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Content

EDITORIAL MESSAGE

Pharmaceutical Sciences have witnessed an enormous growth in diverse fields. It is the need of the hour for pharmacists to keep abreast of the current trends, research and practices with respect to the diversities. Nirma University Journal of Pharmaceutical Sciences (NUJPS) aims to publish research in basic as well as applied pharmaceutical science topic, case studies related to pharmaceutical management, regulatory affairs, clinical reports and review articles. The major emphasis will be given to publish high quality research papers that follow blind review process. Review process will be transparent and will acknowledge all the beneficiaries involved.

We are glad to launch the first issue of NUJPS during NIPiCON 2014 (2nd International Conference). In this issue we have tried to bring forth a blend from different areas of Pharmaceutical Sciences to keep the readers updated about the ongoing research in academia at national and international level as well as opinions & trends from pharmaceutical industries. We are thankful to all the members of national and international advisory board for accepting our invitation and supporting in this new initiative of Nirma University.

In this issue we have received research articles from prestigious US universities titled: 'Strategies to Improve Plasma Circulation of Nanoparticals' by Ameya R. Kirtane, University of Minnesota, Minneapolis and 'Blood-Brain Barrier Permeation of Paclitaxel' by Dr. Vijaykumar Sutariya, University of South Florida. We have also received articles from reputed Indian Pharmaceutical Industries titled: 'Microdosing: A Phase 0 Clinical Trial' by Dr. Jignesh. Patel from Sun Pharmaceutical Industries Ltd, Vadodara; 'Compulsory License in Light of Indian Patent Perspective' by Mr. Sanjaykumar Patel from M/s. Alembic Pharmaceuticals Ltd., Vadodara; 'Identification of a Biomarker: a Necessary Approach in Herbal Industry' by Mr. R. Rajendran, CEO, Green Chem, Bangalore. We have also received articles from prestigious Indian Universities titled: 'Holistic Application of Quality by Design (QbD) for Pharma Product Development Excellence and Regulatory Compliance' by Prof. Bhupindersingh Bhoop, Department of Pharmaceutics, Punjab University, Chandigarh; 'Chemometric Assisted Spectrophotometric and HPLC method for the Estimation of Amlodipine Besylate and Telmisartan Marketed Formulations' by Prof. Sadhna Rajput, Dept of Pharmacy, The Maharaja Sayajirao University of Baroda and 'A Review on Synthesis and Pharmacological Diversity of Isoxazoles & Pyrazolines' by Dr. Kuntal Manna, Department of Pharmacy, Tripura University (A Central University).

We are adorably thankful to all invited authors for timely support and valuable scientific contribution in this first issue of NUJPS and expecting similar contributions in future issues.

We are thankful to all Director, Heads of Department and Faculty Members, Institute of Pharmacy, Nirma University for support and help rendered to shape this new endeavors.

We are specially thankful to Dr. Anup Singh, Director General, Nirma University for sharing his views and experience, which greatly helped us to reach to present form of NUJPS.

We would also like to express deep sense of gratitude to Management Authorities of Nirma University for their tenacity and enthusiasm in bringing this journal.

Happy Reading!!

Editorial Team - NUJPS
editor.nujps@nirmauni.ac.in

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INVITED ARTICLE

STRATEGIES TO IMPROVE PLASMA CIRCULATION OF NANOPARTICLES

Ameya R. Kirtane*
Department of Pharmaceutics, University of Minnesota, Minneapolis, MN, USA 55455

Abstract

There is a tremendous interest in developing long-circulating nanocarriers as treatment and imaging modalities. Enhanced plasma circulation of nanoparticles can improve on-target accumulation and help maintain sustained therapeutic drug levels in the plasma. Nanoparticles, however, are rapidly eliminated from systemic circulation by circulating and stationary macrophages. There have been several approaches devised to overcome this rapid elimination of nanoparticles. Reducing particle size has been shown to reduce opsonization and phagocytic uptake. Particle shape has also been shown to strongly affect the biological interactions of nanoparticles. Coating the surface of nanoparticles with hydrophilic polymers has been one of the most widely used strategies to protect against opsonization. Recent development of facile synthetic approaches has allowed researchers to engineer formulations that mimic blood cells. These strategies have also proven to be successful in producing long-circulating nanoparticles. This review provides a brief description of several approaches that have been used successfully to enhance the plasma circulation of nanoparticles.

Keywords: *RES, opsonization, long circulating, nanoparticles, PEG, biomimetic, particle size, particle shape*

Abbreviations: Mononuclear phagocytic system, MPS; reticuloendothelial system, RES; poly-(ethylene glycol), PEG; red blood cells, RBCs; monosialylganglioside, GM1; poly-(vinyl pyrolidone), PVP; poly-(acrylamide), PAA; poly-(acryloylmorpholine), PAcM; poly-(2-methyl-2-oxazoline), PMOZ; poly-(2-ethyl-2-oxazoline), PEOZ; poly-(lactide-co-glycolide), PLGA; accelerated blood clearance, ABC

***Corresponding Author:** Email: kirta001@umn.edu, Phone: +1-612-624-0443*

Introduction

Nano-sized carriers are rapidly emerging as a useful platform for drug delivery [1, 2]. While nanoparticles offer several advantages over other drug delivery systems, these advantages can be realized only if the carriers are maintained in circulation for prolonged time intervals.

For example, nanoparticles are extensively used to achieve targeted drug delivery [3, 4]. However, when the target has a limited blood supply or is at a very low abundance, nanoparticles need to circulate through the target several times in order to accumulate at high enough concentrations [5, 6]. Nanoparticles are also used as controlled-drug delivery vehicles to sustain high drug levels in the plasma. This too, can be achieved only if the nanoparticles remain in circulation for prolonged periods of time [7]. Hence, there is a considerable interest in improving the plasma residence time of nanoparticles. Once administered, nanoparticles are rapidly eliminated from blood circulation via a two-step process [8]. In the first step serum proteins, of the complement system, are adsorbed on the surface of nanoparticles. This process is known as opsonization and is vital for the elimination of nanoparticles [8-10]. Following opsonization, nanoparticles are engulfed by circulating macrophages or macrophages residing in the liver and spleen (referred to as mononuclear phagocytic system (MPS) or the reticuloendothelial system (RES)) [8, 9, 11]. Hence, in order to prolong the circulation time of nanoparticles, it is essential to subvert either of the two processes.

There have been several attempts to increase the blood circulation of nanoparticles [12, 13]. Initial attempts were directed towards altering the surface of particles [14]. This led to the use of non-ionic hydrophilic polymers [15]. This has been the most widely used strategy till date. Some researchers have also shown that changing the physicochemical properties of the core of the nanoparticles, like size and shape, can also increase their circulation half-life [16]. Recent advances in synthetic techniques have inspired researchers to develop nanoparticles that mimic blood cells [17]. These approaches have also helped prolong the plasma residence of nanoparticles.

In this short review, I have outlined a various strategies that have been used successfully to produce long circulating nanomedicine. I focus, primarily, on the rationale underlying the use of each technique, their advantages and potential shortcomings.

Modifying nanoparticle morphology to enhance circulation time

Particle size

Particle size is an important determinant of the plasma circulation time of nanoparticles [18]. Hence, altering the particle size can be an effective technique to prolong plasma circulation. However, unlike other strategies discussed in this review, the choice of particle size is

peculiar [19]. Nanoparticles are eliminated from plasma circulation via macrophage uptake, distribution to peripheral organs, or urinary excretion. Alterations in particle size can affect each of these processes [19]. Hence, careful consideration of particle size is required. The particle size at which all the three processes are minimized will result in the highest circulation time for nanoparticles. For spherical nanoparticles, macrophage uptake increases with an increase in hydrodynamic diameter. Fang *et al.* compared protein adsorption on 80, 170 and 240 nm nanoparticles incubated in mouse serum [20]. They found that 240 nm nanoparticles showed a 6-fold higher protein adsorption as compared to 80 nm particles. Consequently, larger nanoparticles had a higher macrophage uptake *in vitro*. Finally, 80 nm nanoparticles also showed a longer elimination half-life as compared to 170 and 240 nm nanoparticles [20]. Papović *et al.* compared the circulation half-life of 12, 60 and 125 nm nanoparticles [21]. They also found that the smallest particles had the longest elimination half-life. These results indicate that smaller particles are more efficient at evading the RES [21].

While smaller nanoparticles show lower RES uptake, decreasing the particle size below a certain threshold may result in elimination of particles through other mechanisms [19]. For example, Liu *et al.* showed that decreasing particle size below 50 nm increases their accumulation in the liver [22]. As hepatic capillaries are fenestrated (pore size ~75 nm), they are permissive to the entry of extremely small nanoparticles. Nanoparticles below 5.5 nm can be filtered through renal filtration [23]. Choi and colleagues showed that the circulation half-life of 8 nm quantum dots was longer than that of 4 nm quantum dots. This increased plasma residence for larger particles was because of the lower renal clearance of the larger nanoparticles [23]. Figure 1 shows the half-life of nanoparticles of various sizes [23, 24]. For extremely small particles (<20 nm), the half-life increases with an increase in particle size. The half-life reaches a peak at an intermediate particle size and then declines with a further increase in particle size. However, since RES uptake is often the major mechanism of particle elimination and most studies utilize particles in the size range of ~100 nm, smaller nanoparticles are generally considered favourable [24].

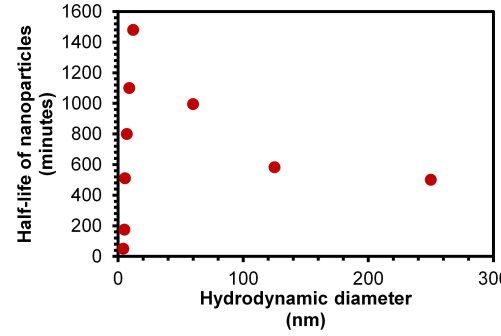

Figure 1: Half-life of various sized nanoparticles

The figure shows plasma half-life of nanoparticles of different sizes. For extremely small nanoparticles, renal clearance decreases with an increase in particle size. This leads to enhanced circulation times. For particles larger than 20 nm, RES uptake increases with an increase in particle size. Thus half-life decreases with an increase in particle size. Data was reported in [23, 24]. Adapted with permission from [23, 24].

Particle shape

There is very limited information available about the performance of non-spherical nanoparticles. This, in part, was due to lack of techniques for the synthesis of non-spherical nanoparticles. However, in recent years, synthesis and characterization of non-spherical nanoparticles has gained tremendous interest and has become a subject of extensive research [16, 25-27]. Geng and co-workers were the first to show that non-spherical nanoparticles have superior pharmacokinetics and anti-tumour efficacy as compared to spherical nanoparticles [28]. This thesis studied the plasma circulation of filamentous micelles composed of block co-polymers of poly-(ethylene glycol) (PEG) and poly-(ethylethylene) and that of poly-(ethylene glycol) and poly-(caprolactone). In a mouse model, filamentous micelles had a significantly longer half-life (~144 hours) than spherical polymerosomes (half-life ~24 hours). The effect of particle size of filomicelles was also studied. Interestingly, filomicelles having an initial length of 8 μm (equivalent to the diameter of red blood cells (RBCs)) had the longest circulation half-life. Filomicelles with a shorter or longer lengths were eliminated faster [28]. While the study by Geng *et al.* provided the first insight into the biological performance of non-spherical nanoparticles, it analysed only one particle shape. A mechanistic understanding of this interaction was first provided by Champion *et al.* [29]. This study focused on the macrophage uptake of polystyrene particles having a variety of shapes such as spherical, oblate ellipsoids, prolate ellipsoids, elliptical discs and rectangular discs. The authors found that the initiation and completion of phagocytosis were strongly affected by the morphology of the nanoparticles. The orientation of nanoparticles relative to the macrophages dictated the initiation of the process. The volume of nanoparticles (a function of particle size) dictated the completion of this process. For example, if the alignment of elliptical discs was perpendicular to the approaching surface of the macrophage, the process of phagocytosis did not initiate at all. However, if the elliptical disc was aligned parallel to the macrophage surface, phagocytosis initiated spontaneously. The completion of this process, however, depended on the volume of the particle relative to the macrophage [29]. Hence, particle shapes that minimize initiation of phagocytosis will have the longest circulation half-life.

Following these studies, there have been a number of experiments which have shown that certain non-spherical nanoparticles have superior pharmacokinetics, efficacy and targeting abilities as compared to spherical nanoparticles [30-33]. However, spherical nanoparticles are still preferred and more popular owing to their ease of synthesis.

Surface functionalization of nanoparticles with hydrophilic polymers

Opsonization and subsequent macrophage uptake of nanoparticles are known to be triggered by the hydrophobicity and charge of the surface of nanoparticles [9, 34]. Hence, initial attempts to decrease MPS mediated nanoparticle uptake were

directed towards masking the surface hydrophobicity and charge of nanoparticles. This has been achieved by using non-ionic hydrophilic polymers, physically adsorbed or chemically grafted, on the surface of nanoparticles.

The use of amphiphilic polymers was first suggested by Illum *et al.* [14]. In this study, polystyrene microparticles were surface functionalized with poloxamer 338, a tri-block co-polymer consisting of a central hydrophobic chain of poly-(propylene oxide), flanked by two hydrophilic chains of poly-(ethylene oxide). The authors showed that poloxamer decreased the rapid initial uptake of microparticles into the liver. This resulted in higher plasma concentrations at early time points. Although the hepatic uptake of particles was decreased considerably, there was no significant increase in terminal half-life of the surface coated particles [14].

In another study, Allen *et al.* hypothesized that increasing surface hydrophilicity and mimicking the cell surface of blood cells may enhance the circulation time of nanoparticles [35]. Earlier studies investigating the long circulation times of RBCs had highlighted the role of surface sialic acid moieties [36]. To replicate this, the liposome surface was modified with sialic acid by incorporating monosialylganglioside (GM1) into the liposome bilayer. The authors found that incorporation of GM1 in the lipid bilayer significantly decreased their hepatic uptake. As the molar ratio of GM1 in the lipid bilayer increased, RES uptake of liposomes decreased. However, increasing the amount of GM1 beyond a certain concentration compromised the stability of the lipid membrane [35]. This limitation could be overcome by using lipids, like sphingomyelin, which showed greater intermolecular interactions [37]. Such lipids increased the rigidity of the liposome membrane and allowed higher incorporation of GM1 [35]. Gabizon *et al.* later compared the tumour accumulation of non-functionalized liposomes and GM-1 functionalized liposomes in a mouse model of lymphoma [38]. GM-1 functionalized liposomes showed 25-fold increase in tumour levels as compared to the non-functionalized liposomes [38]. As GM-1 functionalized liposomes partially evaded the RES system, these liposomes were referred to as “stealth” liposomes.

Poly-(ethylene glycol)

Though GM-1 provided excellent stealth properties to nanocarriers, it was required in large quantities. Additionally, reproducible synthesis of this polymer was also challenging [39]. Hence, there was tremendous interest in developing alternate polymers to substitute GM1. Klibanov *et al.* used poly-(ethylene glycol) (PEG) to increase the circulation of liposomes [40]. This strategy had been successfully implemented to prolong the circulation half-life of proteins [41]. In agreement with those reports, the authors found that PEG functionalized liposomes (half-life: 5 hours) had a significantly higher plasma residence time than non-functionalized liposomes (half-life: 0.5 hours). GM-1 functionalized liposomes had an

intermediate half-life of 1.5 hours [40]. Papahadjopoulos *et al.* later confirmed these results in a mouse model of colon carcinoma [42]. PEG liposomes had an extended plasma half-life (~5 fold higher than the non-functionalized liposomes) and showed lower accumulation in the major RES organs, liver and spleen. Consequently, there was higher tumour accumulation of PEG-liposomes as compared to the plain liposomes [42].

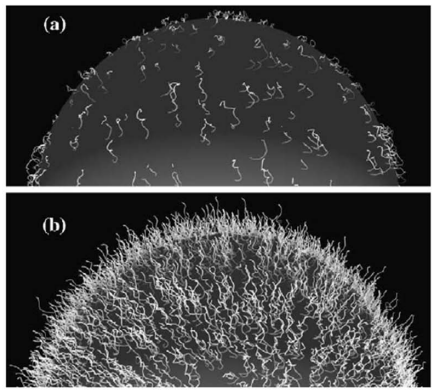

Figure 2: Schematic diagrams of PEG configurations on the upper hemisphere of a polymeric nanoparticle.

In (a), the low surface coverage of PEG chains leads to the "mushroom" configuration where most of the chains are located closer to the particles surface. In (b), the high surface coverage and lack of mobility of the PEG chains leads to the "brush" configuration where most of the chains are extended away from the surface. Reproduced with permission from [9]

PEG provides steric hindrance to the adsorption of opsonins and hence decreases the RES uptake of nanoparticles [9, 43, 44]. Surface density of PEG is an important variable that can affect the efficiency of the polymer [45]. Best steric hindrance is achieved at an intermediate density [46]. At a low surface density, PEG chains exist in a mushroom configuration, while at a high surface density they exist in a brush configuration (Figure 2). As opsonins approach the nanoparticle surface, they cause "brush"-shaped PEG chains to compress. The compressed-high energy conformation of PEG chains is not favourable and as it returns to the preferred elongated conformation, opsonins are pushed away from the nanoparticle surface. Hence, it is important that the surface density of PEG is high enough that they exist in a brush conformation. However, if the surface density is too high, PEG chains lose their flexibility and their protective ability. At low surface coverage, opsonins can get adsorbed in the space between the PEG chains [9, 45, 47].

Many studies have dealt with the effect of molecular weight on the efficacy of PEG [45, 48-50]. Higher molecular weight PEG provides better protection against opsonization [44]. Longer PEG chains can provide higher repulsive forces against the adsorption of opsonins [44]. However, functionalizing nanoparticles with extremely large PEG moieties decreases their interaction with the target cells as well [43]. Additionally, larger hydrophilic polymers are thermodynamically more stable when they are free in solution [51]. Thus, preparing stable nanoparticle formulations with larger PEGs is challenging. Hence, the use of mid-sized PEGs (molecular weight: 1000- 5000 Da) is most common [52, 53]. The method of PEGylation (surface adsorption vs. chemical grafting) and the type of PEG (branched vs. linear vs. star shaped) have

also been found to affect the efficiency of PEG. [54-58]. However, these parameters have not been discussed in this review.

