kg) and crushed. The extraction was carried out using ethyl acetate as a solvent. Bacteria strains were streaked on to Mueller Hinton agar slants, incubated at 37°C for 24 hours. The growth content of each slant was scrapped into 10 ml of sterile distilled water and a uniform suspension was prepared and cell count was adjusted to 10⁴/ml. It was used as an inoculum. The selected fungal test cultures were subcultured onto PDA slants and incubated at 28°C for 4 days. The matured spore crop of each slant was suspended in a solution containing 0.05 % v/v Tween 80 and spore count was adjusted to 1 × 10⁶/ml, which was used as an inoculum. Seed agar plates were prepared by incorporating 1 % level of inoculum into respective media. The Whatman No. 1 filter paper discs (6 mm diameter) were impregnated with 10 ml of each test solution [from the stock of crude extracts (2.5 mg/ml), reference standards (1 mg/ml)] and placed on to the seeded agar plates. The plates were kept in refrigerator at 4°C for 1 h for diffusion of samples and incubated for 18 h at 37°C for bacteria and 96 h for fungi at 30°C. All tests were performed in triplicate and their activities were expressed as the mean of inhibition zone diameters (mm) produced by the test or reference samples. The results of the antifungal activity for the extract showed some degree of protection for all most all the plant pathogens at various concentrations, but the results obtained are not that much comparable with the standard drug, Miconazole (10μg). EEAI showed better antifungal activity against Aspergillus niger, Fusarium sp, Penicillium chrysogenum, Sclerotia rolpsii and showed maximum antibacterial activity against Staphylococcus pyogenes, pseudomonas aeroginosa and Klebsiella pneumoniae. The fibroblast cells of rats in exponential growth phase were harvested and centrifuged at 300 g for 5 minutes and resuspended in Dulbecco’s modified eagle medium (DMEM) supplemented with 10 % fetal calf serum to get a cell count of 1.0 × 10⁴ cells/ml. One ml of this cell culture was added to each well of a flat bottomed 96 well plate with help of multi-channel pipette and incubated for 24 h in 5 % CO₂ humidified incubator at 37°C. Then the test solutions were added to confluent monolayer of L-929 cells and incubated for 24 h. The solvent DMSO was used as a negative control and actinomycin-D was used as positive control. The cell cultures were examined microscopically for cellular responses. The cellular responses were scored accordingly as none, slight, mild, moderate and severe. The plant extract at the concentration of 100mg/ml showed mild cytotoxic activity.

INTRODUCTION
Azadirachta indica A. Juss (Syn. Melia Azadirachta L.) belongs to the Meliaceae (Mahagony) family. It is commonly known as neem, margosa in English, Arishta in Sanskrit, Nimba in Oriya. It is well known in India and neighbouring countries for more than 2,000 years. The Sanskrit name of the neem
tree as Arishta means `reliever of sickness'\(^1\) and hence it is considered as `Sarva roga nivarini'. The tree is regarded as `Village pharmacy' in India. The United State National Academy of Sciences stated as `Neem - a tree for solving global problem. For centuries, fruits, leaves, oil, bark and roots of the tree are being used in Ayurvedic and Unani medicinal treatment. The tree is of moderate to large size (12 to 19 m tall) with a straight trunk that can attain a diameter of 1.8m, the bark is moderately thick, the heart wood is red, hard and durable and leaves are glabrous. Neem bears honey scented white flowers. The fruit is an ovoid, bluntly pointed, smooth drupe, green when young and unripe, yellow to brown when ripe with a very thin epicarp, mesocarp with scanty pulp and a hard bony endocarp, enclosing one seed\(^3\). Chemical investigation on the products of the Neem tree was extensively undertaken in the middle of the twentieth century\(^4\, 5\, 6\). The compounds have been divided into two major classes-isoprenoids and nonisoprenoids\(^7\). Isoprenoids include protomeliacins, limonoids azadirone and their derivatives, gedunin and its derivatives, vilasinin type of compounds and C-seco meliacins such as nimbin, salannin and azadirachtin. Non isoprenoids include proteins, carbohydrates, sulphur compounds, polyphenolics such as flavonoids and their glycosides, dihydrochalcone, coumarin and tannins, aliphatic compounds etc. Several pharmacological activities and medicinal applications of various parts of neem are well known\(^8\) described the medicinal utilities mainly from neem leaf, fruit and bark. Scientifically it has been proved that the plant possesses anti-inflammatory, analgesic, antipyretic, hypoglycaemic, antifertility, antiulcer activities with different extracts.

Based on literature we found that there were no reports on the tender unripe fruits of neem for the biological activity. So we attempt to evaluate the in vitro antimicrobial and cytotoxic activity of the tender unripe fruits of Azadirachta indica, using ethyl acetate as an extractive solvent, which was not reported earlier.

**Materials and Methods**

*Plant material collection and preparation of extracts*

Fresh unripe tender fruits of Azadirachta indica, were collected in the month of October from the thick forest areas of Similipal biosphere reservoir, Mayurbhanj district of Orissa. Taxonomic identification was done by Dr. N. K. Dhal, Sr. Scientist, RRL, Bhubaneswar, Orissa and the voucher specimen is deposited in the herbarium of Dept of Taxonomy, RRL, Bhubaneswar.

The fruits were washed thoroughly with tap water and air dried in shade at room temperature. They were then mechanically powdered and sieved. 1000gm of powdered plant material was extracted with ethyl acetate by soxhlation and dried in a rotary evaporator at 40°C. The extractive yield was found to be 4.764% for the ethyl acetate extract of Azadirachta indica (EEAI).

**Antimicrobial activity test**

*Preparation of inoculum*

**Bacteria:** The test organisms were streaked on to Mueller Hinton agar slants, incubated at 37°C for 24 hours. The growth content of each slant was scrapped into 10 ml of sterile distilled water and a uniform suspension was prepared and cell count was adjusted to 10\(^4\)/ml. It was used as an inoculum.

**Fungi:** The selected fungal test cultures were subcultured onto PDA slants and incubated at 28°C for 4 days. The matured spore crop of each slant was suspended in a solution containing 0.05 % v/v Tween
80 and spore count was adjusted to $1 \times 10^6$/ml, which was used as an inoculum. Seed agar plates were prepared by incorporating 1% level of inoculum into respective media.

**Procedure**

A standardized filter paper disc agar diffusion procedure was adopted for the antimicrobial evaluation\(^9,10\). The Whatman No. 1 filter paper discs (6 mm diameter) were impregnated with 10 ml of each test solution [from the stock of crude extracts (2.5 mg/ml), reference standards (1 mg/ml)] were placed on to the seeded agar plates. The plates were kept in refrigerator at 4°C for 1 h for diffusion of samples and incubated for 18 h at 37°C for bacteria and 96 h for fungi at 30°C. All tests were performed in triplicate and their activities were expressed as the mean of inhibition zone diameters (mm) produced by the test or reference samples.

**In vitro cytotoxicity studies**

In the present investigation, we made an attempt to test the extracts of the selected plants for their cytotoxic activity\(^11,12\) by using fibroblast cell lines of rat (L-929 procured from ATCC). These cell lines were established and well characterized with reproducible results.

**Cell culture**

- The mammalian cell cultures used in this study are fibroblast cells of rat.
- The source of cell line is ATCC (L-929 being the strain).
- The culture medium used is Dulbecco’s modified eagle medium (DMEM), it was supplemented with 10% Fetal calf serum (FCS), 1% sodium pyruvate and 0.06% L-glutamine.
- The cells were grown at 37°C under humidified atmosphere of 5% CO\(_2\).

**Test samples**

The stock solutions were prepared in DMSO to get a concentration at 100 mg/ml of EEAI. From the stock solution, different concentrations (1.5, 3.0, 6.0, 12.5, 25, 50 & 100 mg/ml) were prepared using cell medium as diluents.

**Procedure**

The cells in exponential growth phase were harvested and centrifuged at 300 g for 5 minutes and resuspended in DMEM supplemented with 10% FCS to get a cell count of $1.0 \times 10^4$ cells/ml. One ml of this cell culture was added to each well of a flat bottomed 96 well plate with help of multi-channel pipette and incubated for 24 h in 5% CO\(_2\) humidified incubator at 37°C. Then the test solutions were added to confluent monolayer of L-929 cells and incubated for 24 h. The solvent DMSO was used as a negative control and actinomycin-D was used as positive control.

The cell cultures were examined microscopically for cellular responses. The cellular responses were scored accordingly as none, slight, mild, moderate and severe. The results are presented in the Table 4. From the tabular results we found ethyl acetate extract of tender fruits of *Azadirachta indica* showed cytotoxic activity against L-929 cells.

**Results and Discussion**

The results indicated that EEAI has excellent antibacterial and antifungal activities against all the strains (Table-1). The results of the antifungal activity for the extract showed some degree of protection...
for all most all the plant pathogens at various concentrations, but the results obtained are not much comparable with the standard drug, Miconazole (10µg). EEAI showed better antifungal activity against Aspergillus niger, Fusarium sp, Penicillium chrysogenum, Scleratia rolpsii and showed maximum antibacterial activity against Staphylococcus pyogenes, pseudomonas aeroginosa and Klebsiella pneumoniiae.

The cytotoxic activity testing for EEAI revealed better cytotoxic activity at concentration 100mg/ml comparison to lower concentrations like 12.5mg/ml and 50mg/ml. The extract can able to show mild cytotoxicity to fibroblast cells of rat at the highest concentration taken, where as the standard drug Actinomycin-D showed severe cytotoxic activity (Table-2).