PEG is the preferred polymer for making long circulating nanocarriers [6]. There are several reasons for the popularity of PEG. First, PEG is non-biodegradable. Hence, it does not produce any toxic metabolites and is easily cleared from blood circulation via renal filtration. Additionally, synthesis of PEG polymers with a low polydispersity index is relatively simple. This allows a precise control of the characteristics of the final product. Moreover, PEG chains are flexible. Hence a very small mole% of PEG is required to cover the entire nanoparticle surface and provide stealth properties to the formulation [6, 47]. Finally, the two terminal groups of PEG can be tailored to specification. It is common to conjugate one end group with a hydrophobic polymer (to allow docking on the lipophilic surface of the nanoparticle) and the other end group to targeting moieties [3, 52, 53].

Other polymers

Due to the success of PEG, there was considerable interest in investigating other hydrophilic polymers for stealth properties. Vinyl polymers like poly-(vinyl pyrolidone) (PVP), poly-(acrylamide) (PAA) and poly-(acryloylmorpholine) (PAcM) were one of the first alternate polymers studied [51, 59]. PVP, PAA and PAcM were conjugated to a hydrophobic phospholipid or acyl chain to produce amphiphillic derivatives. Molecular weights of the acyl linker and PVP or PAA were optimized to provide highest encapsulation efficiency and steric protection. However, it was found that these polymers performed similar to PEG and did not provide any additional benefit [51, 59].

Amphiphillic derivatives of poly-(2-methyl-2-oxazoline) (PMOZ) and poly-(2-ethyl-2-oxazoline) (PEOZ) have also been investigated [60]. It was found that both, PEOZ and PMOZ, delayed RES clearance of liposomes in a rat model. The half-life of PEOZ and PMOZ functionalized liposomes was similar to that of PEG functionalized liposomes [60]. Maruyama and colleagues showed that polyglycerols could also be used to delay RES uptake of liposomes [61]. Amphiphillic derivatives of polyglycerol were synthesized by conjugating polyglycerol with dipalmitoylphosphotidic acid. The presence of polyglycerol moieties on the surface of liposomes decreased their uptake in major RES organs like the liver, kidney and spleen. The protective ability of polyglycerol increased with the degree of polymerization of the polymer. However, this study did not compare the polyglycerol derivatives to PEG [61].

Some studies have also shown that polysaccharides can be used effectively to provide stealth properties to nanoparticles [62]. In particular, dextran coating has been widely used to protect iron oxide nanoparticles against RES uptake [63]. Polysaccharides provide a unique advantage over other polymer coatings. Polysaccharides can be used as ‘active targeting’ ligands, as many cells express

receptors for these molecules [62]. While PEG and other synthetic polymers are widely accepted [64-66], there are certain disadvantages associated with them. Some reports have shown the production of anti-PEG IgM antibodies after the first dose of PEGylated nanoparticles [67, 68]. This significantly enhances the blood clearance of the second dose of nanoparticles. In fact, the second dose of PEG nanoparticles are eliminated almost as rapidly as non-functionalized nanoparticles. This phenomenon, termed as accelerated blood clearance (ABC) [67], can have significant ramifications on the use of synthetic polymers. It should be noted, however, that ABC is observed only for a limited time after the first dose of PEGylated nanoparticles. Ishida *et al.* found that if the second dose of PEG nanoparticles is administered 3-7 days after the first, there is 3-5-fold increase in the liver accumulation of second-dose-nanoparticles as compared to the first dose. However, if the second dose is administered two weeks after the first dose, there is no difference in the liver accumulation of the nanoparticles [69]. Thus allowing sufficient time between consecutive doses of PEGylated nanoparticles can help overcome the problem of ABC.

Biomimetic approaches to enhance plasma circulation of nanoparticles

RES mediated uptake of nanoparticles can be significantly reduced by using hydrophilic polymers. But a major fraction of the dose is still found in the liver and spleen. Additionally, immune reaction upon repeated dosing limits the utility of this approach. Hence, alternate approaches for engineering stealth nanoparticles are of tremendous interest. Most nanoparticulate systems show a circulation half-life of ~10-12 hours. In contrast, blood cells (like erythrocytes) remain in circulation for weeks. Thus, several studies are aimed at generating nano-drug delivery systems that mimic blood cells.

Modifying the surface of nanoparticles to mimic blood cells

The mechanism of RES evasion by RBCs is a subject of extensive research. Though RBCs undergo opsonization they are not engulfed by macrophages. RBCs employ various self-markers to evade RES uptake. These include complement receptor 1, decay accelerating factor, C8 binding protein, CD59 and CD47 [70]. CD47 is expressed on the plasma membrane of erythrocytes [71] and often upregulated on circulating tumour cells and stem cells [72, 73]. CD47 interacts with signal receptor protein α (SIRPα), an inhibitory receptor found on the surface of macrophages. Activation of SIRPα leads to an intracellular cascade resulting in the inactivation of myosin. This retards macrophage mediated phagocytosis [73]. Hence, activating SIRPα on the macrophage surface can reduce the phagocytosis of nanoparticles.

Tsai *et al.* were the first to explore this approach [74]. In this report, streptavidin coated polystyrene microparticles were conjugated to biotinylated CD47. *In vitro* studies showed that, in spite of similar levels of opsonization, CD47 coated

microparticles had a significantly lower macrophage uptake than non-functionalized microparticles. Additionally, macrophage uptake decreased with an increase in the surface density of CD47, reaching a plateau at ~200 molecules/μm^2 [74]. Rodriguez *et al.* tested whether presence of CD47 on the surface of 160-nm polystyrene nanoparticles increases their circulation time *in vivo* [75]. The authors found that CD47 functionalized nanoparticles had a longer plasma circulation as compared to the non-functionalized nanoparticles. It is interesting to note that CD47, unlike PEG, has no effect on the opsonization process. CD47 is involved in decreasing phagocytosis via macrophages. Thus, in principle, presence of PEG and CD47 can offer a dual advantage. Consistent with this hypothesis, the authors found that PEGylated polystyrene nanoparticles functionalized with CD47 had a longer plasma residence time and showed lower accumulation in the spleen as compared to PEGylated polystyrene nanoparticles [75].

The presence of CD47 on the surface of nanoparticles improves their plasma circulation. However, the circulation time of these nanoparticles is still not comparable to blood cells. It is possible that the activity of CD47, alone, cannot provide nanoparticles with stealth properties like blood cells. Surface density of CD47 and the nature of interaction of CD47 with SIRPα may also be different for these artificial vessels. In other words, it is difficult to recreate the complex biochemistry of blood cells on the surface of nanoparticles using a single ligand. To overcome this issue, some recent studies have proposed coating nanoparticles with the entire plasma membrane of blood cells.

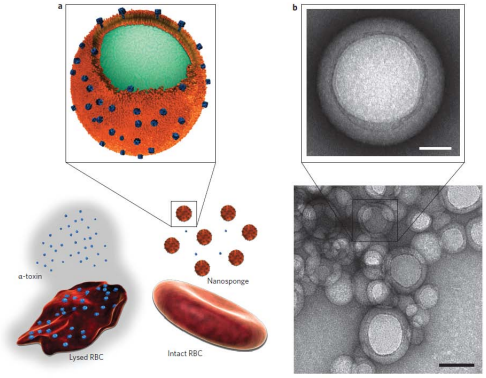

Figure 3: Schematic and actual structures.

a, Schematic structure of toxin nanosponges and their mechanism of neutralizing toxins. The nanosponges consist of substrate-supported RBC bilayer membranes into which toxins can incorporate. After being absorbed and arrested by the nanosponges, the toxins are diverted away from their cellular targets, thereby avoiding target cells and preventing toxin-mediated haemolysis. b, TEM visualization of nanosponges mixed with a-toxin (scale bar, 80 nm) and the zoomed-in view of a single toxin-absorbed nanosponge (scale bar, 20 nm). The sample was negatively stained with uranyl acetate before TEM imaging.
Reproduced with permission from [77]

Hu *et al.* coated poly-(lactide-*co*-glycolide) (PLGA) nanoparticles with the plasma membrane of RBCs [76]. The authors confirmed that major proteins in the RBC membrane were retained even after the coating process. In mouse studies, erythrocyte membrane coated nanoparticles showed an elimination half-life of ~40 hours while PEG nanoparticles showed a half-life of ~15 hours. RBC-coated nanoparticles were later used as decoys to adsorb and eliminate blood

borne-toxins that can cause haemolysis (Figure 3) [77]. In this study, the surface of nanoparticles attracted the blood-borne toxin, while the core of the nanoparticle acted as a 'sponge' to absorb the toxic chemical. This membrane-coating platform has been applied successfully to other nanoparticle systems as well [70, 78].

Parodi *et al.* coated nanoporous silicon microparticles with leukocyte cell membrane [79]. The camouflaged microparticles, termed as leuko-like vectors, showed significantly reduced opsonization and macrophage uptake *in vitro* as compared to plain microparticles. Membrane coating also reduced the *in vivo* hepatic uptake of the microparticles. However, this advantage was transient. While the initial hepatic uptake of coated microparticles was lower, both coated and uncoated microparticles showed similar levels of hepatic uptake 40 minutes post injection. This indicates that the particles lose their coating rapidly and accumulate in the liver. In spite of this, coated microparticles showed a higher tumour accumulation as compared to the uncoated particles [79].

Blood cell mimicking nanoparticles show a longer half-life than PEGylated nanoparticles. However, most PEGylated nanoparticles are produced using single-step processes [53, 66]. Thus PEGylation is a simpler and more reproducible technique than the techniques mentioned above. Additionally, availability of erythrocytes and leucocytes may also significantly limit the translation of these approaches.

Altering the core of nanoparticles to mimic the flexibility of blood cells

Prolonged circulation of RBCs is also attributed to the structure and flexibility of these cells. Erythrocytes are too large to pass through constricted capillaries of the lungs. However, RBCs can pass through capillaries that have a diameter as small as 5μm [80]. Similar to RBCs, circulating tumour cells are also known to be more flexible as compared to normal cells. This flexibility allows circulating tumour cells to extravasate into, intravasate out of circulation and avoid getting trapped in constricted capillaries [81]. Thus, engineering the flexibility of long circulating cells into nanoparticles may allow longer plasma circulation.

Doshi and colleagues showed that the shape and flexibility of RBCs can be built into PLGA microparticles [82]. Spherical PLGA particles were converted to discoid biconcave structures using partial fluidization. Using layer-by-layer coating, bovine serum albumin and haemoglobin were added to the surface of the discoid microparticles. The particles were again exposed to a solvent to fluidize the core even further. The resultant particles were extremely soft and had a remarkably reduced elastic modulus. The pharmacokinetics of these particles was not studied in this report [82]. Merkel *et al.* showed that the plasma residence of 2-hydroxyethyl acrylate microparticles can be changed by altering their bulk modulus [83]. Microparticles were synthesized by cross linking 2-hydroxyethyl acrylate with poly-(ethylene glycol) diacrylate. Bulk

modulus of the microparticles was controlled by modifying the amount of cross-linker in the formulation. The authors found that by changing the modulus from 63 kPa to 8 kPa, the elimination half-life of the microparticles increased from 3 hours to 93 hours. This dramatic increase in half-life was attributed to the deformability of the microparticles and their ability to escape constricted microvasculature. It is interesting to note that though this study utilized microparticles, the half-life achieved was greater than some nanoparticulate systems [83]. Haghgooie *et al.* also showed that similar flexible microparticles can be produced in a variety of shapes and carrying different cargoes [84].

Conclusion

The plasma circulation time of nanoparticle is a key feature that dictates the final performance of these carriers. Rapid macrophage uptake of nanoparticles severely reduces their circulation time. There have been several attempts to reduce RES uptake of nanoparticles. These strategies include modifying particle size, shape, surface characteristics etc. More recently, several studies have attempted to produce nanoparticles that better mimic naturally occurring colloids like RBCs. In spite of considerable improvement in the plasma circulation of nanoparticles, most systems still show a high accumulation in the RES organs like the liver and spleen. Hence, alternate strategies are still of considerable interest in the field of medical nanotechnology.

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INVITED ARTICLE

HOLISTIC APPLICATION OF QUALITY BY DESIGN (QbD) FOR PHARMA PRODUCT DEVELOPMENT EXCELLENCE AND REGULATORY COMPLIANCE

Bhupinder Singh[1,2*], Sarwar Beg[1], Gajanand Sharma[1], Atul Jain[2] and Poonam Negi[1]

[1]University Institute of Pharmaceutical Sciences, UGC Centre of Advanced Studies, Panjab University, Chandigarh-160014, India

[2]UGC-Centre of Excellence in Nano Applications (Biomedical Sciences), Panjab University, Chandigarh-160014, India

Abstract

The realm of optimizing the drug formulations has gained significant momentum towards more systematic approach of "Quality by Design (QbD)" based strategies employing "Design of Experiments (DoE)" from the erstwhile traditional short-gun approach of changing "One Factor at a Time (OFAT)". These traditional approaches are generally associated with multiple intricacies including utilization of greater magnitude of time, money and energy, inconduciveness to plug errors, unpredictability and inability to reveal interactions and only "just workable" solutions. In this regard, the new holistic QbD-based paradigm, i.e., "Formulation by Design (FbD)", applicable especially in the development of drug delivery systems brings about complete understanding of the product and processes based on the sound knowledge of science and quality risk management. Further, the recent regulatory guidance's issued by the key federal agencies to practice QbD has coerced the researchers in industrial milieu to employ these rational approaches during drug product development. Beyond the pharmaceutical formulation development, QbD has diverse applications in API synthesis, analytical method development, dissolution testing, manufacturing and stability testing. The present article describes the principles, methodology and applications of QbD in the entire product development life cycle for attaining product development excellence and regulatory compliance.

Keywords: *Systematic Optimization, Design of Experiments (DoE), Quality by Design (QbD), Formulation by Design (FbD), Design Space*

***Correspondent Author: Email:** bsbhoop@yahoo.com, Phone: +91 172 2534103*

Introduction

Since decades, the pharmaceutical products have been considered as the highly regulated products meant for human use for accomplishing desired therapeutic benefits for treatment of diverse ailments. Despite continuous innovations by the pharma industry, there has been a repeated set back owing their poor quality and manufacturing standards. The adoption of systematic approaches has been originated from a thought provoking article that appeared in *The Wall Street Journal* more than a decade back (i.e., September 2002) was an eye opener for the federal agencies. It stated that "although the pharmaceutical industry has a little secret even as it invents futuristic new drugs, yet its manufacturing standards lag far behind the potato chips and laundry soap makers" [1]. Figure 1 portrays multiple sources of variability during drug product development owing to variability in drug substance(s), excipient(s), process(es), packaging material(s), etc.

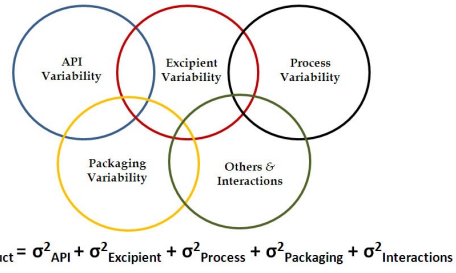

Figure 1: Sources of myriad variability during drug product development

With the consequent growing concern and criticisms, the ICH instituted a series of quality guidances like Q8, Q9, Q10 and Q11, all emphasizing the adoption of systematic principles of Quality by Design (QbD) and Process Analytical Techniques (PAT) as its 21st century quality initiatives. The principal endeavor of ICH has been to accentuate sound science and risk-based understanding of the pharma manufacturing by adopting rational and systematic approaches. Endorsement of such rational paradigms by key global regulatory agencies like USFDA, EMEA, MHRA, Health Canada, TGA and many others is unequivocal testimony to their immense significance for all the potential stake holders, *viz.* patients, industrial scientists and regulators [2-4].

Based upon the Juran's quality philosophy, pharmaceutical QbD embarks upon systematic development of product(s) and process(es) with desired quality. As a patient-centric approach, the QbD philosophy primarily focuses on the safety of patients by developing drug products with improved quality and reduced manufacturing cost by planning quality at first place to avoid quality crisis [5]. Beginning with pre-defined objectives, QbD reveals the pharmaceutical scientists with enhanced knowledge and understanding on the products and processes based on the sound science and quality risk management. Adoption of QbD principles, in particular, tends to unearth scientific minutiae during systematic product development and manufacturing process(es). For pharma industry in particular, QbD execution leads to improved time to market, enhanced knowledge sharing, limited product recalls and rejects, reduced consumer skepticism

towards generics, decreased post-approval changes and efficient regulatory oversight.

One of the integral tools in the QbD armamentarium while developing optimized products and processes has been "Design of Experiments (DoE)" employing apt usage of experimental designs [6]. Amidst a multitude of plausible interactions of the drug substance with a plethora of functional and non-functional excipients and processes, adoption of systematic approaches lead to evolution of the breakthrough systems with minimal expenditure of time, developmental effort and cost. With the objective of developing an impeccable products or processes, earlier this task has been attempted through trial and error, supplemented with the previous knowledge, wisdom and experience of the formulator, termed as the short-gun approach or one factor at a time (OFAT) approach [7, 8]. Using this methodology, the solution of a specific problematic product or process characteristic cannot be achieved and attainment of the true optimal solution was never guaranteed. However, the QbD-based approach usually provides systematic drug product development yielding the best solutions. Such approaches are far more advantageous, because they require fewer experiments to achieve an optimum formulation, reveal interaction among the drug-excipient-process, simulates the product performance and subsequent scale-up. Figure 2 illustrates the QbD-oriented development of drug product embarking upon the comprehensive understanding of the quality traits associated with a product(s) and process(es).

Figure 2: QbD leads to product and process understanding and continual improvement

With the percolation of such systematized approaches, the domain of pharmaceutical product development has endowed a newer look towards drug formulation development and subsequent patient therapy. Owing to the immense benefits, the applications of QbD are galore such as in drug substance manufacturing, formulation development, analytical development, stability testing, bioequivalence trials, etc.

The holistic QbD-based philosophy of product development revolves around five fundamental elements *viz.* defining the quality target product profile (QTPP), identification of critical quality attributes (CQAs), critical formulation attributes (CFAs) and critical process parameters (CPPs), selection of apt experimental designs for DoE-guided, precise definition of design and control spaces to embark

upon the optimum formulation, postulation of control strategy for continuous improvement [9, 10]. Figure 3 illustrates the five step methodology for drug product development employing QbD-based approach.

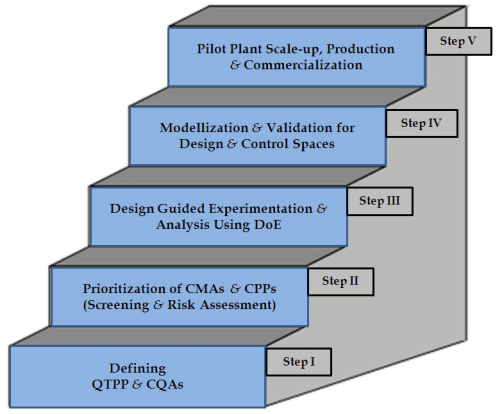

Figure 3: Five-step QbD methodology

Step I: Ascertaining Drug Product Objective(s)

The target product quality profile (QTPP) is a prospective summary of quality characteristics of the drug delivery product ideally achieved to ensure the desired quality, taking into account the safety and efficacy of the drug product. During drug product development, QTPP is embarked upon through brain storming among the team members cutting across multiple disciplines in the industry. Critical quality attributes (CQAs) are the physical, chemical, biological or microbiological characteristic of the product that should be within an appropriate limit, range or distribution to ensure the desired product quality. The identification of CQAs from the QTPP is based on the severity of harm a patient may get plausibly owing to the product failure. Thus after defining the QTPP, the CQAs which pragmatically epitomize the objective(s), are earmarked for the purpose.

Step II: Prioritizing Input Variables for Optimization

Material attributes (MAs) and process parameters (PPs) are considered as the independent input variables associated with a product and/or process, which directly influence the CQAs of the drug product. Ishikawa-Fish bone diagram are used for establishment of cause-effect relationship among the input variables affecting the quality traits of the drug product. Figure 4 illustrates a typical cause-effect diagram highlighting the plausible causes of product variability and their impact on drug product CQAs.

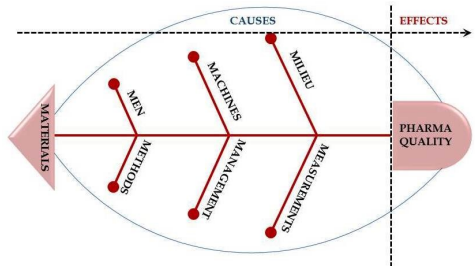

Figure 4: A typical Ishikawa-fish bone diagram depicting sources of variability

Prioritization exercise is carried out employing initial risk assessment and quality risk management (QRM) techniques for identifying the "prominent few" input variables, termed as critical material attributes (CMAs) and critical process parameters (CPPs) from the "plausible so many". This process is

popularly termed as factor screening. Comparison matrix (CM), risk estimation matrix (REM), failure mode effect analysis (FMEA) and hazard operability analysis (HAZOP) are the examples of commonly employed risk assessment techniques. Using these techniques, various MAs and PPs are assigned with different risk levels *viz.* low, medium and high risk based on their severity and likelihood of occurrence. The moderate to high risk factors are chosen from patient perspectives through brainstorming among the team members for judicious selection of CMAs.

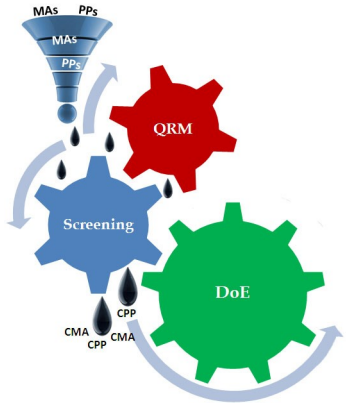

Figure 5: Prioritization using QRM and factor screening is necessary to identify CMAs and CPPs as a prelude to DoE optimization

QRM is rational approach which not only provides holistic understanding of the risks associated with each stages of product development, but also facilitates mitigation of risks too. Experimental designs and risk assessment techniques are used during QRM exercise for factor screening, respectively (Figure 5). Figure 6 portrays the flow layout of overall risk assessment plan employing risk assessment and risk management for identifying the potential CMAs employing a prototype REM model.

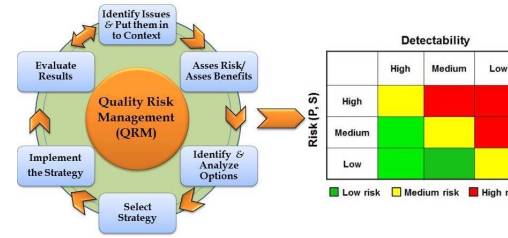

Figure 6: Layout of risk management strategy employing a typical risk estimation matrix

The low-resolution first-order experimental designs (e.g., fractional factorial, Plackett-Burman and Taguchi designs) are highly helpful for screening and factor influence studies. Before venturing into product or process optimization, prioritization of CMAs/CPPs using such QRM and/or screening is obligatory.

Step III: Design-guided Experimentation & Analysis

Response surface methodology is considered as a pivotal part of the entire QbD exercise for optimization of product and/or process variables discerned from the risk assessment and screening studies. The experimental designs help in mapping the responses on the basis of the studied objective(s), CQAs being explored, at high, medium or low levels of CMAs.

Figure 7 provides bird's eye view of key experimental designs employed during QbD-based product development. Factorial, Box-Behnken, composite, optimal and mixture designs are the commonly used high resolution second-order designs employed for drug product optimization.

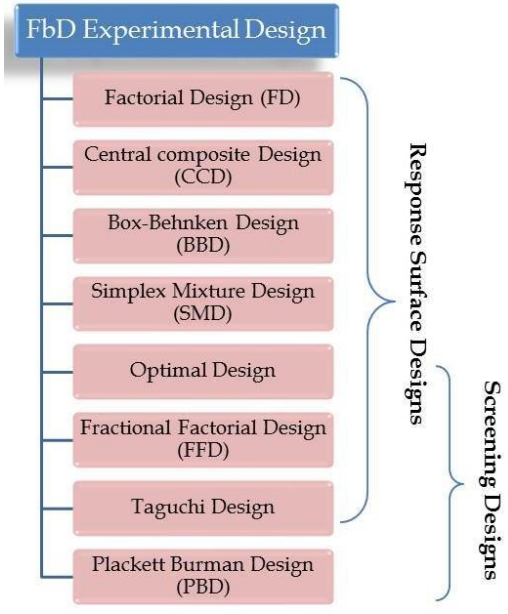

Figure 7: Key instances of experimental designs used during QbD optimization

Design matrix is a layout of experimental runs in matrix form generated by the chosen experimental design, to guide the drug delivery scientists. The drug formulations are experimentally prepared according to the design matrix and the chosen response variables are evaluated meticulously.

Step IV: Modelization & Validation of QbD Methodology

Modelization is carried out by selection of apt mathematical models like linear, quadratic and cubic models to generate the 2D and 3D-response surface to relate the response variables or CQAs with the input variables or CMAs/CPPs for identifying underlying interaction(s) among them. Multiple linear regression analysis (MLRA), partial least squares (PLS) analysis and principal component analysis (PCA) are some of the key multivariate chemometric techniques employed for modelization to discern the factor-response relationship. Besides, the model diagnostic plots like perturbation charts, outlier plot, leverage plot, Cook's distance plot and Box-Cox plot are also helpful in unearthing the pertinent scientific minutiae and interactions among the CMAs too. The search for optimum solution is accomplished through numerical and graphical optimization techniques like desirability function, canonical analysis, artificial neural network, brute-force methodology and overlay plot. Subsequent to the optimum search, the optimized formulation is located in the design and control spaces. Design space is a multidimensional combination of input variables (i.e., CMAs/CPPs) and out variable (i.e., CQAs) to discern the optimal solution with assurance of quality.

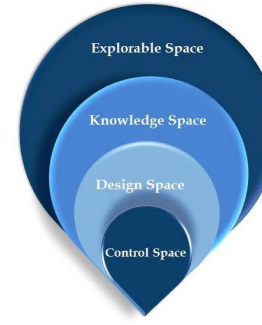

Figure 8: Interplay of knowledge, design and control spaces

Figure 8 illustrates the interrelationship among various spaces like, explorable, knowledge, design and control spaces. Usually in industrial milieu, a narrower domain of control space is construed from the design space for further implicit and explicit studies.

Step V: QbD Validation, Scale-up and Production

Validation of the QbD methodology is a crucial step that forecasts about the prognostic ability of the polynomial models studied. Various product and process parameters are selected from the experimental domain and evaluated as per the standard operating conditions laid down for the desired product and process related conditions carried out earlier, commonly termed as checkpoints or confirmatory runs**.** The results obtained from these checkpoints are then compared with the predicted ones through linear correlation plots and the residual plots to check any typical pattern like ascending or descending lines, cycles, etc. To corroborate QbD performance, the product or process is scaled-up through pilot-plant, exhibit and production scale, in an industrial milieu to ensure the reproducibility and robustness. A holistic and versatile "control strategy" is meticulously postulated for "continuous improvement" in accomplishing better quality of the finished product.

Software Usage during QbD

The merits of QbD techniques are galore and their acceptability upbeat. Putting such rational approaches into practice, however, usually involves a great deal of mathematical and statistical intricacies. Today, with the availability of powerful and economical hardware and that of the comprehensive QbD software, the erstwhile computational hiccups have been greatly simplified and streamlined. Figure 9 enlist the select computer softwares available commercially for carrying out QbD studies in industrial milieu. Pertinent computer softwares available for DoE optimization include Design-Expert®, MODDE®, Unscrambler®, JMP®, Statistica®, Minitab®, etc., are at the rescue, which usually provide interface guide at every step during the entire product development cycle. Softwares providing support for chemometric analysis through multivariate techniques like MNLRA, PCA, PLS, etc. encompass, MODDE®, Unscrambler®, SIMCA®, CODDESA®. For QRM execution using Fish-bone diagrams, REM and FMEA matrices during risk assessment studies, etc., softwares like, Minitab®, Risk®, Statgraphics, FMEA-Pro, iGrafx, etc., can be made use of.

Figure 9: Select computer software used during QbD implementation for product and process optimization

QbD is an inimitable quality-targeted approach for developing efficacious, cost-efficacious, safe and robust drug products, generics as well as innovator's. On industrial fronts, a formulation scientist can derive its stellar benefits at each stage of product development lifecycle and beyond, even after commercial launch and post-marketing surveillance. Figure 10 pictorially illustrates the application of strategic principles of QbD during various stages of drug product development.

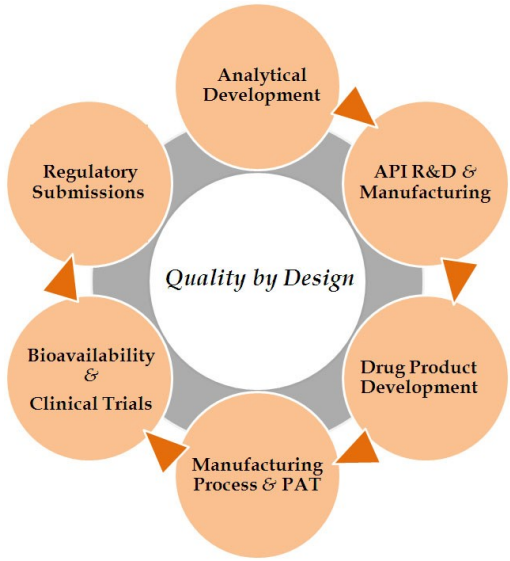

Figure 10: QbD is useful overall product development even after the product launch

Formulation by Design (FbD)

Formulation by Design (FbD) is a recent QbD-based paradigm, applicable exclusively for development of pharmaceutical dosage forms. Product and process understanding are the twin keystones of FbD. It also requires holistic envisioning of the formulation development, including how CMAs and CPPs tend to impact CQAs during laboratory scale, production and exhibit scale leading to a robust and stable drug product [8]. Defining such relationships between these formulation or process variables and quality traits of the formulation is almost an impossible task without the application of FbD model. More the formulator knows about the system, the better he can define it, the higher precision he can monitor it with. Such approach has been widely employed in the development of drug formulations of diverse kinds. Table 1 and Table 2 illustrates the select literature instances on the product and process optimization of drug delivery products employing FbD approach enlisting their QTPP, CMAs, CPPs, CQAs and type of experimental design employed, respectively.

Analytical QbD (AQbD)

AQbD, on the heels of QbD, endeavors for understanding the predefined analytical objectives. These comprise, quality target method profile (QTMP) of an analytical method and identifying the critical method variables (CMVs) affecting the critical analytical attributes (CAAs) for attaining enhanced method performance, like high robustness, ruggedness and flexibility for continual improvement within the ambit of analytical design space [33, 34]. Besides, AQbD helps in reducing and controlling the source of variability to gain in-process information for taking control decisions in a timely manner. This facilitates attaining flexibility in analysis of API and impurities in dosage forms, stability samples and biological samples and to go beyond

Table 1: Select literature instances on QbD-based development of various drug delivery products

Drug	QTPP	CFAs/CMAs/CPPs	CQAs	DoE	Ref. No.
Sumatriptan succinate	Mucoadhesive *in situ* gel	Amount of gellan gum, Lutrol F168	*In vitro* drug release, *ex vivo* permeation	FD	[11]
Iloperidone	Nanostructured lipid carriers	Concentration of lipid, drug and surfactant	Particle size, entrapment efficiency	BBD	[12]
Irbesartan	SNEDDS tablets	Amount of oil, surfactant and cosurfactant	Globule size	PCA	[13]
Tamoxifen Quercetin	SNEDDS	Amount of cremophor RH 40, Labrafil 1944 CS and Capmul MCM	Droplet size, PDI, drug content	D-OD	[14]
Curcumin	Nanoemulsion	Amount of oil, surfactant and co-surfactant	Globule size and zeta potential	BBD	[15]
Tamoxifen	Lecithin organogels	Amount of organic phase, water, Pluronic	Viscosity, gel strength, spreadability consistency	D-OD	[16]
Albendazole	Microspheres	Concentration of chitosan, pectin, carboxymethyl cellulose	Yield, encapsulation efficiency, drug release at 30 and 60 min	FD	[17]
Budesonide	Enteric-coated pellets	Amount of water soluble polymer, amount of water-permeable polymer	Drug release at 3 h, time required for 50% and 85% drug release	FFD	[18]
Carvedilol	Solid SNEDDS	Amount of Capmul MCM and Nikkol HCO-50	Globule size, MDT, dissolution efficiency emulsification time	CCD	[19]
Valsartan	Spray-dried microspheres	Inlet temperature, feed-flow rate and drug-polymer ratio	Yield, particle size, *in vitro* diffusion	FD	[20]
Lamivudine	Gastroretentive tablets	Concentration of Carbopol 971P and HPMC	Drug release in 16 h, buoyancy time, bioadhesion strength	CCD	[21]
Carvedilol	SNEDDS	Amount of Cremophor EL and Transcutol HP	Globule size, MDT, emulsification time	CCD	[22]
Quercetin	SLN	Amount of Compritol 888 and Tween 80	Particle size, drug release in 16 h, zeta potential	CCD	[23]
Tramadol	Controlled release bioadhesive tablets	Amount of Carbopol 971P and HPMC	Drug release in 16 h, bioadhesion strength, release exponent	CCD	[24]
Nimesulide	Liposomes	Amount of phospholipid, cholesterol	Percent entrapment, percent diffused, percent leakage	FD	[25]

QTPP: Quality target product profile; CQA: Critical quality attributes; CMA: Critical material attributes; FD: Factorial design; FFD: Fractional factorial design; CCD: Central composite design; BBD: Box-Behnken design; D-OD: D-optimal mixture design; PCA: Principal component analysis

Table 2: Select literature instances on QbD-based development of process(es) during drug product development

Drug	QTPP	CPPs	CQAs	DoE	Ref. No.
Polypeptide antibiotic	Fermentation process	Incubation time, temperature, pH, aeration rate, nitrogen and carbon concentration	Polypeptide concentration	TgD	[26]
Paclitaxel	Nanoparticles	PLGA amount, Surfactant conc., homogenization rate	Particle size, zeta potential, encapsulation	BBD	[27]
Ursodeoxy cholic acid	High-pressure homogenization technology	Pressure, concentration of ursodeoxycholic acid	Particle size	BBD	[28]
Solid dispersion	Spray drying process	Temperature, Concentration, flow rate, atomization	Yield, outlet temperature, particle size	BBD	[29]
Tinospora cordifolia extract	Extraction of alkaloid palmatine	Extraction temperature, time and cycles	Percent yield	CCD	[30]
Nanoparticles	Media milling process	Motor speed, pump speed, bead volume	time, particle size, yield	CCD	[31]
Matrix metallo proteinase-1	PLGA-PCL nanoparticles	Homogenization time, agitation speed and volume of organic to aqueous phase	Particle size, entrapment efficiency	CCD	[32]

QTPP: Quality target product profile; CQA: Critical quality attributes; CPP: Critical process parameters; FD: Factorial design; FFD: Fractional factorial design; CCD: Central composite design; BBD: Box-Behnken design; TgD: Taguchi design

traditional ICH procedure of method validation. Like FbD, the AQbD also embarks upon risk-assessment studies through REM/FMEA and DoE-guided factor screening and optimization studies for improving the method performance. Instances of CMVs during AQbD optimization include mobile phase composition, flow rate, gradient time, column oven temperature, pH, while CAAs include peak area, retention time, theoretical plates, asymmetry factor and capacity factor. Literature reports on QbD-based analytical method development are enlisted in Table 3.