The results suggest that the ethyl acetate extract of unripe tender fruits of Azadirachta indica possess both cytotoxic and antimicrobial activity. The phytoconstituent responsible for this activity can be isolated and characterized for further studies.

Acknowledgements

The authors are grateful to Regional research laboratory, Bhubaneswar and University Department of Pharmaceutical Sciences, Utkal University, Bhubaneswar, Orissa for assistance in running the experiment and completion of the project successfully.

REFERENCES

Table 1: Antimicrobial activity of the extract (EEAI).

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Microorganisms</th>
<th>Inhibition zone in diameter</th>
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<tr>
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<td>EEA</td>
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Table 4. Cytotoxic activity of the extract (EEAI)

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>Cytotoxic response</th>
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<tr>
<td></td>
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<tr>
<td>100</td>
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<tr>
<td>50</td>
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<tr>
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<td>+</td>
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<tr>
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<td>0</td>
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<tr>
<td>3</td>
<td>0</td>
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<tr>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>Negative control (DMSO)</td>
<td>0</td>
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</tbody>
</table>
| Positive control (Actinomycin-D) | ++++

The cytotoxic responses 0 - none, + - slight, ++ - mild, +++ - moderate and ++++ - severe.
Sustained Release Delivery Of Budesonide From PLGA Microspheres Embedded Within Poly-acid Containing PVA Hydrogel

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ABSTRACT
Budesonide is widely used as antiasthmatic drug having short biological half-life. Since budesonide is poor water soluble, various techniques such as dry elixir, the solid dispersion, water soluble prodrug or complexation have been formulated for enhancement of the solubility. Further, frequent dosing of budesonide is required for therapeutic maintenance because of its fairly fast elimination from the body. Exposure of high levels of budesonide in the stomach may cause gastric damage such as ulceration or bleeding. The development of zero-order release systems capable of delivering drug(s) over extended periods of time is deemed necessary for a variety of biomedical applications. We hereby describe a simple, yet versatile, delivery platform based on physically cross-linked poly (vinyl alcohol) (PVA) microgels (cross-linked via repetitive freeze/thaw cycling) containing entrapped budesonide-loaded poly (lactic-co-glycolic acid) (PLGA) microspheres for controlled delivery over a one-month period. The incorporation of poly-acids, such as humic acids, Nafion, and poly (acrylic acid), was found to be crucial for attaining approximately zero-order release kinetics, releasing 60% to 75% of budesonide within one month. Microspheres alone entrapped in the PVA hydrogel resulted in negligible drug release during the 1-month period of investigation. On the basis of a comprehensive evaluation of the structure-property relationships of these hydrogel/microsphere composites, in conjunction with their in vitro release performance, it was concluded that these poly-acids segregate on the PLGA microsphere surfaces and thereby result in localized acidity. These surface-associated poly-acids appear to cause acid-assisted hydrolysis to occur from the surface inwards. Such systems show potential for a variety of localized controlled drug delivery applications such as coatings for implantable devices.

INTRODUCTION
There is a growing interest in drug delivery systems that can provide site-specific and continuous therapeutic drug levels for extended periods of time. These systems can be envisioned as adhesive patches or implantable devices. The goal of the present research is to obtain an approximately zero-order release profile of budesonide over a 1-month period using a hydrogel system that has potential for future application as a coating for implantable devices such as glucose biosensors. Budesonide is selected for this study. It is a synthetic steroid of the glucocorticoid family, it is a derivative of 16 α-hydroxy-prednisolone, and is reported to have low bioavailability (10.7%) following oral administration. Nasal administration of budesonide is used for the treatment of allergic and seasonal rhinitis, and is also effective for asthma. Therefore, controlled systemic circulation of the drug is expected. Gel-forming polymers, eg, poly(vinyl alcohol) (PVA),1 are of interest as such drug delivery systems, since they can provide soft, permeable, and hydrophilic interfaces with body tissues. In this capacity, PVA hydrogels have been used as drug delivery vehicles, alone,2 as well as in combination with other delivery systems,
to provide desirable release profiles. Typically, the degree of PVA hydrogel cross-linking, which is intimately linked with the mechanical properties and water content, greatly influences drug release properties. Cross-linking has been achieved by annealing at elevated temperatures (120°C-130°C), α-irradiation, and/or by chemical means. For many applications involving both small molecular weight (MW) drugs and protein therapeutics, chemical cross-linking is considered undesirable because the complete removal of any unreacted, often toxic, cross-linking agents is very difficult.

Over the past 2 decades, research aimed at the fabrication of mechanically strong hydrogels has led to the development of a well-established physical cross-linking scheme involving repetitive freezing and thawing of aqueous PVA solutions. This technique promotes the formation of ordered microcrystalline domains as a result of enhanced intra and inter polymer interactions in the unfrozen regions of the PVA-water system. This type of cross-linking circumvents the need for chemical cross-linking agents and has led to PVA gels that are bio-inert and exhibit long-term in-vivo compatibility.

The close resemblance of PVA hydrogels to human soft tissue (high water content, rubbery and elastic nature) has made them a material of choice for many biomedical applications, such as intervertebrate disc nuclei, artificial articular cartilage, contact lenses matrices for cell immobilization and mucoadhesives, as well as for the controlled release of drugs, growth factors, and proteins. Drug release rates from PVA hydrogels tend to be relatively rapid (minutes to weeks) depending on the pore size, extent of cross-linking, and the nature of the incorporated drug and typically follow first-order kinetics. To overcome this limitation, other controlled-release delivery systems (eg, microspheres, nanoparticles, liposomes) can be entrapped within these PVA hydrogels without any detrimental effects that could occur in the presence of organic solvents and chemical cross-linking agents. The incorporation of microspheres and other particulate delivery systems can have advantages, such as isolation of the drug, slower drug release rates, and achievement of different drug release profiles, as well as incorporation of multiple drugs in different microsphere populations. Although microspheres alone can be used to achieve long-term drug release of weeks to months, such systems typically do not result in constant drug release profiles. Microspheres usually exhibit an initial rapid “burst” release as a result of surface-associated drug. Furthermore, microsphere systems based on the commonly used poly(lactic-co-glycolic acid) (PLGA) polymer usually display a “triphasic” release profile, with an initial burst, followed by a minimal release phase, before entering into an approximate superlinear release profile when sufficient polymer degradation has occurred. For many drug delivery applications, such a profile is undesirable, and in some cases the initial burst release can raise safety and efficacy concerns.

The development of a composite (PVA-hydrogel/PLGA-microsphere) drug delivery system having an approximately zero-order controlled-release profile over a period of 1 month is made. These composites are intended for use as biocompatible coatings for implantable devices such as glucose biosensors. Budesonide was selected as a model drug based on its potential for suppressing both early phase bronchospasm and late phase inflammation known as asthma. The incorporation of a small amount of surface active polyacids, poly (acrylic acid) (PAA), humic acids (HAs), and Nafion (perfluorosulphonic acid/polytetrafluoroethylene copolymer) into the composite hydrogel matrix was determined essential in achieving erosion of the PVA-embedded PLGA microspheres, as determined by scanning electron microscopy (SEM) and interfacial tension measurements. Furthermore, a detailed
characterization of the effect of the number of freeze-thaw cycles on the mechanical and hydration properties as well as the drug release profile were conducted.

MATERIALS AND METHODS

Preparation of PVA Gel by the Freeze-thaw Method:

PVA (99% hydrolyzed, MW 133 kd, Polysciences Inc, lot 440424 (Warrington, PA) was used in all experiments as a 5% w/w solution in Millipore (Billerica, MA) deionized water. The PVA films were prepared by casting 5 mL into 5-cm diameter covered Petri dishes and freezing at –20°C for 16 hours, followed by thawing for 8 hours at room temperature. The gels were subjected to as many freeze-thaw cycles (Nf/t) as desired.

Preparation of Budesonide Microspheres:

PLGA (50:50; MW 60 000; Resomer RG504) was a generous gift from Boehringer Ingelheim (Ridgefield, CT). PVA (MW, 30 000-70 000), Budesonide and all other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

100 mg of Budesonide was dispersed in 4 mL methylene chloride containing 1 g of PLGA at 10 000 rpm using a homogenizer. This mixture was poured into 20 mL of 1% w/w PVA solution and further homogenized at 10 000 rpm. The microspheres were hardened in 300 mL of 0.1% w/w PVA solution with vigorous stirring under vacuum, washed with 0.1% w/w PVA solution, and collected by filtration. The microspheres were subsequently dried and stored under vacuum until further use. The encapsulation efficiency of all microsphere batches was determined by dissolving in methylene chloride. Budesonide was analyzed by high-pressure liquid chromatography (HPLC) at 246 nm using a C18 column (Nova-Pak, 4 × 150 mm, Waters, Bedford, MA) at 25°C, and a 1:1 mixture of 2 M acetate buffer (pH 4.8) and acetonitrile as the mobile phase. An encapsulation efficiency of 52.1% (± 0.3) was obtained.

Fabrication of PVA Hydrogel Composites Containing Budesonide and Budesonide-loaded PLGA Microspheres:

20 mL chilled 5% w/w PVA solution (prepared as described above) and 100 mg of Budesonide were homogenized at 10 000 rpm and poured into a mold (24.5 × 57.5 × 0.75 mm). PVA hydrogels containing Budesonide microspheres were prepared with 20 mL of chilled 5% w/w PVA solution and 1 g of Budesonide-loaded microspheres, followed by homogenization at 5000 rpm. The molds containing the Budesonide/PVA dispersions and Budesonide microsphere/PVA dispersions were sealed with parafilm and subjected to freeze-thaw cycling, as described previously. The hydrogel films containing Budesonide or Budesonide encapsulated in microspheres were air dried after 1 to 5 freeze-thaw cycles. The hydrogel films used for subsequent characterization and in vitro release contained either 5 mg of Budesonide in PVA or 50 mg of microspheres loaded with 5 mg of Budesonide.

HAs, Nafion (5% w/w solution of perfluorosulphonic acid/ polyytetrafluoroethylene copolymer in lower aliphatic alcohols (1-propanol and ethanol and water), and PAA (MW, 450 kd) purchased from Sigma-Aldrich (St. Louis, MO) were used as modifiers in the PVA gels. 100 mg polymeric acid (HAs, Nafion, and PAA) was mixed with 100 mL of 5% w/w PVA solution, (approximately 2% w/w additive content with respect to PVA) and processed with the microspheres above.

In Vitro Release Study:

The hydrogel films (24.5 × 57.5 × 0.75 mm), prepared as described above, containing Budesonide
and Budesonide microspheres were immersed in jars containing 100 mL of 0.01 M phosphate-buffered saline (PBS) (pH 7.4) and incubated at 37°C (+ 0.5°C) under constant agitation (100 rpm). The release of Budesonide was periodically monitored by extracting 500-µL aliquots for 2 to 4 weeks and replenishing with 500 µL of PBS. PVA hydrogels without Budesonide were used as a control. The concentration of Budesonide was monitored (using HPLC analysis as described below), and the buffer solution was removed and replaced with fresh buffer as necessary to maintain Budesonide concentration below 10% solubility (sink conditions). Release from the Budesonide microspheres alone was determined by placing 50 mg of microspheres in 3 vials containing 1 mL PBS (pH 7.4) for each of the following time intervals: 2, 4, 7, 8, 9, 10, 12, 14, 16, 18, 23, 28, and 30 days. The concentration of Budesonide was monitored and replenished with fresh buffer to maintain sink conditions, as above. All the in vitro release studies were conducted in triplicate (independently prepared films and different batches of microspheres), and mean values and standard deviations were calculated.

Surface Activity Determination:
The polymeric acids (HAs, Nafion, and PAA) were mixed with PLGA microspheres and dispersed in distilled water at the same concentration as used in the PVA-hydrogel/PLGA microsphere composites (1 g of microspheres in 20 mL of deionized water). These dispersions were subjected to 3 freeze-thaw cycles, following which the microspheres were filtered and rinsed with copious amounts of deionized water. The ability of the polymers to modify the surface of the microspheres was investigated by comparing contact angle and zeta potential before and after the addition of the polymeric acids.

The contact angle studies (20-µL drop size) were performed using a Ramé-Hart goniometer (Mountain Lake, NJ). Freshly prepared and modified PLGA microspheres were annealed in a vacuum at 200°C for 24 hours to form a film. The contact angle measurements were repeated 10 times on several film positions and averaged to account for film inhomogeneities.

Zeta potential studies were conducted using a Zetaplus apparatus (Brookhaven Instruments, Holtsville, NY). The as-prepared and polyacid-modified PLGA microspheres (as above) were dispersed (1 mg/mL) in deionized water or PBS, pH 7.4. This dispersion was then added to the Zetaplus electrophoresis cell, and the electrophoretic mobility was measured and the data converted to zeta potential values. These experiments were repeated 10 times, and the mean values and standard deviations calculated.

The surface tension of the polymeric acids was determined using a Kruss K12 Tensiometer (Hamburg, Germany), in the Wilhelmy Plate mode. Time-dependent changes in the surface tension were monitored automatically. The polymeric acids (2% w/w) were dissolved in either deionized water or PBS buffer, and the surface tension was recorded continuously at 25°C ± 0.5°C until the fluctuation in the data was less than 0.1 mN m⁻¹. These experiments were performed 5 times and mean values and standard deviations calculated.

Characterization of PVA Hydrogel:
The swelling of the air-dried PVA gels was characterized using 2 methods: (1) monitoring the dimensional changes as a function of immersion time under a constant load of 20 mN using a Perkin-Elmer Thermomechanical Analyzer (TMA) (Wellesley, MA), and (2) monitoring weight change. The percentage strain (% strain), as obtained from TMA, was defined as the percentage change in length divided by the original length: (L – Lo)/Lo × 100%. The swelling experiments were performed in PBS.
buffer at room temperature and also at 37°C (± 1°C). The percentage weight change was defined as the percentage change in mass during swelling: \((M - Mo)/Mo \times 100\%\). Air-dried PVA samples (Mo) (approximately 10 × 10 × 0.75 mm) were weighed and immersed either in 20 mL deionized water or in PBS buffer and maintained at 37°C for 96 hours in a thermostated water bath; following which excess fluid from swollen samples was carefully removed and the weight change \((M - Mo)\) with respect to the dry mass recorded.

The Young’s moduli of the as-fabricated and microsphere-loaded PVA gels were obtained from uniaxial tensile testing using TMA. Rectangular samples (approximately 4-mm wide and 15-mm long and 0.75-mm thick) were used for all tensile tests. The force was linearly ramped from 30 to 100 mN at a constant rate of 25 mN min⁻¹ and the Young’s modulus was ascertained from the resulting stress-strain curves. The Young’s modulus was measured for air-dried and fully hydrated samples (PBS buffer).

A Perkin-Elmer differential scanning calorimeter (DSC 7, Wellesley, MA) was used to determine the degree of crystallinity of air-dried PVA gels in the presence and absence of acidic additives and PLGA microspheres. DSC scans were performed at 5°C/min from 25°C to 250°C. The degree of crystallinity \((X)\), \((X = \Delta H / \Delta H_C \times 100\%)\) was calculated by dividing the heat required to melt 1 g of dry sample \((\Delta H)\) obtained by integrating the area under the endothermic peak between 190°C and 240°C by the standard enthalpy \((\Delta H_C)\) for 100% crystalline PVA (138.6 J/g)\(^{31}\). All crystallinity values were normalized with respect to the actual PVA content in the sample.

**Morphological Characterization of Degraded Microspheres:**

Microsphere degradation was monitored using a Phillips environmental scanning electron microscope (ESEM) (FEI Company, Hillsboro, OR) operated at 20 keV. Both as-prepared microspheres and hydrogel-dispersed microspheres were treated as described in the in vitro release experiments, and their morphology was compared at various intervals over a 4-week study period. Microspheres were recovered from the hydrogel by dry-fracturing the polymer matrix and brushing them off the fractured surface. Microscopy specimens were prepared by placing the microspheres on carbon tape-covered microscopy stubs and coating them using a Polaron sputter-coating system (Quorum Technologies, Newhaven, East Sussex, UK) (at 0.04-0.06 Torr for 20 seconds) with a thin layer of Au/Pd to increase conductivity and image quality.

**Mechanical Properties of PVA Hydrogels:**

Prior to freeze-thaw assisted cross-linking, aqueous solutions of 99% hydrolyzed PVA were preheated to c.a. 80°C to facilitate complete polymer dissolution. PVA hydrogels with a lower degree of hydrolysis (below 89%) were soluble in water below 37°C and therefore were deemed inappropriate for use in this study.

**RESULTS AND DISCUSSION**

Figure 1 depicts the degree of swelling of PVA gels, in water and PBS, as a function of freeze-thaw \((Nf/t)\) cycling. In both environments the weight change was inversely related to the \(Nf/t\) cycles, corroborating other reports.\(^{32}\) Typically, hydrogels subjected to 1 \(Nf/t\) cycle had water uptake of up to 500% of their dry weight, whereas hydrogels with 5 \(Nf/t\) cycles exhibited c.a. 350% swelling. This differential water uptake can be related to the extent of physical cross-linking, which decreases porosity\(^7\) and alters fluid uptake.\(^7\) Although the fluid uptake was lower in high ionic strength media (ie, PBS
buffer) for samples subjected to 1 and 2 freeze-thaw cycles, the overall trend was similar to that observed in water. This decreased fluid uptake in high ionic strength media may be a result of antichaotropic effects (i.e., reinforcement of hydrogen bonding within the gel), previously reported in the presence of inorganic (e.g., NaCl, NaF, Na3PO4, Na2SO4) and a few amino acid salts.

The dimensional changes of the PVA gels upon hydration in PBS buffer, as a function of immersion time, are depicted in Figure 2. An initial induction time (varying from 0.5 to 20 minutes), characterized by the plateau before the onset of the dimensional change, was observed for all samples tested at 25°C and is thought to arise from the slower fluid diffusion into the dry film and depends on film thickness and microstructure. Typically, the specimens attained fully hydrated dimensions after approximately 200 minutes of immersion (a 35% increase in length for Nf/t= 3). However, for the same experiments performed at physiological temperature (37°C), not only was no induction time observed but the PVA gels reached the same fully hydrated state substantially faster (in less than 30 minutes) pointing to almost instantaneous swelling at body temperature with equilibrium swelling being attained within 30 minutes.