Other QbD applications in product lifecycle

QbD not only facilitates comprehension of products or processes, but also helps in attaining excellence in federal compliance with phenomenal ease and economy. Hence, besides the drug formulation development and analytical method development, the concept of QbD has slowly been percolating into other diverse interdisciplinary areas like API development, dissolution testing, manufacturing, bioequivalence studies and stability testing.

Table 3: Select literature instances on analytical method development using QbD

Drug	QTMP	CMVs/CMPs	CAAs	DoE	Ref. No.
β-artemether and lumefantrine	Stability-indicating HPLC method	Mobile phase ratio, flow rate	Retention factor, peak symmetry	PBD	[35]
Ebastine	Degradation product characterization	Buffer strength, pH	Peak resolution	FD	[36]
Darifenacin hydrochloride	Stability-indicating UPLC method	Mobile phase ratio, pH, column oven temperature	Peak resolution and retention time of degradation products	CCD	[37]
Rosuvastatin, Telmisartan, Ezetimibe, Atorvastatin	Simultaneous estimation using HPLC method	Mobile phase ratio, buffer strength, flow rate	Peak resolution, peak asymmetry	RCCD	[38]
Protamine sulfate	Simple HPLC method development	Flow rate, temperature, pH	Peak resolution, tailing factor	CCD	[39]

QTMP: Quality target method profile; CMVs: Critical method variables; CMPs: Critical method parameters; CAAs: Critical analytical attributes; FD: Factorial design; CCD: Central composite design; RCCD: Rotatable CCD; PBD: Plackett-Burman design

Developing drug substances employing the systematic QbD-based paradigm has been recently popularized to accomplish the desired objective of producing drug substance with reduced variability, high purity and yield. ICH Q11 guidance, in this regard, provides detailed understanding of the key principles of manufacturing drug substance employing rational paradigms. As per the QbD approach, the quality target profile for drug substance are defined, which includes molecular, physiochemical and biological properties, pharmacokinetics, storage and packaging conditions, etc [40]. The concentration of reactants, solvents, initiators, stabilizers employed during synthesis of drug substance are mainly used as the CMAs, which are subsequently optimized for their impact on CQAs like, API particle size and size distribution, polymorphism, hygroscopicity, density, flow property, aqueous solubility, etc. Table 4 illustrates the select literature reports on development of drug substances employing QbD approach.

QbD in dissolution testing

As dissolution testing is primarily considered as one of the most important quality control test for preparing the release specification for any pharmaceutical dosage form, the QbD approach helps in optimizing the drug product composition for accomplishing analogous drug release profile to that of the reference listed product. Important examples of CQAs which determines the

Table 4: Select literature instances on API development employing QbD approach

Drug	QTPP	CMAs/CPPs	CQAs	DoE	Ref.
Fc fusion protein	Overall yield of protein synthesis	Aggregate level of reactant in load, elution buffer pH	Yield of host cell protein, residual protein, DNA	MVA	[41]
Torcetrapib	Drug substance development	Concentration of reactants	Assay, % purity of drug & intermediates	CCD	[42]
DCBB	Improve reductive sulfonylation process	Sulfite amount, time and temperature	Percent yield	FD	[43]
17 α-methyl-11β-arylestradiol	Optimization of target product yield	Reactant concentration, Reaction temperature	Yield and percent purity	FD	[44]
Calanolide A	optimization for improving yield	Amount of $AlCl_3$ and reaction temperature	% yield of intermediate	FD	[45]

product quality include amount of drug release at specified time intervals, mean dissolution time, dissolution efficiency, release exponent, etc., whereas the concentration of polymers, disintergrants, type of medium are used as CMAs which tend to affect the dissolution profile of drug products.

QbD in bioequivalence testing

Implementation of QbD during bioequivalence study helps in optimizing the drug products (i.e., generics) in obtaining desired pharmacokinetic profile matched with that of the reference listed product. Important pharmacokinetic metric like, C_{max}, T_{max}, AUC, AUC_{0-t}, AUC_{∞}, are considered as the critical quality traits for optimizing the formulation variables like, concentration of release controlling polymer, coating composition, coating percentage, etc.

QbD in stability testing

QbD approach in stability testing furnishes better understanding of the product stability and shelf-life, information on degradation products, compatibility of container(s)/closure(s) with packaging materials. This helps in preparing the specifications related to safety, efficacy of finished product(s) with respect to the concentration of degradants and final qualifications of them for marketing approval.

Conclusion

Today, the federal agencies look for assurance of patient-centric quality "built-in" into the system, rather than through end-product testing. Notwithstanding the enormous utility of QbD-based philosophy in developing optimal drug products, it leads research mindsets to evolve "out-of-box" strategies too. As variability tends to exist at each and every stages of product development life cycle, QbD application needs to be omnipresent. Apt implementation of QbD paradigms, accordingly, would be pivotal in achieving a "win-win situation" for patients, drug industry and regulators. The practice of systematic QbD implementation for

products has undoubtedly spiced up over the past a few decades, yet it is far from being adopted as a standard practice. Federal regulations for generic drug products are already in place. Several initiatives still need to be undertaken to inculcate mundane use of diverse QbD paradigms in the holistic domain. Apart from these, the synergistic use of in-process PAT and RTRT tools in tandem with process engineering approaches like extensometry and chemometry, can also be helpful in ameliorating product and process understanding and enhancing the process capability for efficient manufacturing. With the growing acceptance of QbD paradigms, in a nutshell, it is rationally prophesized that soon these QbD philosophies will be required to be implemented to innovators, biosimilars, analytical development, API development and even beyond.

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INVITED ARTICLE

A REVIEW ON SYNTHESIS AND PHARMACOLOGICAL DIVERSITY OF ISOXAZOLES & PYRAZOLINES

Kuntal Manna *, Udayan Banik, Partha Sakha Ghosh and Manik Das
Department of Pharmacy, Tripura University (A Central University), Suryananinagar, Tripura-799 022. India.

Abstract

Isoxazole is an azole with an oxygen atom next to the nitrogen. It is also the class of compounds containing this ring. Isoxazole rings are found in some natural products, such as ibotenic acid and also found in a number of drugs, including COX-2 inhibitor valdecoxib. Furoxan, a nitric oxide donor is containing isoxazolyl group & found in many beta-lactamase-resistant antibiotics, such as cloxacillin, dicloxacillin and flucloxacillin. The synthetic androgenic steroid danazol also has an isoxazole ring. Pyrazoline is a five-membered heterocyclic having two adjacent nitrogen atoms within the ring. It has only one endocyclic double bond and is basic in nature. Among its various derivatives, 2-pyrazolines seem to be the most frequently studied pyrazoline type compounds. 2-Pyrazolines can be considered as a cyclic hydrazine moiety display a broad spectrum of potential pharmacological activities.

Keywords: *Isoxazole, pyrazoline, synthesis, pharmacological diversity*

***Correspondent Author: Email:** k_manna@tripurauniv.in, Phone: + 91 381 2374801 / 3*

Introduction

Heterocyclic compounds forms a major class of compounds in chemistry. These are cyclic compounds having at-least two different elements as a part of the ring system. On the other hand, a ring system made up of same elements is considered as homocyclic compounds.

Fused saturated 5-membered rings naturally occurring in both carboxylic (Eg. Triquinane) and heterocyclic (Eg. Pyrrolizidin alkaloids) systems have interesting pharmacological importance [1].

Usually 6-membered rings have well defined and readily determined conformations like chair and boat, but 5-membered rings are much flexible and their conformations are difficult to ascertain (Figure 1) [2].

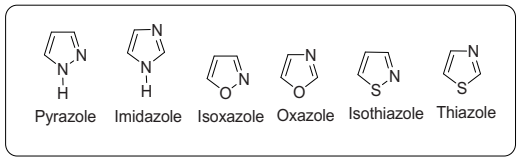

Figure 1: Structures of Various 5-membered Heterocycles

Pyrazolines are well known and important nitrogen containing 5-membered heterocyclic compounds. 2-Pyrazolines show a broad spectrum of pharmacological activities and are present in a number of pharmacologically active molecules such as phenazone, amidopyrine, methampyrone (analgesic and antipyretic), azolid, tandearil (anti-inflammatory), indoxacarb (insecticide) and anturane (uricosuric) [3]. Numerous pyrazoline derivatives have been found to possess considerable biological activities, which stimulated the research activity in this field. They have several prominent effects, such as antimicrobial, antimycobacterial, antifungal, anti-amoebic, antiinflammatory, analgesic, antidepressant and anticancer activities. They also possess some potent, receptor selective biological activity like nitric oxide synthase (NOS) inhibitor and cannabinoid CB1 receptor antagonist activity [4]. The work provides an insight view to pyrazolines synthesis and its biological activities. Isoxazole (C_3H_3NO) is an azole with an oxygen atom next to the nitrogen. Isoxazolyl is the univalent radical derived from isoxazole. Isoxazole ring is found in some natural products, such as ibotenic acid. Isoxazoles also form the basis for a number of drugs, including the COX-2 inhibitor valdecoxib (Bextra) (Figure 2). A derivative, furoxan, is a nitric oxide donor. An isoxazolyl group is found in many beta-lactamase-resistant antibiotics such as cloxacillin, dicloxacillin and flucloxacillin. The synthetic androgenic steroid danazol also has an isoxazole ring [5].

Isoxazoles are unsaturated aromatic heterocyclic compounds containing a ring with three carbon atoms, one oxygen atom and one nitrogen atom. The trivial name for the title five-membered fully unsaturated heterocycles as “isoxazole” was originally proposed by Hantszch as it was the isomer “oxazole” discovered first. The trivial name follows the Hantszch-Widman system of nomenclature: the prefix “iso” represents isomer, “oxa”

represents the oxygen atom "aza" represents the nitrogen atom. The suffix "ole" denotes the ring size as five-membered; altogether the derived name is "isoxazole" [6]. This name has been accepted in IUPAC and has been used in Chemical abstracts. In Chemical Abstracts, the other systematic name 1,2-azole, is also used. Isoxazole being an azole with an oxygen atom next to the nitrogen, exhibits broad spectrum of biological activity and also forms a part of various biodynamic agents [7].

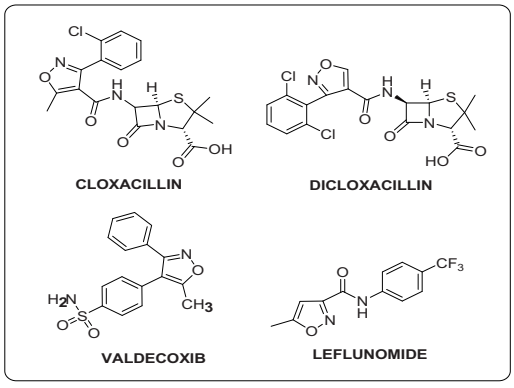

Figure 2: Isoxazole derivative having established pharmacological activity

The substituted isoxazoles are also considered to be important synthons due to their versatility towards chemical transformations to useful synthetic intermediates. Isoxazole derivatives show hypoglycemic, analgesic, antiinflammatory, antifungal, anti-bacterial and HIV-inhibitory activities [8]. Synthesis of hybrid natural products has gained momentum in recent years. It is expected that combining features of more than one biologically active natural segment in a single molecule may result in pronounced pharmacological activity while retaining high diversity and biological relevance [9-11]. The nitrogen hetero atom is more pronounced for electron withdrawing effect, while the oxygen atom is more pronounced for electron donating effect. As neutral molecules, isoxazoles undergo electrophilic substitution rather more readily at the position-4 than benzene. Effects of substituents can modify their behavior.

Substituents at the position-5 apparently have more activating and deactivating effect than substituents at the position-3. In natural product synthesis, isoxazoles are used as latent synthons, such as masked new heterocyclic rings, masked fused rings, masked aromatic rings and masked aldol and related moieties. The capability of isoxazole undergoing reaction is diverse: protonation, quaternization, complexation, oxidation, reduction, carbanionic condensations, thermolysis, photolysis, transformations into other heterocyclic ring systems and reaction with electrophiles, nucleophiles and Grignard reagents [12].

The naturally occurring antibiotic cycloserine [13]; the monoamine oxidase inhibitor isocarboxazide; isoxazole steroids, ibotenic acid; muscimol isolated from *Amanita muscaria* [14] and isoxazoline-5-ones isolated from Legume seed [15] are potential isoxazole derivatives.

Methods of synthesis of isoxazoles

Figure 3 showed regioselective cyclo-condensation of acrylophenone dibromide derivatives with hydroxylamine

hydrochloride giving corresponding isoxazoles [16].

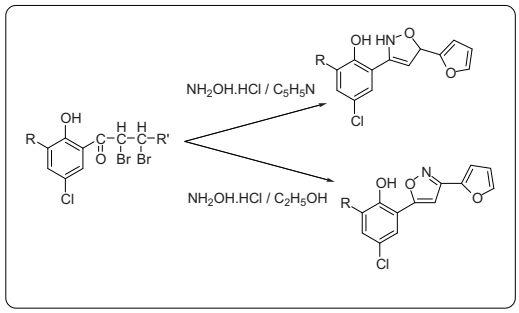

Figure 3: Synthesis of isoxazoles from acrylophenone dibromide

3-Alkyl, 5-aryl isoxazoles can be prepared from aryl cycolpropanes (Figure 4) with $NaNO_2$ in CF_3COOH [17].

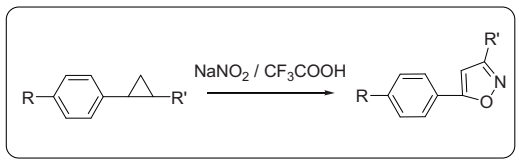

Figure 4: Synthesis of isoxazoles from aryl cyclopropanes

Solid phase synthesis of isoxazole derivative from diaryl 1,3-diketones (Figure 5) can be carried out in presence of hydroxylamine hydrochloride and silica gel [18].

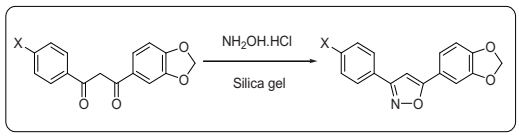

Figure 5: Solid phase synthesis of isoxazoles from diaryl 1,3-diketones

Reaction of various substituted acetophenones with diethyl oxalate in the presence of sodium ethoxide forms resulting 2,4-diketo esters which on treatment with hydroxylamine hydrochloride furnishes substituted 3-isoxazole esters (Figure 6) [19].

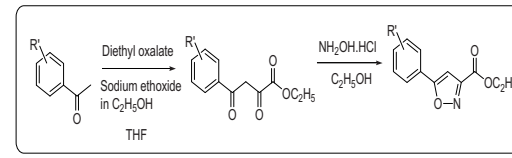

Figure 6: Synthesis of 3-isoxazoles from acetophenones

Pharmacological aspects of isoxazole

Anti-convulsant activity

The search for antiepileptic compounds with more selective activity and lower toxicity continues to be an area of investigation in medicinal chemistry [20]. Many patients with epilepsy fail to experience adequate control of their seizures, despite the optimal use of available antiepileptic drugs. Other patients do so only at the expense of significant toxic side effects. In recent years it has been established that inhibitors of GABA transport and in particular as troglial uptake can act as anticonvulsant agents and several isoxazole derivative (compound-A) (Figure 7) [21]. Compound-B is also a synthesized isoxazole derivative which affects the sodium channel to show its activity [22].

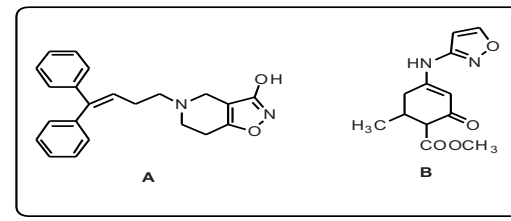

Figure 7: Isoxazoles having anti-convulsant activity

Anti-cancer activity

The effects of curcumin and of its isoxazole analogue in breast cancer cell line and in its multidrug-resistant (MDR) variant were examined. The isoxazole analogue Compound A has shown more potent antitumor and molecular activities both in parental and in MDR tumor cells. Isoxazole derivatives produces significantly higher direct inhibition of the COX-2 catalytic activity than curcumin. The isoxazole derivatives proved better because of minimum metal chelation when compared to curcumin [23]. The compound B has been found highly effective against human tumor cell lines especially on renal cancer, CNS cancer cell and ovarian cancer cell lines [24]. Recently NO-NSAID has been established as potent anti-cancer agents rather than their anti-inflammatory property [25]. Compound C is a NO donating compound used as anti-cancer agent.

Anti-microbial activity

Drazoxolon is a commonly used fungicidal agent. Acetyl Sulfisoxazole is another important anti-microbial agent from the isoxazole family which is widely used in pediatric suspensions (Figure 8). Cycloserine is a well-established molecule widely known for its potency against Mycobacterium tuberculosis [26-28].

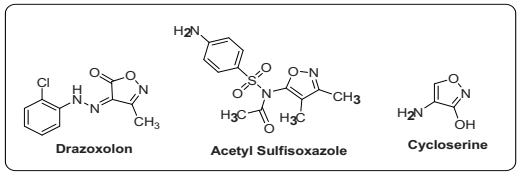

Figure 8: Isoxazoles having antimicrobial activity

Pyrazoline

Pyrazole is a π-excessive aromatic monocyclic heterocycle containing two nitrogen atoms in a five membered 1,2-diazole ring. Pyrazoles exhibit aromatic character with properties resembling those of both pyrrole and pyridine. 1-pyrazoline, 2-pyrazoline and 3-pyrazoline are the three partially reduced forms of the pyrazole structure with different positions of the double bonds and exists in equilibrium one with the other [29]. 2-pyrazoline exhibits the monoimino character and hence more stable than the rest. Pyrazole is freely basic and forms salts with inorganic acids. The imino hydrogen may be replaced by an acyl group. Pyrazole is very resistant to oxidation and reduction, but may be hydrogenated catalytically, first to pyrazoline and then to pyrazolidine [30].

Phenylbutazone is a pyrazoline derivative known for its analgesic and anti-inflammatory and anti-pyretic properties (Figure 9). Muzolimine is a pyrazoline derivative used as a diuretic which differs from the structures of other conventional diuretic compounds.