Figure 3 depicts the Young's modulus for dry and fully hydrated PVA films in the presence and absence of microspheres as a function of Nf/t cycles. In general, these gels have an order of magnitude larger modulus after the first freeze-thaw cycle, and reach an asymptotic value after Nf/t = 3 cycles, corroborating observations of Takamura et al. and Takamura and Ishii. The modulus for the dry samples changed from 2 (Nf/t= 1) to 20 to 40 MPa (Nf/t= 5), which can be attributed to the gradual ordering and crystallization of the macromolecular chains upon repetitive freezing and thawing. The crystalline regions within the gels act as stress transfer points, redistributing external stress similarly to chemically or irradiatively induced cross-links. Additionally, the PLGA microspheres dispersed within the dry PVA matrix act as a filler, which is apparent from the slight reinforcement of gels prepared with 1, 2, and 3 freeze-thaw cycles. In contrast to dry samples, the fully hydrated gels exhibit an order of magnitude smaller modulus, which is comparable with that of soft human tissue. Surprisingly, the reinforcing effect of the microspheres was not observed in the hydrated state, where the Young’s modulus was lower than that of the fully hydrated PVA gels prepared with 3, 4, and 5 freeze-thaw cycles. This
discrepancy may be related to the poor adhesion between the microspheres and the solvated polymer matrix, and possibly dewetting (slippage at the interface), which may occur owing to limited hydration of the relatively hydrophobic PLGA microspheres.\textsuperscript{36}

**In Vitro Release From Hydrogels and Hydrogel/Microsphere Composites:**

Based on the above results, PVA gels subjected to either 1 or 2 freeze-thaw cycles were determined to be inadequate in terms of their mechanical properties and therefore were not considered suitable for further investigation. Consequently, the in vitro release studies were conducted on samples that had undergone 3 to 5 freeze-thaw cycles. The in vitro release of Budesonide from the PVA hydrogels alone (no microspheres) was determined to be inversely proportional to the number of freeze-thaw cycles (shown in Figure 4) for $N_f/t = 3$ and 5, respectively. The effect of the number of freeze-thaw cycles on drug release rates is in agreement with literature data on release from such PVA hydrogels.\textsuperscript{20} The release of Budesonide from these gels into pH 7.4 PBS reached 100% release within 10 days for $N_f/t = 3$ and followed approximately first-order release kinetics.

The release of Budesonide from these gels into pH 7.4 PBS reached 100% release within 10 days for $N_f/t = 3$ and followed approximately first-order release kinetics. PLGA microsphere-encapsulated Budesonide embedded within the PVA matrix (for $N_f/t = 3$) released only approximately 6% in pH 7.4 PBS after 30 days (Figure 5). For the purpose of comparison, the release profile of Budesonide-loaded microspheres into pH 7.4 PBS in the absence of the PVA gel is also shown in Figure 5. As expected, release of Budesonide from the microspheres alone into PBS revealed an initial “burst” followed by a slower release phase. Apparently, the presence of the surrounding PVA hydrogel has a profound effect on the release profile of PLGA microspheres and, as shown in Figure 4, this extremely slow release profile cannot be attributed to drug diffusion through the PVA hydrogel matrix, since release of drug alone (no microspheres) from the PVA was complete in 10 days. Such behavior may result from a dramatic decrease in the acid-catalyzed, self-accelerated ester bond cleavage that has been established as a prevalent mechanism for PLGA microsphere degradation and consequent drug release.\textsuperscript{39} Typically, as the PLGA degrades there is a build up of acidic oligomeric by-products of PLGA, and these prefer to remain within the nonpolar environment of the PLGA microspheres rather than partition into aqueous release media. These by-products then self-catalyze bulk degradation of the PLGA.\textsuperscript{39-42} However, in the presence of the PVA hydrogel, the oligomeric by-products are speculated to partition into the hydrogel (since this presents an environment that is more polar than PLGA, yet less polar than water) and, therefore, are unavailable for self-catalysis of the PLGA. A second possible explanation for the decreased release of Budesonide from the microspheres embedded in the hydrogels is that microcrystalline PVA domains form at the PLGA microsphere surfaces, thereby significantly decreasing the ability of water to partition into the microspheres and, therefore, decreasing polymer degradation and drug release.

**SEM Study:**

In order to further investigate the effects of polyacids on PLGA microsphere degradation, when embedded in PVA microgels, a morphological study was conducted using HAs as a model polyacid. Figures 6-A1 and 6-A2 illustrate low and high magnification ESEM images of nondegraded PLGA microspheres. As can been seen from these photomicrographs, there is a considerable particle size variation with a volume-weighted mean size of $\sim$ 40 micrometers. The as-prepared microspheres appear round with a fairly smooth surface and no evidence of significant porosity. Following 4 weeks
Figure 3. Young’s modulus for PVA Hydrogels determined at 25°C as a function of the number of freeze-thaw cycles for: (E%) dry gels as-fabricated; (ië) dry gels MS-loaded; (º%) fully hydrated gels as-fabricated, and (½%) fully hydrated gels MS-loaded. (n = 3, where n is the number of repeat experiments conducted. P < .05 shows significant difference between PVA and MS/PVA at different freeze-thaw cycles under dry and wet conditions as analyzed using Student t test.)

Figure 4. Cumulative release of Budesonide from PVA hydrogels as a function of the number of freeze-thaw cycles. (PBS buffer, p\(\text{H} \) 7.4, 37°C; n = 3, where n is the number of repeat experiments conducted.)
Figure 5. Cumulative release of Budesonide (Bud) from (I%) PLGA microspheres (MS); and MS incorporated into the PVA hydrogel matrix: with (E%) no additives; and in presence of (A) PAA; (I%) HAs; and (*) Nafion (Nf/t = 3; PBS buffer, pH 7.4, 37°C; n = 3, where n is the number of repeat experiments conducted.)

Figure 6. ESEM photomicrographs of PLGA microspheres: (A1, A2) as-prepared, nondegraded, no-gel; (B1, B2) after 4 weeks submersion in PBS; (C1, C2) embedded in PVA gel, no additives after 4 weeks submersion in PBS; and (D1, D2) embedded in PVA gel with humic acids after 4 weeks submersion in PBS.
submersion in PBS buffer, (Figures 6-B1 and 6-B2), these microspheres show a significant degree of degradation. As expected, degradation proceeds faster internally than externally for the as-prepared microspheres in PBS buffer due to internal build up of acidic by-products that self-catalyze the PLGA degradation process, resulting in the formation of hollow cores.\textsuperscript{39,42} On the other hand, the PLGA microspheres embedded in PVA hydrogels, which have been immersed in PBS for 4 weeks, show similar or less external porosity with little internal degradation as evident from the fractured microspheres shown in Figures 6-C1 and 6-C2. This finding is consistent with the minimal drug release rate observed from these systems when embedded in neat PVA hydrogels. However, in the case of PLGA microspheres that had been embedded in HAs-modified PVA hydrogels and immersed in PBS for 4 weeks, an entirely new surface morphology is witnessed. As can be seen in Figure 6-D1, these microspheres exhibit significant surface pitting, which appears as crater-like dimples a few micrometers in diameter. This finding is in agreement with HAs surface association (presented above), followed by pronounced surface catalyzed degradation. Degradation also occurs in the interior of these microspheres (see Figure 6-D2), and although it is not as extensive as in the case of the non-PVA embedded systems, it is clearly greater than in the absence of polyacids.

CONCLUSION

A study was performed on PVA hydrogels to delineate their structure-property relationships in conjunction with their in vitro release performance using Budesonide as a model drug. The physicochemical and mechanical properties of these gels (ie, modulus, swelling, crystallinity, and drug release) was dependent on the number of freeze-thaw cycles ($N_{f/t}$) used to physically cross-link the PVA matrix and was determined to plateau at $\sim 3 N_{f/t}$. These gels, in their dried state exhibit sufficient hardness to withstand the forces associated with handling and implantation and rapidly hydrate and swell to c.a. 350% in aqueous environments, resulting in pliable gels with Young’s modulus comparable with that of soft human tissue (0.1 to 4 MPa).

When Budesonide was incorporated into plain PVA hydrogels, 80%-100% release was attained within 2 weeks depending on the number of $N_{f/t}$ cycles. On the other hand, PLGA microsphere-encapsulated Budesonide, embedded within PVA hydrogels, resulted in minimal release (approximately 6%) over a period of 1 month. Incorporating polyacids within gels containing PLGA microspheres resulted in an order of magnitude increase in the Budesonide release rate (to 60% to 75% release over a 1-month period). Polyacid-induced acceleration of drug release was linked to the surface activity of these polyacids towards PLGA microspheres. This appeared to cause a localized increase in acidity in the vicinity of the microsphere surfaces, which in turn appeared to alter their degradation characteristics from an inside-out (in the case of PLGA microspheres alone) to an outside-inwards model when embedded in polyacid modified PVA hydrogels. These systems show potential for a variety of localized controlled drug delivery applications, such as biocompatible coatings for implantable devices.

ACKNOWLEDGEMENT

The authors like to thank Dr. Gautam Kumar Dalapati, Sr. Scientist, Institute of Materials Research & Engineering, Singapore for his valuable suggestion and support for this research.

REFERENCES

1. Hassan CM, Peppas NA. Structure and applications of poly(vinyl alcohol) hydrogels produced


Evaluation Of Antioxidant Activities Of Selected Indian Plants

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¹Natural Products Laboratory, Department of Chemistry, University of Rajasthan, Jaipur
²Birla Institute of technology, Mesra, Ranchi

ABSTRACT:

The methanolic extracts of seeds of Ipomoea hederacea (Convolvulaceae) and Strychnos potatorum (Loganiaceae), leaves of Zanthoxylum alatum (Rutaceae) and stem bark of Alstonia scholaris (Apocynaceae) were subjected to evaluation of antioxidant potential by using DPPH method. Out of these selected plants Zanthoxylum alatum was found to show maximum antioxidant potential. Whereas methanolic extracts of Alstonia scholaris and Strychnos potatorum were found to show moderate antioxidant potential.