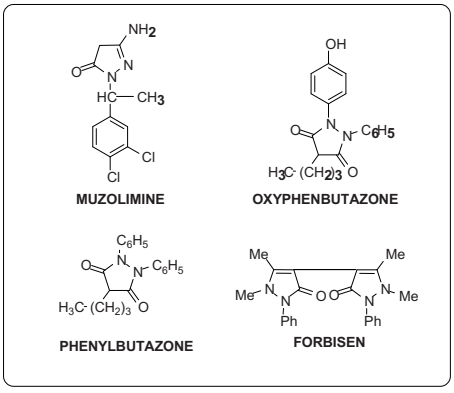

Figure 9: Pyrazoline derivative having established pharmacological activity

Forbisen is a byproduct separated during synthesis of antipyrine. This pyrazoline is used in bovine analplasomosis. Oxyphenbutazone is a pyrazoline derivative similar to phenylbutazone. It is an active metabolite of phenyl butazone. The difference with phenyl butazone is the presence of *p*-hydroxyphenyl group instead of phenyl at position 1 of phenylbutazone [31].

Methods of synthesis of pyrazolines

α, β-unsaturated carboxylic acid esters reacts with diazomethane to give 2-pyrazolines (Figure 10). The primary product of this reaction is a 1-pyrazoline, formed by 1, 3-dipolar cyclo addition, which spontaneously isomerizes into its thermodynamically more stable 2-pyrazoline isomer by a 1, 3-H shift [32].

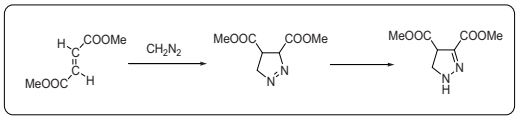

Figure 10: Synthesis of pyrazolines from α, β-unsaturated carboxylic acid esters

Figure 11 showed an example of synthesis of a pyrazoline from the reaction of an α,β-unsaturated ketone and diazomethane. Benzylidene-acetone on reaction with diazomethane by 1,3- dipolar cycloaddition yield 2-pyrazolines [33-35].

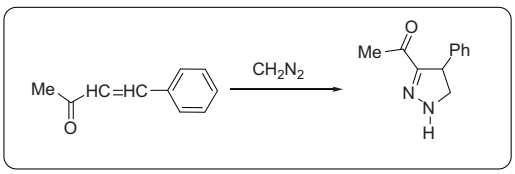

Figure 11: Synthesis of pyrazolines from α, β-unsaturated ketone

Cycloaddition reaction (Figure 12) of substituted styrenes with *p*-anisyl diazomethane at low temperature yield *trans*-3, 5-bis-(*p*-anisyl)-1-pyrazoline [36].

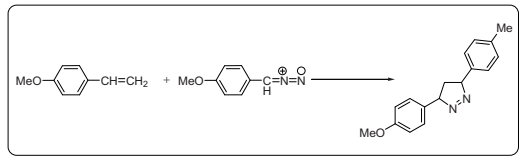

Figure 12: Synthesis of pyrazolines by cycloaddition reaction

Chalcone derivatives on reacting with hydrazine hydrate and glacial acetic acid, form corresponding N-phenyl-3,5-diphenyl pyrazoline & 3,5-diphenyl pyrazoline derivatives respectively (Figure 13) [37].

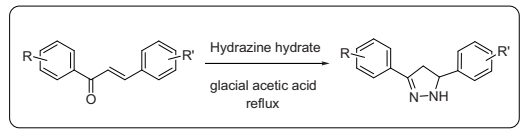

Figure 13: Synthesis of pyrazolines from Chalcone derivatives

Pharmacological aspects of pyrazolines:

2-pyrazoline is insoluble in water but soluble in propylene glycol because of its lipophilic character [38]. 2-pyrazolines display a broad spectrum of potential pharmacological activities and are present in a number of pharmacologically active molecules such as phenazone, amidopyrene, methampyrone (analgesic and antipyretic), azolid/ tandearil (anti-inflammatory), indoxacarb (insecticidal), anturane (uricosuric), etc. Considerable interest has been focused on the pyrazoline structure. The discovery of this class of drugs provides an outstanding case history of modern drug development and also

points out the unpredictability of pharmacological activity from structural modification of a prototype drug molecule. It is having a variety of medicinal applications. Pyrazoline derivatives were found to have potential antipyretic-analgesic, tranquilizing, muscle relaxant, psycho analeptic, antiepileptic, antidepressant, anti-inflammatory, insecticidal, antimicrobial and anti-hypotensive activities. Their derivatives were also found to exhibit cytotoxic activity, inhibitory activity of platelet aggregation, herbicidal activity and cannabinoid CB1-receptor modulators. Pyrazoline interest is also extended to dyes and dye couplers [39]. Given below is a brief account of various modifications reported on chalcones, which resulted in a variety of biological and pharmacological activities.

Anti-cancer agents

Cancer is a leading cause of death worldwide, accounting for 8.7 million deaths (around 14% of all deaths) in 2012. Although many chemotherapeutic agents, such as cisplatin, 5 fluorouracil and taxol, have been developed to treat different kinds of cancer effectively, some side effects could happen simultaneously. Therefore, it is important and urgent to develop novel compounds as anticancer agents with higher bioactivities and lower side effects [40, 41]. It is well known that a number of multi-cyclic compounds containing heterocycle fragments exhibit a wide variety of biological activities. Pyrazoline and pyrazole are important structural fragments of many bioactive compounds.

As the diverse pharmacological importance of pyrazoline have already been explained in the earlier sections, some of the pyrazoline derivatives showed potent anti-cancer activity (Figure 14).

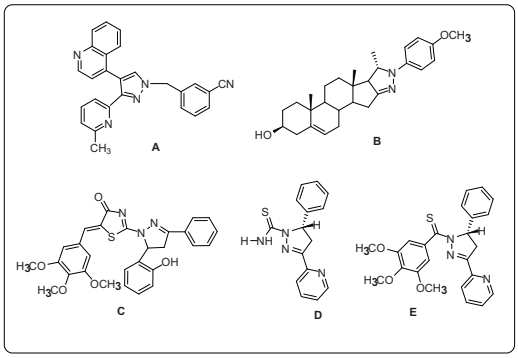

Figure 14: Pyrazolines having Anti-cancer activity

Compound A showed potential ALK5 inhibitory activity as transforming growth factor-β type 1 receptor kinase inhibitors [42]. But this compound showed some extent of cytotoxicity. This problem is eradicated when *p*-methoxy phenyl pyrazoline fragment is introduced to the ring-D of steroid in Compound B [43]. Compound C is proven to be active with selective influence on colon cancer cell lines [44]. The above mentioned compounds A and B have been examined for anti-proliferative activity against human colon carcinoma and highly metastatic human breast carcinoma [45].

Anti-microbial agents

The pyrazoline nucleus is a ubiquitous feature of various compounds possessing many pharmacological and physiological activities and therefore they are useful materials in drug research. It was reported

in the literature that different substituted 2-pyrazolines possess antimicrobial activities (Figure 15).

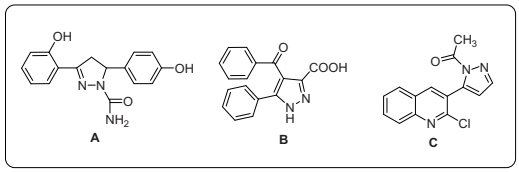

Figure 15: Pyrazolines having Anti-microbial activity

Compound A have structural similarities to siderophores and evaluated as novel antimicrobials against *Mycobacterium tuberculosis* and *Yersinia pestis* [46]. Compound B and C possess antibacterial activities against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas putida* [47].

Hypotensive agent

Clonidine and Clonidine like thiazolo-imidazoline derivatives (Figure 16) encouraged the development of pyrazoline derivatives of this class which are proven to have hypotensive activity. These derivatives also bear structural and isosteric relationship to Clonidine [48].

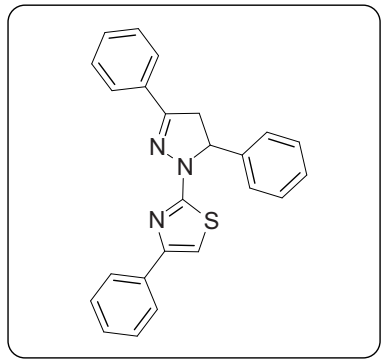

Figure 16: Pyrazoline having hypotensive activity

Analgesic agent

Pain is one of the most prevalent conditions that limits productivity and diminishes quality of life. Although there is an arsenal of effective and widely used analgesics, there is some concern regarding their safety and side-effects, making their clinical use problematic. In the recent years pyrazoline has emerged as Class of analgesics having very high pharmacological activity [49]. Dipyrone (metamizol) is a drug that belongs to the pyrazole derived family. Reports in the literature have shown that dipyrone, although generally considered as belonging to the nonsteroidal anti-inflammatory drug family, seems not to present the typical side effects of this drug class. Antipyrine (phenazone) is also a widely used analgesic belonging to pyrazoline family.

Anti-depressant activity

Depression is a serious disorder with estimates of lifetime prevalence as high as 21% of the general population in some developed countries [50]. Treatment for this disease is possible with antidepressant medications and psychotherapy for some patients [51].

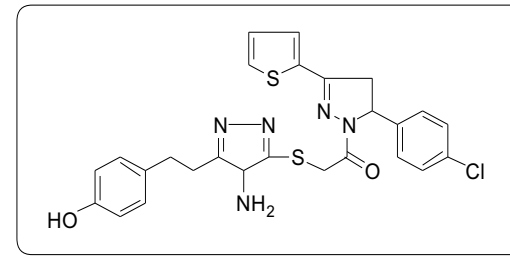

Figure 17: Pyrazoline having anti-depressant activity

Although antidepressants have been used in the clinic for several decades, most of them are inadequate in efficiency and have many serious adverse side effects. Pyrazolines were also reported as potential antidepressant agents (Figure 17) which act by MAO inhibition. The compound shown above has been proven as a potent anti-depressant activity involving serotonergic system for its activity [52].

Conclusion

Review emphasizes on detailed information of Isoxazole and Pyrazoline. Isoxazole, an azole with an oxygen atom next to the nitrogen, is found in many natural products and also found in number of drugs, including COX-2 inhibitor valdecoxib. Furoxan, is a nitric oxide donor is containing isoxazolyl group is found in many beta-lactamase-resistant antibiotics, such as cloxacillin, dicloxacillin and flucloxacillin. The synthetic androgenic steroid danazol also has an isoxazole ring. Pyrazoline, a five-membered heterocycle having two adjacent nitrogen atoms within the ring, has only one endocyclic double bond and is basic in nature. Among its various derivatives, 2-pyrazolines seem to be the most frequently studied pyrazoline type compounds. 2-Pyrazolines can be considered as a cyclic hydrazine moiety display a broad spectrum of potential pharmacological activities. Both ring systems are having many pharmacological activity including but not limited to anti-cancer, anticonvulsant, antimicrobial, etc. These ring systems can further exploit in future to design novel pharmacologically active molecules.

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INVITED ARTICLE

COMPULSORY LICENSE IN LIGHT OF INDIAN PATENT PERSPECTIVE

Sanjaykumar Patel*
Patent Attorney, Deputy Manager at Alembic Pharmaceuticals Ltd, Vadodara.

Abstract

The compulsory license is defined as an involuntary license between a willing licensee and an unwilling licensor, imposed and enforced by the controller of patents. Compulsory license is a very effective legal tool which can allow the third parties to produce and manufacture the intellectually protected product or process at reasonable cheaper price. Till date, healthcare or pharmaceutical sector is the only sector of interest for the compulsory. Very recently India has also joined the list of such countries after the grant of first compulsory license to M/s Natco for producing Nexavar® (Sorafenib Tosylate) which is patented by M/s Bayer. The very main purpose of the compulsory license is to prevent the patentee from abusing intellectual right and to rectify the unfair trade practice and to consider the easy availability of the basic health need of the society at reasonable affordable price. Department of Industrial Policy and Promotion (DIPP) initiated the thought process of issuing compulsory licenses for Herceptin® (Trastuzumab) which is used in the treatment of breast cancer, Ixempora® (Ixabepilone) which is used in treatment of chemotherapy and Sprycel® (Dasatinib) which is used in treatment of leukemia. The present review gives an overview about the compulsory licensing aspects pertaining to India.

Keywords: *Compulsory license, Indian Patent Act, Natco vs. Bayer*

***Correspondent Author:** E-mail- patentagent2007@gmail.com, Phone: +91-9825867635*

Introduction

Compulsory license is a self-explaining word meaning a license agreement under any compulsion and not by willingness. The compulsory license can be better defined as an involuntary license between a willing licensee and an unwilling licensor, imposed and enforced by the controller of patents. Compulsory license is a very effective legal tool which can allow the third parties to produce and manufacture the intellectually protected product or process at reasonable cheaper price and at a constant sufficient quantity through license so that the society does not remain deprived from any product or have to pay very high amount for the same.

The compulsory license were introduced and implemented by many international arrangements like WTO Agreement on Trade Related Aspects of Intellectual Property Rights (TRIPS) and World Intellectual Property Organization (WIPO) [1].

Compulsory license and Pharma sector

Till date, healthcare or pharmaceutical sector is the only sector of interest for the compulsory license or in other words, it is the only sector which has the requirement of compulsory licensing.

As per the survey, during 2001 to 2007, nearly 52 developing and least developing countries have allowed compulsory license for anti-cancer and anti-HIV drugs which are otherwise priced very high by the innovator due to patent protection [2]. Very recently India has also joined the list of such countries after the grant of first compulsory license to Natco for producing Nexavar® (Sorafenib Tosylate) which is patented by Bayer [3].

When we talk about pharmaceutical industry, we cannot forget India as India is one of the largest supplier of pharmaceutical products all over the globe and majority of Indian pharmaceutical organizations are involved in the generic drugs and Indian Patent Act which includes the statutory provision of compulsory license.

Compulsory license provisions in Indian Patent Act

As per Indian Patent Act, compulsory license related provisions are covered under section 84 to section 94.3 [4].

Section 84(1) says at any time after the expiration of three years from the date of the grant of a patent, any person interested may make an application to the Controller for grant of compulsory license on patent on any of the following grounds, namely:

(a) that the reasonable requirements of the public with respect to the patented invention have not been satisfied, or

(b) that the patented invention is not available to the public at a reasonably affordable price, or

(c) that the patented invention is not worked in the territory of India.

It is crystal clear from above clause that a very first requirement is expiration of three years after the grant of patent. The reason

behind it may be to give respect to the invention and exclusive right to patentee to get benefit from the exclusive right till three years from the grant of patent.

Secondly, the third party seeking for compulsory license can apply for a compulsory license showing fulfillment of any of the above three criteria along with the proper justification and proof to support the argument. However while considering any application for compulsory license, the controller will look in to many other factors too, like the ability and capacity of the applicant to work for the invention in public benefit at reasonable price and also whether the applicant has made an efforts to obtain a license from the patentee on reasonable terms and conditions and such efforts have not been successful within a reasonable period of at least as six months.

As per section 92, if the Central Government is satisfied, in respect of any patent in force in circumstances of national emergency or in circumstances of extreme urgency or in case of public non-commercial use, it gives notification in the official gazette and subsequently on receipt of the application, compulsory license is granted as per the terms and conditions as the controller thinks fit.

In Indian patent act, there is one more special provision under Section 92-A, which is meant for the compulsory license for export of patented pharmaceutical products in certain exceptional circumstances. In this case the compulsory license can be granted to manufacture any patented product and process in India with condition that such products is to be exported to any country having insufficient or no manufacturing capacity in the pharmaceutical sector for the concerned product to address public health problem provided compulsory license has been granted by such country or such country has, by notification or otherwise, allowed importation of the patented pharmaceutical products from India.

The very main purpose of the compulsory license is to prevent the patentee from abusing intellectual right and to rectify the unfair trade practice and to consider the easy availability of the basic health need of the society at reasonable affordable price.

Indian patent act has inserted certain check point to prevent abuse of this provision by the third party by way of possibility of revision of terms and condition of license and revocation of license. The controller can revise the terms and condition after receipt of an application by any party after one year of grant of license based on reevaluation of the factors considered at the time of granting compulsory license. The controller can also revoke granted compulsory license on receipt of an application any time after two years from the grant of license by patentee on the ground that the patented invention has not been worked in the territory of India or that reasonable requirements of the public with respect to the patented invention has not been satisfied or that the patented invention is not available to the public at a reasonably affordable price.

First compulsory license of Indian patent scenario since 1970: M/s Natco Vs M/s Bayer: M/s Bayer is patentee holding compound patent IN215758 which covers the Sorafenib Tosylate which is marketed as Nexavar® for the treatment of hepatocellular carcinoma and renal cell carcinoma. M/s. Natco applied for compulsory license of IN215758. Natco has argued that a) drug was not easily available to the public based on the Form-27 filed by M/s Bayer in year 2009 and 2010 b) M/s Bayer was selling Nexavar® at a price of INR 2,80,000 per month in India.

After reviewing the application for compulsory license from M/s Natco in light of the evidence provided and justifications, the controller has finally granted the first compulsory license of Indian patent history in March 2012 based on terms and condition from which few are listed here [5].

- The price of the licensee shall not exceed Rs.8880 for a pack of 120 tablets.
- The licensee shall manufacture the drug at his own manufacturing facility and shall not outsource the production.
- The license is non-exclusive and non-assignable.
- The licensee shall pay royalty at the rate of 6% (Later revised to for 7% by IPAB) of the net sales of the drug on a quarterly basis.
- The licensee shall supply the drug covered by the Patent to at least 600 needy and deserving patients per year free of cost.
- The licensee is solely and exclusively responsible for its product and for all associated product liability.

The decision of granting the compulsory license was welcomed by Indian generic pharmaceutical industry as one of the approach to cope up the patent related hurdles but they have to move over with caution and as per statutory requirement laid down in legislation. If not, the road will end with dead end.