INTRODUCTION:

It was suggested recently that generation of free radicals play a major role in the progression of a wide range of pathological disturbances such as brain dysfunction, cancer and cardiovascular diseases¹,². Free radicals, together with other derivatives of oxygen are inevitable by products of biological redox reactions. Reactive oxygen species such as superoxide anion (O²⁻), hydroxyl radical (OH⁻) and nitric oxide inactivates enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation and thus have been shown to augment collagen synthesis and fibrosis. Antioxidants are supposed to protect cell membranes against free radical oxidative damages³,⁴.

The synthetic antioxidants may create health problems. So scientists have focused on isolation and characterization of natural antioxidants. The natural antioxidants have replaced the synthetic ones because they are beneficial for our health and they are soluble in oil and water. There is growing interest to replace synthetic antioxidants by natural antioxidants. There is also a worldwide trend towards the use of natural additives in food and cosmetics. For this reason an extensive search for different types of plant based antioxidants reason an extensive search for different types of plant based antioxidants is taking place.

A large number of plants have shown potent antioxidant activities. The increasing interest in alimentary applications i.e. dietary, nutraceuticals, flavoring etc. of plant matrices rich in antioxidants is due to the possible correlation between the oxidant action of free radicals and the onset of some important pathologies like ischemia-reperfusion injury, atherosclerosis, thrombolic and cancer diseases⁵. This kind of correlation has led the health nutrition industry to seek antioxidants from plant matrices that can be used in the formulation of preventive phytocomplexes. Strict legislation on the use of synthetic food additives and consumer preferences has also shifted the attention of manufacturers from synthetic to natural antioxidants⁶. Noteworthy is that natural matrices rich in antioxidants have proved to be less toxic than synthetic molecules such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are highly volatile and unstable at high temperature making their industrial scale application difficult. They might possibly induce carcinogenesis despite the fact that they inhibit lipid peroxidation⁷-⁹. Therefore, in the present investigation various extracts of different plants were screened for antioxidant activity using DPPH method.
MATERIALS AND METHOD

1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from HiMedia Laboratories Limited, Mumbai, India. Methanol (analytical grade) was purchased from Samir tech-chem. Pvt. Ltd. Vadodara, India.

The seeds of Ipomoea hederacea (P1), Strychnos potatorum (P2) and stem bark of Alstonia scholaris (P3) and Zanthoxylum alatum (P4) were collected from various regions of India. The materials were identified in the Department of Botany, University of Rajasthan, Jaipur. The voucher specimens of the plants are stored in the herbarium of this Department for reference.

Extraction Of Plant Material

The various parts of the plants were dried at room temperature, grounded and 4 K.g. of each plant material was defatted by extraction with Pet. Ether and then were successively extracted with chloroform, ethyl acetate and methanol. The extracts were concentrated to dryness under reduced pressure and controlled temperature. All the extracts were preserved in a refrigerator till further use.

Determination Of The Antioxidant Activity By Dpph Method

Free radical scavenging activity of the crude extracts was determined using a method based on the reduction of methanolic solution of the colored free radical DPPH solution. A methanolic DPPH solution (250μM) was mixed with methanolic solutions of extract concentrations 0.009 gm/ml, 0.008 gm/ml, 0.007 gm/ml and 0.006 gm/ml respectively. The stock solutions were diluted with methanol to obtain lower dilution. Then samples were shaken vigorously and kept in dark for 0.5 hour. At room temperature, sample absorbency was measured at 517nm with spectrophotometer. A methanolic solution of DPPH was employed as blank. Antioxidant activity of each extract was determined according to the percentage of DPPH decoloration.

\[
\text{Inhibition percentage} = \left[1 - \frac{\text{absorbance with compound}}{\text{absorbance of the blank}}\right] \times 100
\]

All determination were performed in triplicate and averaged.

RESULTS AND DISCUSSION

The antioxidant activity of all extracts was evaluated with the DPPH method. This technique is particularly fit for the evaluation of the antioxidant activity of crude extracts. Moreover, this method is rapid, simple, sensitive, reproducible and require conventional laboratory equipments. Application of this method to these extracts provided interesting and homogenous results. The DPPH assay showed that all extracts exhibited minimal antioxidant activity even at the lowest test concentration of 0.005gm./ml. Antioxidants serve hydrogen to free radicals and scavenge radicals. The scavenging effect of four extract is shown in Table 2. All extracts exhibited free radical scavenging ability at various concentrations.

Acknowledgement

The authors wish to thank D. K. Gupta, Incharge of Sophisticated Instrumentation Centre, Department of Chemistry, U. O. R., Jaipur, for his encouragement.

REFERENCE


Table 1. Phytochemical details of the (P₁-P₄)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Popular name</th>
<th>Botanical name</th>
<th>Family</th>
<th>Habitat</th>
<th>Actions</th>
<th>Constituents</th>
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</thead>
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<tr>
<td>P₁</td>
<td>Ivy leaf morning glory</td>
<td>Ipomoea hederacea</td>
<td>Convolvulaceae</td>
<td>India</td>
<td>Purgative, anticancer, antitubercular</td>
<td>kaladasterone, muristerone A, makisterone</td>
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<td>P₂</td>
<td>Nirmali</td>
<td>Strychnos potatorum</td>
<td>Loganiaceae</td>
<td>India</td>
<td>Demulcent⁹, antihypercholesterolemic¹¹</td>
<td>Diaboline¹², angustine, 11-hydroxydiaboline</td>
</tr>
<tr>
<td>P₃</td>
<td>Tumbru</td>
<td>Zanthoxylum alatum</td>
<td>Rutaceae</td>
<td>India</td>
<td>Antioxidant, carminative, stomachic, antihelminthic</td>
<td>1,8-cineole, sabinene, linalool, α-terpine</td>
</tr>
<tr>
<td>P₄</td>
<td>Saptarni</td>
<td>Alstonia scholaris</td>
<td>Apocynaceae</td>
<td>India</td>
<td>Hepatoprotective, antimalarial, immunostimulating</td>
<td>alschamine, alstonamine, dittamine, venoterpine</td>
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Table 2. Scavenging effect of various extracts of different plants on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>0.006 gm/ml</th>
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<th>0.008 gm/ml</th>
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<tr>
<td>P₁</td>
<td>68.81 ± 1.3</td>
<td>79.81 ± 1.2</td>
<td>81.23 ± 0.1</td>
<td>90.13 ± 0.8</td>
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<tr>
<td>P₂</td>
<td>78.26 ± 0.2</td>
<td>84.89 ± 1.1</td>
<td>70.12 ± 0.6</td>
<td>73.98 ± 1.3</td>
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<tr>
<td>P₃</td>
<td>11.95 ± 1.3</td>
<td>41.95 ± 0.1</td>
<td>56.63 ± 1.7</td>
<td>58.18 ± 1.2</td>
</tr>
<tr>
<td>P₄</td>
<td>44.37 ± 1.3</td>
<td>96.18 ± 0.9</td>
<td>98.11 ± 1.4</td>
<td>81.73 ± 0.3</td>
</tr>
</tbody>
</table>
A Quantitative analysis of Gemfibrozil in human plasma using Ultra Performance Liquid Chromatography/Tandem Mass Spectrometry

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ABSTRACT
The aim of this study was to compare single-dose oral bioavailability of two formulations of Gemfibrozil 600 mg tablets (A & B) developed by OHM Laboratories (a subsidiary of Ranbaxy Pharmaceuticals inc, USA) with Lopid 600 mg tablet of Parke Davis (A division of Pfizer Inc. USA), under fasting condition. The study was carried out as 3-way crossover design on 18 subjects. The plasma samples were obtained over a 12 hour post dose in each period. Plasma Gemfibrozil samples were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with negative ion electro spray ionization using multiple reaction monitoring (MRM). A sensitive, reproducible, accurate and validated LC-MS/MS method with limit of quantification (LOQ) 0.157 µg/ml was used to analyze gemfibrozil. ln-transformed AUC$_{0-t}$, AUC$_{0-\infty}$, and C$_{max}$ were assessed for bioequivalence using 90% confidence interval (CI). Ratio of least-squares mean and its 90% confidence intervals for ln-transformed AUC$_{0-t}$, AUC$_{0-\infty}$ and C$_{max}$ were within the regulatory acceptance criteria of 80-125%.

INTRODUCTION
Gemfibrozil is fibric acid derivative chemically 5-(2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid and it is an oral lipid lowering agent with short plasma half-lives and available in 300, 600 and 900mg over which gemfibrozil disposition is linear$^{1-4}$. Gemfibrozil decreases serum triglycerides and very low density lipoprotein (VLDL) cholesterol, and increases high density lipoprotein (HDL) cholesterol. While decrease in total low density lipoprotein (LDL) cholesterol may be observed with gemfibrozil therapy, treatment of patients with elevated triglycerides due to Type IV hyperlipoproteinemia often results in a rise in LDL-cholesterol. LDL-cholesterol levels in Type IIb patients with elevations of both serum LDL-cholesterol and triglycerides are minimally affected by gemfibrozil treatment; however, gemfibrozil usually raises HDL-cholesterol significantly in this group.

Gemfibrozil increases levels of high density lipoprotein (HDL) subfractions HDL2 and HDL3, as well as a polipoproteins AI and All thus acting as an agonist for peroxisome proliferation activated receptors (PPARs) thereby reducing incidence of coronary heart diseases$^{5-8}$. In vitro gemfibrozil is highly bound to serum albumin and over 95% of drug is protein bound.

Gemfibrozil is completely absorbed after oral administration of LOPID tablets and peak plasma concentrations were reached within 1 to 2 hours. Gemfibrozil pharmacokinetics is affected by the timing of meals relative to time of dosing. In humans the plasma half-life is 90 minutes and over 95%
of the drug is protein bound. Gemfibrozil mainly undergoes oxidation of a ring methyl group to successively form a hydroxymethyl and a carboxyl metabolite^9. Approximately seventy percent of the administered human dose is excreted in the urine, mostly as the glucuronide conjugate, with less than 2% excreted as unchanged gemfibrozil^10.

Gemfibrozil was estimated previously by UV, flurimetric detection, gas chromatographic mass spectrometric analysis method, but run time was found lengthy. In order to develop a rapid lcms assay technique to estimate gemfibrozil from human plasma a simple, sensitive and robust method was developed with a low level of quantification. The method was validated successfully and used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence. Our cost effective simple, reproducible method using reverse phase chromatography with UPLC and Micromass was able to achieve a run time of 1 minute and made it possible to analyze a throughput of more than 500 human plasma samples per day.

Experimental

Study Design

A single oral dose of two different formulations of Gemfibrozil 600 mg tablets (A & B) developed by OHM Laboratories (a subsidiary of Ranbaxy Pharmaceuticals inc, USA) and Lopid 600 mg tablets of Parke Davis (A division of Pfizer Inc. USA) were administered to the subjects with 240 mL of drinking water at ambient temperature after an overnight fast of at least 10 hours as per randomization schedule^12.

Clinical Design and Sample Collection

The study was designed as an open label, balanced, randomized three-treatment, three-period, three-sequence, single-dose, crossover bioavailability study in 18 healthy, adult, human, male subjects under fasting condition. A total of fifty-one, 5-mL blood samples were collected from each subject in EDTA vacutainers through indwelling cannulae placed in forearm veins. The blood samples were collected at pre-dose and at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 hours post dose in each period. The pre-dose blood samples in each period was collected within a period of 1.5 hours before dosing and the post-dose samples were collected within 2 minutes of the scheduled time. The actual end-point time of collection of each blood sample was recorded.

Chemical and Reagents

Gemfibrozil was purchased from USP (Rockville, MD, USA), Gemfibrozil-d6 was procured from C/D/N ISOTOPS INC (88 Leacock Street, Pointe-Clair, Quebec, Canada) and methanol was purchased from Qualigens fine chemicals (A division of GSK Ltd, Mumbai, India). Tertiary Butyl Methyl Ether was purchased from Sisco Research laboratories (Mumbai, India). Human plasma containing ethylene diamine tetra acetic acid (EDTA) was collected in-house which were free from HIV and Hepatitis. All other solvents & reagents of analytical grade were purchased from S.d. fine chem. Ltd (Mumbai, India).

Stock Solution, calibration curve (CC) and Quality Control (QC) Sample Preparation

Approximately 2000µg/ml of stock solutions for Gemfibrozil were prepared using methanol. Sub stocks of 2000.0, 1600.0, 800.0, 320.0, 160.0, 64.0, 19.0, 8.0µg/ml solutions were used to prepare spiking standards. The calibration range selected to measure the expected sample concentrations was 0.157 to 39.018µg/ml for gemfibrozil in EDTA plasma. Calibration curve consisted of single replicate
of eight non-zero standards, with two standards below the low QC and two standards above the high QC. Quality control concentrations of 0.407, 7.535 and 30.142 µg/ml were prepared by spiking 100 ml of blank plasma with an appropriate gemfibrozil stock/substock solutions. In addition, precision & accuracy batches included lower limit of quantification (LOQ QC) samples. The spiking volume did not exceed 2% of the total calibration curve & quality control sample volumes. The QC samples were mixed and redistributed into 0.5 ml aliquots for storage at -15°C.

**LC-MS/MS**

Chromatographic separation was achieved by maintaining the column oven temperature at 35°C and by using the Aquity BEH C18 column (Waters corporation, Milford, Massachusetts, USA; 2.1x 50 mm, 1.7µm ) using the mobile phase comprised of acetonitrile - ammonium acetate buffer (pH 6.8 ; 2mM )(90:10 v/v) which was pumped by using waters micro pump at a flow rate of 0.5 mL/min. The samples were loaded in the Waters Aquity UPLC autosampler and temperature of the auto sampler was set at 10°C. 5µl of sample extract was injected and the eluent was monitored by tandem mass spectrometry with an electro spray ionization (ESI) inter face of Quattra Premier (Waters ,UK) The chromatographic data was acquired and processed using computer based MASSLYNX software Version 4.1. The Negative ions were monitored in the multiple reaction-monitoring (MRM) mode. The following ion transitions (m/z) were monitored 249.09 (parent) and 121.02 (product) for gemfibrozil and 255.04 (parent) and 121.20 (Product) for gemfibrozil- d6.

**Bioanalytical Sample Preparation**

An aliquot of human plasma containing both analyte and its internal standard was extracted by using a liquid-liquid extraction. 50 µL of internal standard (gemfibrozil- d6) stock dilution (approximately 100.0µg/ml) was pipetted into stoppered tubes and 300 µL aliquot of each plasma sample was added into these tubes and vortexed. Then 4ml of Tertiary Butyl Methyl Ether was added in each tube and placed in reciprocating shaker for 20 minutes at 100 rpm. Tubes spun in refrigerated centrifuge for 5 minutes at 3500 rpm at temperature 2-10°C. 3ml of clear supernatant organic layer was transferred to another glass tube and evaporated to dryness at 50°C under nitrogen gas pressure. The dried residue was reconstituted with 400 µL of mobile phase ammonium acetate buffer, (2 mM :pH 6.8±0.2): acetonitrile (10:90v/v)) and transferred into the autosampler vial for injection.

The weighted linear least square regression analysis (weight: 1/Concentration²) was performed in order to obtain a best-fit line using peak area ratio of gemfibrozil and internal standard. The concentrations of gemfibrozil in plasma samples were calculated using linear regression parameters by corresponding calibration curve.

**Bio analytical Method Validation**

The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, linearity of response, accuracy, precision, recovery, stability,dilution integrity, matrix effect. The described LC-MS/MS method to quantify gemfibrozil in human plasma was validated and found to be specific and sensitive for gemfibrozil. For selectivity six lots of plasma with EDTA as anticoagulant were evaluated and none showed significant interfering peaks at the retention time of gemfibrozil and gemfibrozil- d6. Figure 1, 2 depicts chromatogram of blank and blank with internal standard. For sensitivity the limit of quantification (LLOQ) was 0.157 µg/ml. Figure 3 depicts chromatogram of
LLOQ and with internal standard from extracted plasma sample. The method was validated for the analytical range of 0.157 to 39.018 µg/mL and linearity was determined by weighted least square regression analysis of standard plot associated with eight point standard curve. Six sets of Quality control samples at different concentrations levels of lower limit of quantification (LOQ QC) 0.163 µg/mL, 0.407 µg/ml (LQC), 7.535 µg/ml (MQC) and 30.142 µg/ml (HQC) prepared in human plasma, were analyzed to ensure acceptable assay precision and accuracy.

The accuracy of the assay was defined as an absolute value of the calculated mean concentration of the quality control sample to their respective nominal values, expressed as percentage. The intra and inter batch accuracy using internal standard area ratio method ranged from 98.0 to 110.4% and 98.1 to 109.2% respectively. Deviation of the assay accuracies was well within ±15.0% for the analyte.

The recovery was based on the comparison of the peak areas of extracted plasma QC samples at high, medium and low concentrations with un-extracted aqueous samples. The mean recovery of Gemfibrozil and its internal standard from plasma was more than 60%. Gemfibrozil proved to be stable in biological samples for at least three Freeze-and thaw cycles, Bench top stability for 8 hours, In-Injector stability for 47 hrs, Long term storage stability for 217 days at -15°C, proved no matrix effect and met the acceptance criteria for dilution integrity as per USFDA method validation guidance.

Figure 1: Representative Chromatogram of Gemfibrozil and its internal standard from extracted plasma blank.