Earlier in January 2013, Department of Industrial Policy and Promotion (DIPP) has initiated the thought process of issuing compulsory licenses for Herceptin® (Trastuzumab) which is used in the treatment of breast cancer, Ixempora® (Ixabepilone) which is used in treatment of chemotherapy and Sprycel® (Dasatinib) which is used in treatment of leukaemia [6]. However as per the recent development in this matter, the approval was given only to Sprycel® (Dasatinib) and remaining two drugs are either under discussion or rejected for time being form the consideration of compulsory license.

Interestingly M/s BDR Pharma, a Mumbai based pharmaceutical company had applied for compulsory license for Sprycel® (Dasatinib) in March 2013 before the thought process of DIPP. More interestingly the compulsory license application of M/s BDR Pharma has been

rejected by the Controller General and provided the statement that M/s BDR Pharma was not successful to establish the "prima facie" case in support of their application for compulsory license [7]. As discussed above regarding provision of compulsory license, the controller also considers the reasonable efforts made by the applicant to obtain voluntary license from the patentee and in case of M/s BDR Pharma, the failure to establish this, was one of the ground reasons for rejection.

Future prospective

Either failure or success, it is a good move by Indian generic pharmaceutical companies approaching for compulsory license and contributing towards the corporate social responsibility in one or other way. The effective use of the provision of compulsory license can reduce the cost of healthcare products protected by patent up to affordability of the public at large for developing and least developed countries. Compulsory license application filed by M/s BDR Pharma is a good example for big Indian generic companies to think in this direction and target all anti-cancer and anti-HIV drugs which are available at a very high cost by patentee.

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INVITED ARTICLE

CHEMOMETRIC ASSISTED SPECTROPHOTOMETRIC AND HPLC METHODS FOR THE ESTIMATION OF AMLODIPINE BESYLATE AND TELMISARTAN MARKETED FORMULATION

Khushboo Khatri, Prachi R. Bhamre and S. J. Rajput*

Quality Assurance Laboratory, Centre of Relevance and Excellence in Novel Drug Delivery System, Pharmacy Department, G. H. Patel Building, Donor's Plaza, The Maharaja Sayajirao University of Baroda, Fatehgunj, Vadodara – 390 002, Gujarat, India.

Abstract

Chemometric assisted spectrophotometry and HPLC methods have been developed for the simultaneous determination of Amlodipine besylate and Telmisartan. The two chemometric methods i.e. Inverse least square (ILS) and Classical least square (CLS) methods were successfully applied to quantify each drug in the mixture using the information included in the UV absorption spectra of appropriate solutions in the range of 230-302 nm with the intervals of 4 nm at 19 wavelengths. The HPLC method was developed and the three methods (ILS, CLS and HPLC) were successfully applied to marketed formulation and the results were compared statistically which showed no significant difference among the results.

Keywords: *HPLC, chemometrics, spectrophotometric, amlodipine besylate, telmisartan, CLS, ILS*

***Correspondent Author:** Email: sjrajput@gmail.com, Phone: +91-9998309662*

Introduction

Amlodipine besylate (AML) is chemically described as (3-Ethyl 5-methyl (4RS)-2-[(2-aminoethoxy) methyl] - 4 - (2-chlorophenyl) - 6 - methyl - 1, 4 - dihydropyridine - 3, 5 - dicarboxylate benzene sulfonate). AML is a calcium-channel blocking agent; a dihydropyridine derivative with an intrinsically long duration of action and can be given once daily.AML is an antihypertensive and used as a prophylaxis for angina.

Telmisartan (TEL) is an angiotensin II receptor (type AT1) antagonist used in the management of hypertension. TML prevents the constriction (narrowing) of blood vessels (veins and arteries). It is a non-peptide molecule and chemically described as 4'-[(1,7'-Dimethyl-2'-propyl-1H,3'H -2,5'-bibenzimidazol-3'-yl) methyl]-2-biphenyl carboxylic acid. The combination of AML and TEL has been reported to show substantial and sustained 24 hour blood pressure reduction and is well-tolerated in a range of patients with hypertension and at risk of cardiovascular events. AML and TEL are available in combined tablet dosage form for the treatment of hypertension.

Literature review reveals several analytical methods for the estimation of AML and TEL alone or in combination with other drugs. Several spectrophotometric methods [1-3], HPTLC method [4] chromatographic methods [5-15], stability indicating analytical methods [16, 17] and methods for determination of AML and TEL in human plasma [18-20] have been reported.

The UV spectra of AML and TEL are highly overlapping and hence very few spectrophotometric methods are available in the literature for their simultaneous estimation. In the present study the chemometric approach has been applied to resolve the spectra and enable the simultaneous determination of AML and TEL in the marketed formulation alongwith the HPLC method. The results obtained by chemometric methods have been statistically compared with the HPLC method to confirm the results.

Materials and Methods

Instrumentation

Chemometric spectrophotometry

A double-beam Shimadzu UV-1700 spectrophotometer (Kyoto, Japan) connected to a computer loaded with Shimadzu UV Probe 2.10 software was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were carried out in 1cm quartz cells over the range of 200-400 nm. The numerical calculations for ILS and CLS methods were performed by using MATLAB R2007a Software and Excel.

HPLC

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV absorbance detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20µL. Separation and quantitation were made on a reversed-phase, Hypersil BDS

C_{18} column (250×4.6 mm i.d, 5 µm particle size). Detector was set at 238nm. Data acquisition and integration was performed using Spinchrome software (Spincho Biotech, Vadodara).

Materials and Reagents

AML and TEL (Bulk drugs) were kindly gifted by Cadila pharmaceutical limited, Ahmedabad. HPLC grade methanol (Qualigens fine chemicals, Mumbai) was used for HPLC and AR grade methanol (Qualigens fine chemicals, Mumbai) was used for spectrophotometry. Potassium dihydrogen orthophosphate KH_2PO_4 (AR grade: Qualigens fine chemicals, Mumbai), ortho phosphoric acid (AR grade: Qualigens fine chemicals, Mumbai), acetonitrile (HPLC grade, Qualigens fine chemicals, Mumbai) were used for HPLC. Double distilled water filtered through nylon filter paper 0.2 µm pore size and 47 mm diameter (Pall Lifesciences, Mumbai, India), was used throughout the analysis. The marketed formulation of Telma-AM tablet (Glenmark Pharmaceuticals Ltd.) containing 5mg AML and 40mg TEL per tablet was taken for the study.

Experimental conditions
Chemometric spectrophotometry

The UV absorption spectra of appropriate solutions were recorded in the wavelength range 200-400nm with the intervals of 4nm ($\Delta\lambda$ = 4nm) at 19 wavelength points. The scanning range selected was 230-302nm.

HPLC

The mobile phase was prepared by mixing acetonitrile and 0.01M phosphate buffer. The phosphate buffer was prepared by dissolving 0.27 g of potassium dihydrogen phosphate in sufficient double distilled water to produce 100 mL, 0.4 ml of triethylamine (TEA) was added and the pH was adjusted to 2.5 with orthophosphoric acid and filtered through 0.2 µm nylon filter paper. Acetonitrile and phosphate buffer were mixed in a ratio of 45:55% v/v and degassed by sonicating for 5 min in ultrasonic bath. The flow rate was maintained at 1.0 mL/min. All determinations were performed at ambient temperature. Quantitation based on peak area was achieved with UV detection at 238nm. The injection volume was 20 µL.

Standard solutions and calibrations

AML stock solution (1000 ppm) was prepared by dissolving 13.9mg of Amlodipine Besylate (equivalent to 10 mg of AML) in 10 mL methanol. TEL stock solution (1000 ppm) was prepared by dissolving 10 mg of TEL in 10 mL methanol. The stock solutions were ultrasonicated for 1 min. The working solutions of AML and TEL (100 ppm) were prepared by transferring 1 mL aliquot from stock solution to a 10 mL volumetric flask and making up the volume with mobile phase in case of HPLC and making up the volume with methanol in case of chemometric spectrophotometry.

Preparation of solution mixture sets for chemometric estimation

A calibration set of 24 mixtures was prepared in methanol, applying a multilevel multifactor design in which two levels of concentrations of AML and TEL

Table 1: Calibration Set and Validation Set

Calibration Set			Validation Set		
Sr. No.	AML	TEL	Sr. No.	AML	TEL
1	2	16	1	5	2
2	4	16	2	5	4
3	6	16	3	5	6
4	8	16	4	5	8
5	7	2	5	2	14
6	7	4	6	4	14
7	7	6	7	6	14
8	7	8	8	8	14
9	2	0	9	30	4
10	4	8	10	30	6
11	0	16	11	30	8
12	0	2	12	30	10
13	25	4	13	15	5
14	25	6	14	25	5
15	25	8	15	35	5
16	25	10	16	45	5
17	25	12	17	1	8
18	0	7			
19	10	7			
20	20	7			
21	30	7			
22	40	7			
23	50	7			
24	60	0			

Concentrations in µg/mL

within the linearity range were introduced as shown in Table 1. A validation set containing 17 synthetic mixture solutions was prepared as shown in Table 1. The linearity range of AML was found to be 1-60 µg/mL and of TEL was found to be 1-18 µg/mL. UV spectra were recorded in the wavelength range 200-400 nm versus solvent blank and digitalized absorbance was recorded at 4 nm intervals.

Preparation of calibration curve for HPLC

Aliquots ranging from 0.1 mL to 1 mL of AML and 0.8 mL to 8 mL of TEL were

taken, from working stock solution, in 10 mL volumetric flask and diluted upto the mark with the mobile phase to obtain the final concentration of binary mixtures as marketed available formulation. Triplicate 20 µL injections were made for each concentration and chromatogram was obtained under the specified chromatographic conditions described previously. The calibration graph was constructed by plotting peak area versus concentration of each drug and the regression equation was calculated.

Sample preparation

Twenty tablets were weighed and finely powdered. The powder equivalent to one tablet (5 mg AML and 40 mg TEL) was accurately weighed and transferred to volumetric flask of 100 mL containing 50 mL of the methanol and sonicated for 15 min at cool temperature. The above solution was carefully filtered through whatman filter paper (No. 41) to 100mL volumetric flask and residue were washed thrice with methanol , the combined filtrate was made up to the mark with methanol.

For chemometric spectrophotometry, an aliquot of 0.2 mL was pipette out from above prepared solution and transferred to volumetric flask of 10 mL. Volume was made up to the mark with methanol to give a solution containing 1: 8 µg/mL of AML:TEL. This solution was used for the estimation of AML and TEL.

For HPLC, an aliquot of 1 mL was pipette out from above prepared solution and transferred to volumetric flask of 10 mL. Volume was made up to the mark with mobile phase to give a solution containing 5: 40 µg/mL of AML:TEL. This solution was used for the estimation of AML and TEL.

Results and Discussion

Chemometric spectrophotometry

Figure 1 shows the overlain zero-order absorption spectra of AML, TEL and their binary mixture solution in methanol in the 200-400nm absorption region.

The ratio of AML and TEL in commercial tablet is 1:8. The zero-order absorption spectra (Figure 1) of AML and TEL completely overlap with each other making it difficult to use conventional spectrophotometric techniques for simultaneous analysis. Thus the ILS and CLS chemometric spectrophotometric methods were found to be appropriate for determination of AML and TEL in binary mixture. A training set (calibration set) of 24 synthetic binary mixture solutions (Table 1) and a validation set containing 17 synthetic binary mixture solutions (Table 1) in the possible combinations were prepared with methanol (AR grade). The UV absorption spectra of appropriate solutions were recorded in scanning range of 200-400nm and the wavelength range selected was 230-302nm with the intervals of 4nm ($\Delta\lambda$ = 4nm) at 19 wavelength points.

CLS method

CLS is one of the simplest methods, based on a linear relationship between the absorbance and the component

concentrations at each wavelength. In matrix notation, the Beer's law models for m calibration standards containing l chemical components with spectra of n digitised absorbance is given by

$A = C \times K + EA$ ..... *(Eq. 1)*

where A is the m x n matrix of calibration spectra, C is the m x l matrix of component concentrations, K is the l x n matrix of absorbance-concentration proportionality constants (absorptivity-pathlength) and EA is the in m x n matrix of spectral errors or residuals not fit by the model.

In this method, the coefficient matrix (K) was calculated by using the linear equation system between the absorbance data and training set. The observed absorbance values of the compounds in the binary mixture solutions, at the 19 wavelength points with the interval of 4nm ($\Delta\lambda$ = 4nm) in the spectral region from 230nm to 302nm, were replaced in the equation 1 and the amount of AML and TEL in the synthetic mixtures (validation set) were calculated.

ILS method

In this method, the coefficient matrix (P) was obtained from the linear equation system using the absorbance data and the calibration set. Introducing (P) into the linear equation system we obtain the calibration for ILS, as this method treats concentration as a function of absorbance. The inverse of Beer's law model for m calibration standards with spectra of n digitised absorbance is given by

$C = A \times P + Ec$ ..... *(Eq. 2)*

Where C and A are as before, P is the n x l matrix of unknown calibration co-efficient relating the l component concentrations of the spectral intensities and Ec is the m x l vector of errors. Since in ILS the number of wavelengths cannot exceed the total number of calibration mixtures, stepwise multiple linear regressions have been used for the selection of wavelengths. The observed absorbance values of the compounds in the binary mixture solutions, at the 19 wavelength points in the spectral region from 230nm to 302nm, were replaced in the equation 2 and the amount of AML and TEL in the synthetic mixtures (validation set) were calculated.

Validation of chemometric methods

The tested mixtures for ILS and CLS were subjected to recovery studies. The results obtained were in good agreement with the true values. These results of percentage recoveries and standard deviation are shown in (Table 2) which determines the accuracy of the chemometric methods. The numerical values of the statistical parameters (Table 3), such as plots of predicted versus true value (their regression coefficient), RMSEP (Root mean square error of prediction) value indicate that the proposed techniques are suitable for the determination of these drugs in the tablet formulation as excipients do not interfere. Moreover the plots of residual value versus predicted concentration show that the residual values for the model are close to zero and more randomly distributed (Figure 2). The ILS and CLS models were then applied to estimate AML and TEL in commercial

tablet preparations. The results are shown in (Table 4).

Selection and optimization of chromatographic condition

Optimization of chromatographic procedure

Different solvents in mixtures of varied proportions, at different flow rate and wavelength were tried to select the mobile phase. The mobile phase with water:acetonitrile 50:50 gave broad peaks. Hence trials were carried out with different pH of phosphate buffer and acetonitrile at pH 2.5, 4.5 and 6. Among these trials the most appropriate peak shape and resolution alongwith appropriate system suitability parameters (according to ICH guidelines) were obtained with pH 2.5 with a ratio of phosphate buffer:acetonitrile 55:45 with 0.4% TEA (Table 5). TEA was added to minimise the tailing of the peaks. The optimized chromatogram of the laboratory sample solution has been shown in Figure 3.

Selection of common wavelength

The sensitivity of HPLC method that uses UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. In the present study individual drug solutions of 1µg/mL of AML and 8µg/mL TEL were prepared in methanol. As the absorbance and concentration of AML is less than TEL its absorbance maxima 238nm (Figure 1) was selected as the common wavelength for simultaneous estimation of the two drugs.

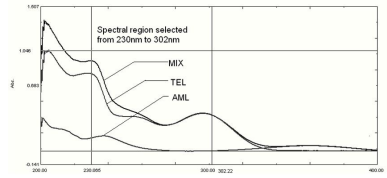

Figure 1: The overlain zero-order absorption spectra of AML(1 ppm), TEL(8 ppm) and their binary mixture(AML:TEL 1:8 ppm) in methanol

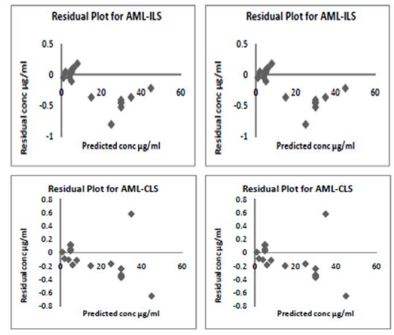

Figure 2: Concentration residuals vs actual concentration plot for AML and TEL for CLS and ILS

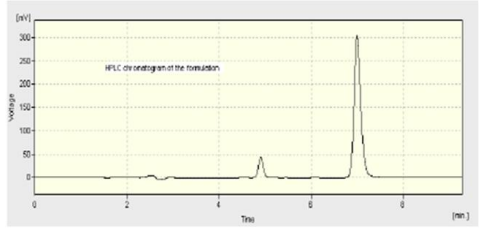

Figure 3: HPLC chromatogram of the formulation

Validation of HPLC method

Validation of the HPLC method was carried out according to ICH guideline [21].

Linearity and Range

The linearity study was carried out for both drugs at ten different concentration levels. The linearity of AML and TEL were in the

range of 1-10 µg/mL and 8-80 µg/mL respectively.

Precision

Precision was estimated by the determination of the repeatability of the method. Binary mixtures containing three different concentrations were prepared with mobile phase. 20 µL of the standard solutions were injected and chromatograms were recorded. The peak area of AML and TEL were calculated for each trial. The experiment was repeated three times in a day (intra-day precision) and repeated on three different days (inter-day precision). The average % RSD (relative standard deviation) values of the results were calculated which were found to be less than 2%, which confirms that the method is precise.

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at three level of standard addition from 80 % to 120 % of label claim. The results are shown in Table 4. Recovery greater than 98 % with low SD (standard deviation) justifies the accuracy of the method.