Figure 2: Representative Chromatogram of Gemfibrozil and its Internal standard from extracted plasma blank + Internal Standard.
**Figure 3:** Representative Chromatogram of Gemfibrozil and its internal standard from extracted plasma at LLOQ concentration

**Table 1:** Intra & Inter-Batch Precision & Accuracy of Gemfibrozil in human Plasma.

<table>
<thead>
<tr>
<th>QC Samples</th>
<th>LOQQC</th>
<th>LQC</th>
<th>MQC</th>
<th>HQC</th>
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<tr>
<td>Concentration(µg/ml)</td>
<td>0.163</td>
<td>0.407</td>
<td>15.071</td>
<td>30.142</td>
</tr>
<tr>
<td>Intra -batch mean</td>
<td>0.1597</td>
<td>0.4270</td>
<td>15.8688</td>
<td>32.7758</td>
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<tr>
<td>Intra -batch % CV</td>
<td>5.0</td>
<td>1.6</td>
<td>5.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Intra -batch % SD</td>
<td>0.00799</td>
<td>0.00693</td>
<td>0.82469</td>
<td>0.74757</td>
</tr>
<tr>
<td>Inter -batch mean</td>
<td>0.1599</td>
<td>0.4309</td>
<td>15.8969</td>
<td>32.9084</td>
</tr>
<tr>
<td>Inter -batch % CV</td>
<td>4.5</td>
<td>1.9</td>
<td>4.8</td>
<td>1.9</td>
</tr>
<tr>
<td>Inter -batch % SD</td>
<td>0.00721</td>
<td>0.00808</td>
<td>0.75924</td>
<td>0.62516</td>
</tr>
</tbody>
</table>

**Statistical Analysis**

Appropriate statistical analysis for a three-way crossover design was performed to assess the bioequivalence. The analysis was performed using SAS software Version 9.1 (SAS Institute Inc., USA). Analyses of variance (ANOVA) was performed on the log-transformed AUC$_{0-t}$, AUC$_{0-inf}$ and C$_{max}$ using a mixed effects ANOVA model with the main effects of sequence, formulation and period as fixed effects and subject nested within sequence as a random effect. The sequence effect was tested at the 0.10 level of significance using the subjects nested within sequence mean square as the error term. All other main effects were tested at the 0.05 level of significance against the residual error (mean square error) from the ANOVA model as the error term. Ratios of least-square means (LSM) were calculated using the exponentiation of the LSM from the analyses on the log-transformed AUC$_{0-t}$, AUC$_{0-inf}$ and C$_{max}$. Consistent with the two one-sided test for bioequivalence, 90% confidence intervals for the ratios were derived by exponentiation of the confidence intervals obtained for the difference between formulation LSM resulting from the analyses on the log-transformed AUC$_{0-t}$, AUC$_{0-inf}$ and C$_{max}$. The confidence intervals were expressed as a percentage relative to the reference formulation.

Gemfibrozil was readily absorbed from the gastrointestinal tract and was measurable at the first sampling time (0.25hr) in the majority of volunteers. Mean Plasma concentration profiles of gemfibrozil attained after the administration of test and reference products are shown in Figure 4 and 5. Mean plasma concentration profiles of gemfibrozil were comparable and closely similar between the three formulations. The summary results of pharmacokinetic parameters are summarized in below table 2:
Table 2: Summary statistics of pharmacokinetic parameters for Gemfibrozil in Plasma

<table>
<thead>
<tr>
<th></th>
<th>Test Formulation A (mean ± sd)</th>
<th>Test Formulation B (mean ± sd)</th>
<th>Reference Formulation R (mean ± sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmax (hr)</td>
<td>1.8056±1.0694</td>
<td>1.8611±0.6978</td>
<td>2.0417±1.10230</td>
</tr>
<tr>
<td>Cmax (µg/ml)</td>
<td>29.2892±7.6261</td>
<td>29.2439±8.0405</td>
<td>27.3398±7.2572</td>
</tr>
<tr>
<td>AUC_{0-t} (µg/ml.h)</td>
<td>98.8374±24.8099</td>
<td>96.7480±26.3669</td>
<td>97.2337±22.4100</td>
</tr>
<tr>
<td>AUC_{0-inf} (µg/ml.h)</td>
<td>106.5009±24.0419</td>
<td>102.0616±26.3184</td>
<td>102.7684±22.8182</td>
</tr>
<tr>
<td>t_{1/2} (hr)</td>
<td>4.2191±3.7993</td>
<td>2.6898±1.6015</td>
<td>3.0493±1.7636</td>
</tr>
</tbody>
</table>

The estimated value of $\text{AUC}_{0-t}$ being more than 80% of the estimated value of $\text{AUC}_{0-inf}$ implied that the LOQ of this method and sampling schedule was adequate.

The most important objective of this bioequivalence study was to assure the safety and efficacy of generic formulations. If two formulations of the same drug are equivalent in the rate and extent to which the active drug becomes available at the site of drug action, they are bioequivalent and thus considered therapeutically equivalent\textsuperscript{14,15}. It is generally accepted that the standard equivalence range for basic pharmacokinetic characteristics such as $\text{AUC}_{0-t}$, $\text{AUC}_{0-inf}$ and $\text{C}_{\text{max}}$, is 0.80-1.25\textsuperscript{19}. Pharmacokinetics parameter estimates were statistically analyzed following log transformation and the results of statistical analysis are shown in Table 3A and 3B.

Table 3A: Comparison A vs. R

<table>
<thead>
<tr>
<th></th>
<th>Ratio of Least Square Means (90% CI)</th>
<th>Power</th>
<th>Intrasubject CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AUC}_{0-t}$ (µg/ml.h)</td>
<td>101.13 (93.45-109.44)%</td>
<td>99.38</td>
<td>13.63</td>
</tr>
<tr>
<td>$\text{AUC}_{0-inf}$ (µg/ml.h)</td>
<td>103.52 (96.46-111.10)%</td>
<td>99.8</td>
<td>12.18</td>
</tr>
<tr>
<td>$\text{C}_{\text{max}}$ (µg/ml)</td>
<td>107.90 (96.42-120.74)%</td>
<td>90.12</td>
<td>19.50</td>
</tr>
</tbody>
</table>
Table 3B: Comparison B vs. R

<table>
<thead>
<tr>
<th></th>
<th>Ratio of Least Square Means (90% CI)</th>
<th>Power</th>
<th>Intrasubject CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{0-t} (µg/ml.h)</td>
<td>98.41 (90.94-106.49)%</td>
<td>99.38</td>
<td>13.63</td>
</tr>
<tr>
<td>AUC_{0-inf} (µg/ml.h)</td>
<td>98.54 (91.82-105.76)%</td>
<td>99.80</td>
<td>12.18</td>
</tr>
<tr>
<td>Cmax (µg/ml)</td>
<td>106.39 (95.07-119.05)%</td>
<td>90.12</td>
<td>19.50</td>
</tr>
</tbody>
</table>

The ratios of least square means and its 90% confidence intervals for ln-transformed C_{max}, AUC_{0-t} and AUC_{0-inf} for the comparison A vs. R and B vs. R were well within the regulatory acceptance range of 80-125% suggesting bioequivalence between Gemfibrozil 600 mg tablets (Formulation A & Formulation B) developed by the OHM Laboratories and Lopid 600 mg tablet of Parke Davis.

RESULTS AND DISCUSSION

Total 18 subjects were enrolled and all subjects completed all three periods of the study, pharmacokinetic and statistical analysis was performed on the same. The test and reference products were well tolerated by the study subjects as there were no serious adverse events.

Gemfibrozil was readily absorbed from the gastrointestinal tract and was measurable at the first sampling time (0.25 hr) in the majority of volunteers. Plasma concentrations profiles of Gemfibrozil attained after the administration of test and reference products were shown in Figure 4 and 5. Mean plasma concentration profiles of Gemfibrozil were comparable and closely similar between the two treatments. The pharmacokinetic parameters of Gemfibrozil for the test and reference treatments were
shown in Table 2. Peak concentrations of 202.527 ± 65.625 ng/ml and 200.383 ± 52.624 ng/ml for Gemfibrozil were attained at 2.238 ± 1.239 and 2.086 ± 0.967 hours after administration of test (A and B) and reference (R) products respectively. The estimated value of AUC$_{0-t}$ being more than 80% of the estimated value of AUC$_{0-inf}$ implied that the sampling scheme was adequate. In addition, the sample size of 18 subjects was close enough to give 100% power and to detect the difference of at least 20% in C$_{max}$, AUC$_{0-t}$ and AUC$_{0-inf}$ between the two treatments.

The most important objective of bioequivalence testing is to assure the safety and efficacy of generic formulations. When two formulations of the same drug are equivalent in the rate and extent to which the active drug becomes available at the site of drug action, they are bioequivalent and thus considered therapeutically equivalent\textsuperscript{14,15}. It is generally accepted that the standard equivalence range for basic pharmacokinetic characteristics such as AUC$_{0-t}$, AUC$_{0-inf}$ and C$_{max}$, is 0.80-1.25 \textsuperscript{15}. Pharmacokinetics parameter estimates were statistically analyzed following log transformation and the results of statistical analysis were shown in Table 3A and 3B. Geometric mean ratios and 90% confidence intervals for C$_{max}$, AUC$_{0-t}$ and AUC$_{0-inf}$ for Gemfibrozil were 107.90(96.42-120.74) \%, 101.13(93.45-109.44) \% and 103.52(96.46-111.10)\% respectively and the intra-subject CV (%) for C$_{max}$, AUC$_{0-t}$ and AUC$_{0-inf}$ for Gemfibrozil was 19.50, 12.18 and 13.63 respectively. Gemfibrozil 600 mg tablet developed by OHM Laboratories is therefore bioequivalent to Lopid 600 mg tablet of Parke Davis (A division of Pfizer Inc., USA).

Conclusion

Gemfibrozil was determined in plasma and other biological fluids by several methods such as gas chromatography – mass spectrometry (LOQ- 0.5µg/ml; RT-3.8mins)\textsuperscript{16}, high pressure liquid chromatography coupled to fluorimetric detection (LOQ 0.1µg/ml; RT 7min\textsuperscript{17} and LOQ 0.05 µg/ml; RT5.9min\textsuperscript{18}), spectrofluorometric detection (LOQ 0.02 µg /ml)\textsuperscript{19}, high-pressure liquid chromatography coupled to ultra-violet detection (LOQ 0.05 µg/ml; RT9min\textsuperscript{20}), high-pressure liquid chromatography coupled to ultra-violet detection (LOQ-0.5 µg/ml; RT10.4 min)\textsuperscript{21}. Our method had a suitable LOQ of 0.157 µg /ml along with the very short retention time of 1 minute was useful for analyzing more than 500 samples in a single day which is the most advantageous aspect in comparison to other analysis techniques available in published literature. Our method employs a very simple cost effective liquid liquid extraction procedure finally requiring 5µl injection volume resulting in high throughput bioanalysis technique\textsuperscript{22}. The current investigation demonstrates that the two tablet formulations of Gemfibrozil displayed similar rate and extent of bioavailability under fasting condition. The Test/Reference ratio (using Least Square Means) and its 90% Confidence interval for In-transformed C$_{max}$, AUC$_{0-t}$ and AUC$_{0-inf}$ were entirely within the bioequivalence acceptance range of 80%-125%. The plasma concentration profiles of Gemfibrozil from the test formulation and reference formulation were identical and the efficacy resulting from their pharmacokinetics profiles will also be considered identical. As a result Gemfibrozil 600 mg tablet developed by OHM Laboratories is bioequivalent to 600 mg tablets of Parke Davis, USA and may be used interchangeably in medical practice.

ACKNOWLEDGEMENT

The authors would like to thank Ranbaxy Laboratories Ltd. for giving permission to write this paper and to use in-house data.
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Efficient Trifluoroacetic acid Mediated Synthesis Of 4 -Nitroimidazoles

Ganguly S and Vishwanathan K.
Birla Institute of Technology, Mesra, Ranchi

ABSTRACT
Amides of 2-methyl-4 -nitroimidazole have been synthesized in high yields by treatment of 2-methyl-4 (5)-nitroimidazole with appropriate chlorpropionamides in presence of trifluoroacetic acid. All the compounds were characterized by spectral and elemental analyses.

INTRODUCTION
The nitroimidazole moiety is found in various bioactive compounds having anti protozoal, anti bacterial and radiosensitizing activities1-3. Recently, 1-(2-{(diarylmethoxy)-ethyl}]-2-methyl-5-nitroimidazoles (DAMNIs) has been discovered as a novel class of NNRTIs4,5. The conditions for selective alkylation of 4(5) nitroimidazoles is already well established6. Alkylation under alkaline conditions gives primarily the 4-nitro isomer, whereas in strongly acidic solutions the 5-nitro isomer is predominantly formed. In continuation of our work on some biologically active derivatives of imidazoles7-9, we hereby report the synthesis of a number of new amides of 2- methyl -4-nitroimidazole.

MATERIALS AND METHODS
General procedure for preparation of propionamides of 2-methyl-4-nitroimidazole 3a-f:
To a solution of 2-methyl-4(5)-nitroimidazole (0.0169 mole) in dimethyl formamide (3ml), trifluoroacetic acid (0.0846 mole) was added dropwise and stirred well. To this mixture a chlorpropionamide (0.020 mole) was added slowly. The reaction mixture was refluxed for 8-10 hr at 80-85°C. The solvent was evaporated under reduced pressure and the residue was extracted with chloroform. The precipitated 2-methyl-4(5)-nitroimidazole if any was filtered off. The filtrate was washed with aqueous ammonia (2x5ml), ice cold water (2x10ml), brine solution (2x10ml) and dried over anhydrous sodium sulphate. Further processing of the chloroform extract yielded a residue, which was crystallized from mixture of dichloromethane and hexane.

RESULTS AND DISCUSSION
Treatment of corresponding chlorpropionamides 2 with 2-methyl-4(5)-nitroimidazole 1 in presence of trifluoroacetic acid yielded the 4-nitro isomers 3 (Scheme 1). The products were formed in high yields (70-92%) (Table 1, entry 3a-f). The structures of the products were determined from their spectral (1H NMR, IR and MS) data.

In conclusion, trifluoroacetic acid has been employed here for the first time as an effective reagent for the preparation of 1-substituted 2-methyl 4-nitroimidazoles in high yields.

The spectral (1H NMR, IR and MS) data of the synthesized nitroimidazoles are given below.

Compound 3a
1H NMR (CDCl3 + DMSO-d6): δ 10.08 (1H, s), 7.04-7.52 (5H, t), 4.27-4.30 (2H, t), 2.86-2.89 (2H, t),
2.40 (3H, s); IR (KBr): ν 3345, 672, 1540, 1519, 815 cm⁻¹; MS: m/z 275 [M+H]+; Anal. Calcd for C₁₄H₁₅N₃O₃: C, 61.53; H, 5.53; N, 15.38; Found: C, 6.33; H, 5.22; N, 15.26.

**Compound 3b**

¹H NMR (CDCl₃ + DMSO-d₆): d 9.97 (1H, s), 8.24 (1H, s), 7.07-7.38 (4H, s), 4.24-4.27 (2H, t), 2.81-2.84 (2H, t), 2.39 (3H, s), 2.23 (3H, s); IR (KBr): ν 3326, 1685, 1537, 1528, 815 cm⁻¹; MS: m/z 289 [M+H]+; Anal. Calcd for C₁₅H₁₇N₃O₃: C, 62.71; H, 5.96; N, 14.63. Found: C, 62.62; H, 5.49; N, 14.56.

**Compound 3c**

¹H NMR (CDCl₃ + DMSO-d₆): d 9.95 (1H, s) 8.29 (1H, s), 7.14-7.43 (4H, d), 4.25-4.28 (2H, t), 2.39 (3H, s), 1.15-1.16 (6H, s); IR (KBr): ν 3252, 1692, 1532, 1541, 830 cm⁻¹; MS: m/z 317 [M+H]+; Anal. Calcd for C₁₇H₂₁N₃O₃: C, 64.74; H, 6.71; N, 13.32; Found: C, 64.62; H, 6.49; N, 13.56.

**Compound 3d**

¹H NMR (CDCl₃ + DMSO-d₆): d 10.03 (1H, s), 8.29 (1H, s), 7.20-7.50 (4H, s) 4.25-4.28 (2H, t), 2.84-2.87 (2H, t), 2.40 (3H, s), 2.36 (3H, s); IR (KBr): ν 3265, 1682, 1542, 1510, 827 cm⁻¹; MS: m/z 321 [M+H]+; Anal. Calcd for C₁₅H₁₇BrN₃O₃: C, 47.74; H, 4.01; N, 11.93; Found: C, 47.43; H, 4.17; N, 11.56.

Acknowledgements

The authors thank Dr. Uma Ramachandran, Vice President-New Drug Discovery, Orchid Research Laboratories Ltd., Chennai, India for providing all facilities required to carry out this work. We thanks UGC, New Delhi for a fellowship also.

REFERENCES


Table 1: Synthesis of 2-methyl 4-nitroimidazoles using trifluoroacetic acid

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Chlorpropionamide</th>
<th>Product</th>
<th>Time (h)</th>
<th>Isolated Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td><img src="image1" alt="Structure" /></td>
<td><img src="image2" alt="Structure" /></td>
<td>8</td>
<td>96</td>
</tr>
<tr>
<td>3b</td>
<td><img src="image3" alt="Structure" /></td>
<td><img src="image4" alt="Structure" /></td>
<td>8</td>
<td>93</td>
</tr>
<tr>
<td>3c</td>
<td><img src="image5" alt="Structure" /></td>
<td><img src="image6" alt="Structure" /></td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>Compound No.</td>
<td>Chloropropionamide</td>
<td>Product</td>
<td>Time (h)</td>
<td>Isolated Yield (%)</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------</td>
<td>---------</td>
<td>----------</td>
<td>-------------------</td>
</tr>
<tr>
<td>3d</td>
<td>Cl (\text{O} \ \text{NH} \text{C-} \text{SCH}_3)</td>
<td>O(_2\text{N}) (\text{N} \ \text{CH}_3 \ \text{O} \ \text{NH} \text{C-} \text{SCH}_3)</td>
<td>8</td>
<td>90</td>
</tr>
<tr>
<td>3e</td>
<td>Cl (\text{O} \ \text{NH} \text{C-} \text{Br})</td>
<td>O(_2\text{N}) (\text{N} \ \text{CH}_3 \ \text{O} \ \text{NH} \text{C-} \text{Br})</td>
<td>8</td>
<td>92</td>
</tr>
<tr>
<td>3f</td>
<td>Cl (\text{N} \ \text{O} \ \text{N} \ \text{C-} \text{Br})</td>
<td>O(_2\text{N}) (\text{N} \ \text{CH}_3 \ \text{O} \ \text{N} \ \text{N} \ \text{C-} \text{Br})</td>
<td>8</td>
<td>94</td>
</tr>
</tbody>
</table>

\[
\begin{align*}
&1 \quad \text{Cl} \quad \text{O} \ \text{N} \ \text{R} \ \text{R'} \quad + \quad \\
&2 \quad \text{O}_2\text{N} \ \text{N} \ \text{C-} \text{H} \ \text{CH}_3 \quad \xrightarrow{\text{CF}_3\text{COOH}} \\
&3 \quad \text{O}_2\text{N} \ \text{N} \ \text{C-} \text{R} \ \text{R'} \quad 80-85^\circ \text{C}, \ 8-10\text{h} \quad 90-94\%
\end{align*}
\]

Scheme 1
Instructions to Authors

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