Table 2: Percentage recovery results of AML and TEL in synthetic mixtures (validation set) by the proposed ILS and CLS chemometric methods

Sr. No.	Mixture Composition (µg/mL)		%Recovery			
			ILS		CLS	
	AML	TEL	AML	TEL	AML	TEL
1	5	2	102.08	98.11	97.54	97..62
2	5	4	98.46	99.06	97.8	99.77
3	5	6	98.96	100.55	98.97	99.71
4	5	8	99.15	98.78	99.388	98.28
5	2	14	97.68	96.88	104.37	99.4
6	4	14	101	98.23	102.64	98.59
7	6	14	98.16	99.04	103.025	98..08
8	8	14	97.75	98.8	101.4	98.56
9	30	4	101.75	101.38	101.22	101.77
10	30	6	101.36	101.73	100.79	100.56
11	30	8	101.52	102.19	101.1	102.44
12	30	10	101.5	101.69	101.17	102.62
13	15	5	102.43	101.65	101.29	102.31
14	25	5	101.22	101.99	100.66	102.1
15	35	5	101.04	102.99	98.33	102.38
16	45	5	100.48	97.12	101.43	101.76
17	1	8	104.79	98.27	99	97.31
	AVG		100.5488	99.90941	100.5955	100.504
	SD		1.925714	1.949287	1.872517	1.807041
	%RSD		1.915203	1.951054	1.861433	1.797979

Table 3: Statistical parameters of AML and TEL in synthetic mixtures (validation set) using the proposed ILS and CLS chemometric methods

Statistical Parameter	AML		TEL	
	CLS	ILS	CLS	ILS
Range (µg/mL)	2-60		1-18	
Regression equation*	y =1.007x - 0.013	y=1.014x - 0.043	y=0.979x + 0.147	y=0.975x + 0.164
Regression Coefficient	0.999	0.999	0.998	0.998
RMSEP	0.176	0.344	0.155	0.176

*Regression equation of predicted Vs true concentration

Table 4: Summary of the validation parameters.

Parameters	AML	TEL
Wavelength (nm)	238nm	
Linearity range (µg/mL)	1-10	8-80
Accuracy*	100.45 ± 0.69	100.72 ± 1.33
Precision(%RSD)		
Intraday	0.60	0.40
Interday	0.71	0.40
Regression Equation: y=mx+c	y = 23.8663x + 64.5460	y = 57.6180x - 3.5013
Correction coefficient	0.9990	0.9998
LOD (µg/mL)	0.210	0.041
LOQ(µg/mL)	0.639	0.125

*Average of 80, 100 and 120% standard addition

Table 5: Results of system suitability parameters

Parameters	AML	TEL
Theoretical Plates ± RSD	9080.9±1.81	9666.3 ± 1.76
Asymmetry Factor± RSD	1.155± 0.162	1.189± 0.345
Resolution ± RSD	--	9.05± 0.458

Table 6: Assay results of the commercial tablet preparation (Telma-AM) by the proposed ILS, CLS chemometric methods and HPLC method

Formulation	TELMA-AM (Glenmark)					
Drug	**Amlodipine**			**Telmisartan**		
Labeled claim	5(mg/tab)			40(mg/tab)		
Methods	HPLC	CLS	ILS	HPLC	CLS	ILS
Amount Found (mg)	5.007	5.09	5.06	40.032	39.65	39.8
% Amount Found	100.14 ± 1.05	101.8 ± 1.74	101.13 ± 2.04	100.08 ± 1.37	99.13 ± 1.96	99.50 ± 1.80
P-value		0.615			0.528	
F-value		0.527			0.712	

Average of 5 determinations for HPLC
Average of 3 determinations for CLS and ILS
F-crit value 5.14

Limit of detection (LOD) and Limit of quantitation (LOQ)

Calibration curve was repeated 5 times and the SD of the intercepts was calculated. Then LOD and LOQ were measured as follows

$$LOD = \frac{3.3 \times S.D.}{Slope\ of\ calibration\ curve} \quad (Eq.\ 3)$$

$$LOQ = \frac{10 \times S.D.}{Slope\ of\ calibration\ curve} \quad (Eq.\ 4)$$

SD = Standard deviation of intercepts

The theoretical values were assessed practically. The detection limits were found to be 0.21 and 0.041 µg/mL for AML and TEL, respectively. The quantitation limits were found to be 0.639 and 0.125 µg/mL for AML and TEL, respectively. (Table 4)

Robustness

Robustness of the method was examined by changing factors at three levels (-1, 0, 1) such as: flow rate (0.9, 1.0, 1.1 mL/min), mobile phase ratio (acetonitrile composition: 44, 45, 46%) and pH of the buffer (2.4, 2.5, 2.6). One factor at the time was changed to estimate the effect. Thus replicate injections (n=3) of standard solution at three concentration levels were performed under small changes of three chromatographic parameters (factors). Results indicate that the selected factors remained unaffected by small variation of these parameters. It was also found that acetonitrile of different lots from the same manufacture had no significant influence on the method. Insignificant difference in asymmetric factor and less variability in retention time were observed.

Stability in sample solutions

Binary mixtures containing two different concentrations of AML and TEL were prepared from standard stock solution and stored at room temperature for 24hrs. They were then injected in to LC system. There was no change in signal intensity and no additional peak found in chromatogram indicating the stability of AML and TEL in the sample solution.

System suitability

The system suitability parameters including resolution (Rs), asymmetric factor (As) and theoretical plates were established by 10 replicates of binary mixture of AML and TEL (1:8 μg/mL, respectively).

Analysis of commercial tablet preparation

The proposed multivariate ILS and CLS spectrophotometric methods and HPLC method were applied to the simultaneous determination of AML and TEL in commercial Telma-AM tablet preparation. Satisfactory results were obtained for each compound which were in good agreement with the label claim. The assay results of the proposed ILS and CLS spectrophotometric methods were compared to those of the proposed HPLC method. The results obtained were statistically analyzed by one way ANOVA at 95% confidence level. The results are shown in Table 6. The calculated values did not exceed the theoretical ones; indicating that there was no significant difference between the methods compared.

Conclusion

RP-HPLC techniques are generally used for separation and determination of components in final pharmaceutical preparations and are superior with regard to identification and specificity. However, the chemometric methods are less expensive by comparison and do not require sophisticated instrumentation nor any prior separation step. The proposed chemometric-assisted spectrophotometric methods are applicable, prompt and specific for the simultaneous determination of AML and TEL in their synthetic mixtures and commercial pharmaceutical tablets. The results obtained were compared with the proposed RP-HPLC method and good coincidence in the means of recovery was observed as there was no significant difference between the methods compared. The three proposed methods were accurate, precise with good reproducibility and sensitivity; hence can be used for the routine analysis of AML and TEL in their combined pharmaceutical formulations.

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INVITED ARTICLE

BLOOD-BRAIN BARRIER PERMEATION OF PACLITAXEL

Aditya Grover, Anjali Hirani, Vijaykumar Sutariya*
Department of Pharmaceutical Sciences, College of Pharmacy,
University of South Florida, Tampa, FL 33612, USA

Abstract

The glutathione-conjugated methylene blue loaded nanoparticles and glutathione-conjugated coumarin loaded nanoparticle were constructed in the present work as biodegradable carrier for brain-specific drug delivery along with evaluation of its *in-vitro* permeation properties. Nanoparticles of PLGA was prepared by reacting it with glutathione using EDAC as a linker, which was also characterize for particle size analysis. The *in-vitro* study performed for permeation property of developed nanoparticles using Trans well co-culture of C6 (rat astrocytoma) cells and RBE4 (rat brain endothelial) cells showed significant permeability of glutathione conjugate nanoparticle. Significant *in-vitro* permeability suggest that glutathione serve as a better vehicle to deliver paclitaxel in brain during brain cancer therapy.

Keywords: *Glutathione, Paclitaxel, Brain cancer, Nanoparticle*

***Correspondent Author:** Email: vsutariy@health.usf.edu, Phone: +1-813-974-1401*

Introduction

Brain cancers, primarily glioblastomas, present with poor prognoses in most patients and have very high clinical malignancies [1]. Central nervous system (CNS) tumors are associated with a number of undesirable side effects, namely nausea and vomiting, headaches and seizures [2]. Gliomas are mainly primary tumors which arise from glial cells; however, there are instances of metastasized breast and lung cancers that induce the formation of brain tumors [2,3]. Gliomas are classified into three main categories by the World Health Organization (WHO): astrocytomas, oligodendrogliomas and oligo-astrocytomas; and each of them is graded from I to IV with an increasing level of malignancy [3]. Although, a number of drugs express promising *in-vitro* efficiency against brain cancers, the therapeutic outcome of these drugs are observed to be limited [4]. This may be due to the Blood Brain Barrier (BBB), which develops through the interactions of endothelial cells, astrocytes and pericytes; resulting into formation of tight junctions in the endothelial cells, which prevents the entry of the drug and other substance into brain. BBB is the major hurdle for variety of anti-cancer drug in the treatment of brain cancer.

Paclitaxel is proven potent anti-cancer drug for brain tumor; however, the permeability through the blood brain barrier is the rate limiting parameter for paclitaxel drug delivery. Paclitaxel is commercially administered as Taxol, belongs to the taxane family of drugs, which is derived from the bark of the pacific yew tree (*Taxus brevifolia*) and has been clinically used against a number of cancers including ovarian and breast cancers and particularly significantly active against malignant gliomas and metastasis [1,2,5,7]. Paclitaxel has a unique mechanism of action that it promotes the assembly of microtubule which in turn prevents depolarization for cell division in the G2 or M phase of cell mitosis causing the cell death [3]. However, specific structural property of brain anatomy and limited permeability of paclitaxel reduces the access of drug at brain tumor. Additionally, Paclitaxel structure is also the subtract for multidrug resistant protein, which express p-glycoprotein (p-gp). Such expressed proteins like p-gp transporter efflux-out the paclitaxel with their transport mechanism [7, 8]. Hence, in conclusion, variety of research it has been proved that permeability of paclitaxel in brine is limiting parameter for brain cancer therapy. According to Fellner et al [6] 90 % of brain tumor was observed to be reduced by administration of paclitaxel with p-gp inhibitor as compare to paclitaxel alone.

The poor aqueous solubility of paclitaxel due to macro-molecular structure, limits the development of conventional intravenous injections to improve bioavailability at site of action. To improve the solubility of paclitaxel, the co-solvency approach have been explored, whereby 1:1 volume ratio of Cremophor EL and ethanol has shown improved pharmaceutical

perspectives of formulation [4]. However, cremophor EL has been shown to cause hypersensitivity reaction, which limits the clinical application. Although, paclitaxel has been successfully used in a number of different cancers, its use in brain cancers has been limited due to the blood-brain barrier (BBB), which impedes the permeation of possible toxins into neural tissue. To surmount the BBB's challenge to brain-targeted drug delivery, many attempted has been like liposomes, nanoparticles, micelles and conjugates etc. [9]. After variety of attempts, few formulation (i.e. Xyotax) has been proved as a promising against in phase-II and phase-III clinical trials for brain metastasis [10]. However, the review from the scientist does not expect its efficiency to deliver paclitaxel in to the brain for brain cancer. Therefore, the in-depth research is still required to deliver paclitaxel in brain with high efficiency. The Aim of this present work is to develop efficient carriers, which are able to deliver anti-cancer drug in to the brain for brain cancer therapy.

In present investigation, glutathione was explored as a coating material for efficient delivery of paclitaxel nanoparticle in to the brain for brain cancer. Out of number of mechanisms proposed to induce the trans-BBB permeation of drugs, a glutathione method has shown success in *in-vitro* and *in-vivo* studies [11, 12]. Glutathione is the substrate for p-gp protein which is express in brain and therefore the nanoparticles conjugated with glutathione may be able to cross BBB. Therefore, it was predicted that conjugation of glutathione with nanoparticles loaded with methylene blue as well as nanoparticles loaded with coumarin may improve the permeation of drug through BBB.

Material and method

Material

PLGA was supplied by CHS pvt. Ltd., Acetone and Poloxamer 188 were purchased from sigma eldritch, EDAC was procured from MP Biomedical. Glutathione, Coumarine, Methylene blue were supplied by Venus Chemicals. Methanol used was of analytical grade. Deionized water used for further experiments was obtained from Millipore.

Preparation of methylene blue loaded nanoparticles

The methylene blue loaded PLGA nanoparticles were prepared by nanoprecipetation method proposed by Patel N et al. [13] Initially, the PLGA was dissolved in acetone with pluronic F-108. Methylene blue was was separately mixed with acetone using magnetic stirrer. Organic solvent containing PLGA, pluronic F-108 and methylene blue were added drop wise at the rate of 2ml/min into the deionized water under continuous stirring at 900 RPM on magnetic stirrer. The organic solvent was allowed to evaporate at room temperature on constant stirrer, resulting in formation of methylene blue loaded nanoparticles of PLGA. Further, the dispersion was centrifuge at 5000 RPM to separate the nanoparticles from the suspension and were lyophilized to obtain free flowing powder.

Preparation of coumarin loaded nanoparticles

The coumarin loaded PLGA nanoparticles were prepared by method proposed by Vanya B et al. [14] The 1:10 proportion of coumarin: PLGA were dissolved in 5 mL of acetone. The solution was mixed with 10mL of aqueous poloxamer 188 solution. The resulting suspension was stirred alongwith heating at 60°C using a magnetic stirrer for 3 hours to remove the organic solvent from dispersion. Further, the suspension was filtrated through a "white tape" paper filter and lyophilized to get free flow powder.

Conjugation of glutathione with nanoparticles

Conjugation of glutathione with nanoparticles was carried out as per the methodology proposed by Acharya S et al [15]; whereby 60 mg of nanoparticles were added in 2.4 mL phosphate buffer (pH 4.0) followed by drop-wise addition of an equal volume of EDAC solution having concentration of 50 mg/mL. The resultant mixture was incubated at room temperature for 45 minutes. The un-reacted EDAC was removed by centrifugation and the nanoparticles were re-suspended in 2.4 mL phosphate buffer (pH 7.4). The same volume of glutathione solution having the concentration of 50 mg/mL was added to the dispersion containing the activated nanoparticles and the mixture was stirred gently at room temperature for 6 hours. The conjugated nanoparticles were centrifuged for 30 minutes at 15000 RPM to remove excess of glutathione. Finally the glutathione conjugated nanoparticles were lyophilized to get free flowing powder. Nanoparticles were prepared using a nanoprecipitation method and were coated with enough reduced glutathione to yield a 2% w/v coating on the nanoparticles.

Particle size measurement of nanoparticles

Particle size and particle size distribution was measured by Malvern's zeta-sizer. The nanoparticles were dispersed in suitable solvent and sonicated to prevent the agglomeration. Later on, the particle size was characterized after appropriate dilutions with deionized water.

In-vitro study

The *in-vitro* permeation of glutathione conjugated nanoparticles of PLGA was performed through establishing a Trans-well co-culture of C6 (rat astrocytoma) cells and RBE4 (rat brain endothelial) cells [12] as shown in Figure 1, whereby the cell culture was allowed to developed on both the sides of permeable support.

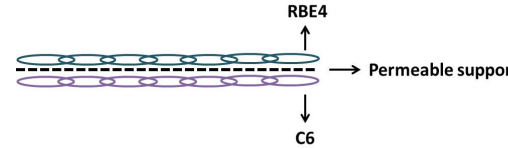

Figure 1: Representation of the Transwell apparatus set-up to investigate the in-vitro permeability.

The comparative trans-BBB permeation of 10 μM each of MB free drug solution, uncoated MB nanoparticle and 2% glutathione-coated MB nanoparticle was

carried out by taking readings at predetermined intervals (0, 3, 6 and 24 hours).

Results

PLGA Nanoparticles

Experimental variations like process related parameters (i.e. stirring speed, rate of addition, evaporation rate, evaporation temperature, centrifugal force, incubation time, etc.) as well as the formulation parameters (i.e. concentration of polymers; type, volume and ratio of organic solvent; etc.) have shown to have the significant effect on the formulation and development of nanoparticles.

Particle Size measurement

Mean diameter of glutathione conjugated methylene blue loaded nanoparticle was found to be 220 nm, whereas glutathione conjugated coumarin loaded nanoparticle was 250 nm. Moreover, the particles size distribution for the glutathione conjugated methylene blue loaded nanoparticle was ranging between 190 mm to 250 nm, whereas glutathione conjugated coumarin loaded nanoparticle was between 200 nm to 300 nm.

In-vitro study

In-vitro study shown that the glutathione conjugated nanoparticles showed a significant increase in their trans-BBB permeation over 24 hours, while the free drug solution showed almost nil permeation across the Transwell system (Figure 2). Similar result pattern was observed for both the types of nanoparticles.

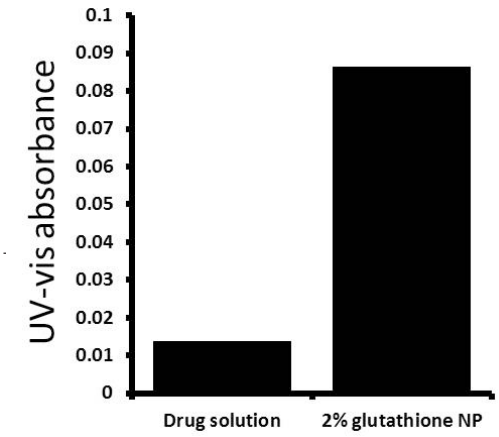

Figure 2: Trans-BBB permeation of MB drug solution as compared to MB-loaded, glutathione-coated nanoparticles after 24 hours of treatment in Transwell apparatus.

Discussion

PLGA Nanoparticles

The results revealed that formulation of nanoparticle is very complicated process and variety of process related parameters and the formulation related parameters significantly affects the physico-chemical properties of nanoparticles, which in turn affect the therapeutic outcome of the nanoparticles. Hence, careful design and optimization of each variable is essential to achieve desired properties of nanoparticles.

Particle size measurement

Experimental variables like amount of polymer, amount of surfactant content and amount of drug affect significantly to particle size distribution. The amount of

PLGA content is directly proportional to particle size diameter. According to Quintanar Guerrero et al. [16] study of PLGA content influence on nanoparticle size distribution, which suggest that as higher polymer amount promotes the aggregation due to less repulsive force between two particles, leading to increase in the mean particle size by aggregating with each other. The drug loading also showed direct relation with particle size distribution, due to higher entrapment efficiency of carrier and free drug tries to absorb on surface of nanoparticle causing aggregate [17]. Glutathione content also increases bulkiness of particle after conjugation, therefore, over-time of conjugation increases particle size [18].

In-vitro study

The *in-vitro* study of the glutathione conjugated coumarin loaded nanoparticles shown significant tran-BBB permeation as compare to unconjugated nanoparticle, which suggest that p-gp transporter efflux-out unconjugated nanoparticle successfully; however, the glutathione conjugated nanoparticles are able to cross the BBB. Overall uptake study reveals the vital role of glutathione as a carrier for trans-BBB permeation. Therefore glutathione may serve as an better vehicle for paclitaxel to deliver it in brain for the cancer treatment.

Conclusion

The *in-vitro* permeation study reveals that the glutathione conjugated nanoparticles of PLGA are able to cross the BBB. The success of this glutathione method should be investigated first *in-vivo* in animal study and then in human subjects to determine its clinical efficacy. This newer approach may provide a valuable therapeutic outcome and may provide the biomedical benefits in the number of therapies that are targeted to the brain and facilitates reduction of the morbidity faced by patients with CNS malignancies.

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INVITED ARTICLE

IDENTIFICATION OF A BIOMARKER: A NECESSARY APPROACH IN HERBAL INDUSTRY

R. Rajendran*
CEO, Green Chem (100% EOU), Banglore, India

Abstract

Herbs have now become a source of medicinal drugs that can be used as supplements or as - drugs for ailments of various disorders. Moreover, complementary medicines (i.e a combination of standard drug and herbal drug) may help in reducing side effects and increasing the drug potency. The market for herbal drugs is growing rapidly due to its use in different industries like medicinal, nutraceutical, cosmetic and textile . Due to the large scale usage of herbal drugs, government and private organizations are searching for ways to determine their quality, consistency, efficacy and safety. Hence, in the preliminary stage herbal ingredients have to be standardized and potential biomarkers in these herbs needs to be identified. Also, research is being conducted to find low cost detection methods using common spectroscopic instruments for specific biomarkers. This review provides a brief overview of the importance of biomarkers in the herbal industry.

Keywords: *Biomarker, consistency, herbal drug, identification, standardization*

Abbreviations: World Health Organization, WHO; Department of Ayurveda, Yoga and Neuropathy, Unani, Siddha and Homeopathy, AYUSH; Indian Pharmacopeia, IP; United States Phamacopeia, USP; European Pharmacopeia, EU; Therapeutic Good Administration of Australia, TGA

***Correspondent Author:** Email: rajendran@greenchem.biz, Phone: +91-080-25359569*

Introduction

Herbs are different in its own unique way; each herb has its own physiological, chemical, biological characteristics with a unique cultivation method required for each. Earlier some crops were only available in regions that were best suited for it, regions that had high yield and high active chemical constituents.

But, human beings changed the habitat of crops and consequently the genes of the seeds also changed, resulting in an increase in the chemical constituent content and a reduction in the growth period of these plants. Herbal drugs were foremost used in ancient times for treating various ailments and these drugs had a rapid onset of action and targeted specific efficacy. Nowadays, these herbs do not show such significant effects in clinical assessments. There are many reasons that may be causing a reduction in potency such as spurious seeds, non-favorable cultivation environments, usage of toxic chemical pesticides during cultivation, adulterated or spurious herbal drugs, inability to identify the correct solvent for extraction, herb-herb interaction and many more [1]. To overcome these limitations it is important to identify biomarkers.. A biomarker helps to identify a given herb both qualitatively and quantitatively. Moreover, the biomarker helps in identifying that the sample is the same as the prescribed by comparing with the said standard of the same species and this process may help in determining the presence of adulterated or spurious drugs. A biomarker can be easily identified by simple spectroscopic analysis, a cost effective method.[2].

Herbs are also identified by their physical characteristics (macroscopically and microscopically), chemical analysis and other physicochemical parameters [3]. There are many agencies that have standards available for these parameters such as the Indian Pharmacopeia (IP), United States Pharmacopeia (USP), European Pharmacopeia (EU) and Therapeutic Goods Administration of Australia (TGA). Recently, the Department of Ayurveda, Yoga and Neuropathy, Unani, Siddha and Homeopathy (AYUSH), under the Ministry of Health and Family Welfare(Government of India) has started developing monographs for herbs and herbal extracts by collaborating with different companies such as Green Chem, Bangalore, India. This will help in setting up a database that could be used as the standard for determining the herbs [4]. The World Health Organization (WHO) has also given different standards for research and evaluation in traditional medicine for different herbs [5].

Herbal fingerprinting

Research on herbal medicine is carried out to check the consistency, safety, quality, efficacy and its mode of action. Herbal drugs have been proved to have significant drug activity in different formulations but its consistency is sometimes questionable. To overcome this problem some standards need to be followed in steps such as identifying required species, maintaining the consistency of plant source (plant environment and cultivation pattern),

selecting the season of collection of plant parts and washing and storing these herbs. Herbal drugs are generally considered to be safe due to its natural origin. But, in today's world the use of chemical pesticide and different chemicals for the faster germination of crops with higher quantity output is generally preferred to increase profits. Due to the use of these chemicals the plant may absorb some of these chemicals and become toxic and the plant needs to be checked for its microbial load, heavy metal analysis and toxicity level and drug-drug interaction before usage [6]. In the health care system quality is an important criterion that has to be maintained.

WHO has given different standard monographs of different plant drugs to maintain the quality standard of a given plant drug.

Efficacy of a plant extract or a plant drug depends on the above characteristics of consistency, safety and quality. Various analyses should be carried out to know the effectiveness of the drug for a given symptom. Herb-Herb interactions and Herb-Drug interactions; if present, need to be specified to avoid adverse drug reactions. Also, it is conceptualized that the slow mode of actions of herbal drugs may be due to fact that the above criteria are not followed [7]. Moreover, the active chemical constituent from the plant for the required activity needs to be isolated. This will help in determining the higher efficacy and identification of the constituent giving the said activity [8].

Application of Biomarkers

Biomarkers help in the identification of a given disorder and detect the presence of a compound in a given product. In the herbal industry, biomarkers play an important role in identifying herbs and in identifying the chemical constituent giving the respective herb activity. In herbal drugs, sometimes adultered and spurious drugs are also used. The low quality/sub-standard drugs are generally mixed with the pure drugs to increase the profit margin. In order to identify the correct drug and its constituent, the biomarkers play an important role [9]. Biomarkers ensure that the distributor is providing the correct sample. There are various detection methods for identification of biomarkers such as the ultraviolet visible spectra, high performance thin layer spectroscopy spectra, high performance liquid chromatography spectra, infrared spectra and many more. The simpler the method, the faster and more cost effective it is.

Herbal drugs have complex constituents and identification of these active constituents can be difficult. There are some markers that are present in all the species of one genus so identification of the correct biomarker is important [10].

Regulatory Requirement

Regulations may vary from country to country as they are influenced by their environmental conditions. Earlier the herbal industry didn't have any regulatory body. Nowadays, with use of plant hybridization techniques and other

chemicals for growth of herbs, the regulations have become necessary to maintain the safety and efficacy of these drugs [11].

The WHO has set guidelines to detect the botanical characters, sensory evaluation, foreign organic matter, microscopic, histological, histochemical assessment and quantitative measurement. The WHO has also set guidelines for, the physical and chemical identity, fingerprints chromatography, ash values, extractive values, moisture content, volatile oil and alkaloids tests. Also guidelines for the quantitative estimation protocols, estimation of biological activity, the values of bitterness, astringency hemolytic index, a factor swelling, foaming index, detail-toxicity pesticides residues, heavy metals, microbial contamination as viable count, total pathogens such as *E. coli, Salmonella, P. aeroginosa, S. aureus, Enterobacteriaceae*, microbial contamination and radioactive contamination needs to be followed. In India, the herbal products must satisfy the requirements specified by the Department of AYUSH, a department that is preparing the standards for herbs and herbal extracts. The other requirements that need to followed are specified in the Food, Drug and Cosmetic Act. Now, regulatory authorities have started to be focused on labeling requirement such as the botanical source of the herbs, the quantity used and the expiry of the same [4].

In the United States, herbal drugs come under the purview of the Dietary Supplement Health and Education Act, which states that any herb, botanical and natural concentrate, metabolite and constituent of extract, is classified as a dietary supplement. Moreover, dietary supplements do not require approval from Food and Drug Administration (US FDA). USFDA considers dietary supplements safe and only requires that the label is not misleading or adulteration/spurious drugs are not added. The label should specify all the GLP criteria like the drug intended use, side effect if any, drug-herb interaction and dosage of the product [12]. The European Directive provides the guidelines for the use of herbal medicines pertaining to Europe. European laws are very stringent towards the use of herbal medicine. They have stringent measures such as mandating that the herbal medicinal products need to be medically used for atleast a period of 30 years in the European Union, at least 15 years within the EU and 15 years elsewhere for products outside the EU.

The monographs of the products should also be attached with comparison to the standards [13].

Perspective

Since ancient times, herbs have been used as medicines for treating various disorders and as supplements for prevention of the disorder. Medications currently available are generally derived from plants or from the bases of plant drugs [14]. So, it is necessary to prove the safety and efficacy of these herbs with scientific evaluations. Due to different plant cultivation regions and hybridization of plant parts, the identification of the plant material is

important for better efficacy and activity [15]. Also, the consistency of the herbs needs to be evaluated with the available spectroscopic techniques. For this reason, a biological fingerprinting or a biomarker is required to ensure the consistency, safety, quality, efficacy and mode of action of these herbs. Ayurveda knowledge and traditional usage information are excellent, but using these in a scientific way and incorporating a pharmaceutical approach will be more meaningful to ensure the safety of consumers.

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INVITED ARTICLE

MICRODOSING: A PHASE 0 CLINICAL TRIAL

Aman Romesh Khanna, Jignesh L. Patel*
Clinical Pharmacology Unit, Sun Pharmaceutical Industries Ltd.,
Tandalja, Vadodara-390020, India.

Abstract

The process of new drug development requires extensive non-clinical safety testing in order to generate sufficient data to move to clinical development and then for market entry. Not only clinical development, but also non-clinical safety and pharmacology testing are time consuming, costly and require lot of resources. Many-times, after clearing non-clinical safety and pharmacology testing, the drug candidate may fail to demonstrate desired pharmacokinetic in clinical development phase. Suboptimal pharmacokinetic is one of the reasons for failure of the drug in demonstrating desired clinical efficacy and safety in later clinical development phase. Therefore, it is very important to have idea of pharmacokinetic of the drug candidate in human at least to make early "go/no-go" decision during development in order to reduce the time and cost of development. Microdosing is a new technique to obtain human pharmacokinetic information before the usual expensive clinical phase of drug development starts. Microdose studies uses less than 1/100th of the dose calculated to yield a pharmacological effect of the drug and hence, risk to trial subject is very less in microdose studies and it can be initiated early after completing limited non-clinical studies. It uses highly sensitive and specific analytical methods for estimating drug and metabolite concentrations in picogram to fentogram range. Experience of various drug developers with microdose studies so far suggests that microdosing is a better tool for predicting human pharmacokinetics early and may be helpful in order to make early "go/no-go" decision in drug development.

Keywords: *Microdosing, Phase 0 Clinical trial, drug developement*

***Correspondent Author:** *Email: jignesh.patel@sunpharma.com*

Introduction

Drug development is a scientific endeavor and is highly regulated because of legitimate public health concerns. Drug development process involves integration of different expertise, conduct of many experiments and involvement of lot many resources, considerable time and significant cost. It takes about 2 to 12 years for a new drug for development [1] and the cost of a new drug development range from USD$92 million to USD$883.6 million [2]. During the drug development process, many animals are used for experiments and during clinical trials, many human subjects are exposed to the drug at various dose. Even after spending lot of resources, time, cost and experimentation on animals and humans; the drug may fail to provide desired clinical efficacy and safety. Only one in 10,000 compounds ever reaches the market and of those, only one in three ever recaptures its development costs [1]. There are many reasons for failure of the drug candidate during drug development or after market entry. They are Poor pharmacokinetic (39%), Lack of efficacy (30%), Animal toxicity (11%), Adverse effects in man (10%), Commercial reasons (5%), Miscellaneous (5%) [3,4] and are appeared at different stage of drug development or sometimes after market entry of the drug. Hence, at each stage of drug development, the developer needs to make "go/no-go" decision based on available information. On one side, it is good to make "go" decision at each stage of development based on the available data, but on other side, it is highly desirable to identify limiting factor (if any) as early as possible so as to make earliest "no-go" decision with the drug candidate in order to save time, money and efforts. The drug development phase which tests the drug's performance in humans is call clinical development. The drug's fate, eventually the developer's fate, depends upon the performance of the drug in target population (humans) during clinical development and then in market use. Hence, it is very important to have data of drug's performance in humans as early as possible in order to make "go/no-go" decision.

Microdosing

Microdosing is a new technique to study new drug especially for human pharmacokinetic before the usual expensive clinical development phase starts. A microdose is defined as less than 1/100th of the pharmacological (predicted) dose of a test drug candidate or a maximum dose of < 100 μg. Due to differences in molecular weights as compared to synthetic drugs, the maximum dose for protein products is d" 30 nanomoles to be said as microdose [5]. Studies using such a microdose are called microdose studies. In microdose studies, sub-pharmacological doses of prospective drug candidates are administered to human subjects in order to obtain pharmacokinetic and if possible some pharmacodynamic information (e.g. mechanisms of a drug action). European and USFDA guideline now permit microdosing studies in human subjects very early in the drug development process. The preclinical

toxicology data required for microdosing studies are minimal and hence these studies can be used as a drug candidate selection tool to effectively eliminate drug candidates that show sub-optimal human pharmacokinetic before spending time and effort in the kind of extensive toxicology that is required prior to conventional Phase 1 and other conventional clinical pharmacokinetic studies for such drug candidates. The term "Phase 0" is therefore used to refer to such microdose studies.

To measure drug concentration in microdose studies, ultrasensitive analytical methods like Accelerator Mass Spectrometry (AMS) and Positron Emission Tomography (PET) are used. AMS is the most sensitive analytical method being used in microdosing study using radiolabeled substance. AMS differs from other forms of mass spectrometry in that it accelerates ions to extraordinarily high kinetic energies before mass analysis. The special strength of AMS among the mass spectrometric methods is its power to separate a rare isotope from an abundant neighboring mass. AMS measures individual atom of the isotope and not radioactive decay events, this makes AMS highly sensitive than conventional Liquid Scintillation Counting and upto 100000 times more sensitive than liquid chromatography mass spectrometry (LC-MS). In studies using high performance liquid chromatography-AMS, limits of detection of 0.0008 dpm/fraction have been reported. Using AMS, drug developer can generate human pharmacokinetic data of drug candidate using microliter or milligram quantities of sample and minute levels of ^{14}C tracer, which is virtually undetectable and of nanocurie level of radioactivity [6,7]. PET is 3-dimensional imaging technique using radioactive tracer to label drug. PET can be used to study pharmacodynamic information like receptor selectivity, receptor occupancy profile etc. ^{11}C or ^{18}F are generally used for radiolabeling which generate images using gamma cameras and can be used to study distribution of labeled drug candidate in body in real time including penetration in central nervous system, cross of blood brain barrier etc [6-8]. Sometimes, liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) is also used in microdose studies. Below are some of the advantages and limitation of microdosing technique (Table 1).

Microdosing technique is significant technological advancement in the field of clinical development which generates some human pharmacokinetic and pharmacodynamic data to improve and speed-up drug development process. It greatly helps in generating early pharmacokinetic data to make early "go/no-go" decision in order to save cost, time and efforts. Lappin and Garner [9] had reviewed literature comparing pharmacokinetics at a microdose with a therapeutic. They concluded that of the 18 drugs reported, 15 demonstrated linear pharmacokinetics within a factor of 2 between a microdose and a therapeutic dose. There are currently a total 35 compounds where microdose and therapeutic dose data have been compared (oral, intravenous, human and animal) [10]. Of these 35 compounds (human and

animal), 27 tested orally showed scalable pharmacokinetics between a microdose and a therapeutic dose (79%) and 100% of those tested intravenously [10]. Reference of microdosing has been included in guidelines of ICH [5], the European

Table 1.: Advantages and limitations of microdosing technique

Advantages	Limitations
• It helps to generate human pharmacokinetic data early in order to make "go/no-go" decision early for the drug candidate. • It requires minute quantities of the drug substance for study. • It reduces cost of drug development phenomenally. • It reduces time of drug development significantly. • As it helps to take "go/no-go" decision early and hence, it reduces unnecessary exposure of human volunteers to new drug candidates for phase I studies if the drug candidate is not have desired human pharmacokinetic properties. • It requires smaller toxicology package for initiation in human. • It helps to avoid unnecessary exposure of animals for additional toxicology studies for non-promising drug candidates. • The microdose can be administered via any route of drug administration for study. • It involves minimal risk to the volunteers due to extremely low dose.	• Because it uses very less does of the drug, the predictive accuracy of microdosing has limitation and microdose studies cannot replace traditional pharmacokinetic studies to establish pharmacokinetic of the drug candidate. • It has limitation in case the drug candidate has dose dependent dissolution and absorption characteristics, have non-linear pharmacokinetic and pharmacokinetic properties are influenced by enzyme saturation, transporter saturation, binding site saturation, dose dependent drug distribution, tissue saturation or any other dose dependent factor etc. and may lead false positive or false negative conclusions. • It requires radiolabeling of test drug, which further add to regulator and ethical clearance. • It requires AMS and PET set-up, which itself is a big capital investment. • It does not give any direct therapeutic or efficacy evaluation of the drug candidate. • As it uses very less dose than predicted pharmacologically active dose, it does not give any therapeutic benefit to the volunteer.

• Data of microdose studies are helpful in establishing likely pharmacological dose and thereby determining the first dose for the subsequent phase-1 study. • Data of microdose studies are helpful in selection of best animal species for long term toxicology studies. • It may be an attractive approach for the study of new and existing drugs in vulnerable populations (children, pregnant women, elderly, hepatically and renally impaired), who are routinely excluded from clinical trials due to safety concerns.	• Experience with microdosing studies are emerging; and as of today, it has been used for BDDCS (Biopharmaceutical Drug Disposition and Classification System) class 1 drug only. No information exists for experience with other BDDCS class drugs.

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