

UNIVERSITAT DE BARCELONA

FACULTAT DE FARMÀCIA

DEPARTAMENT DE FARMÀCIA I TECNOLOGIA FARMACÈUTICA
Unitat de Farmàcia i Tecnologia Farmacèutica

TARGETING OF ANTILEISHMANIAL DRUGS PRODUCED BY
NANOTECHNOLOGIES

GEORGINA PUJALS I NARANJO

Barcelona, 2007

BIBLIOGRAPHIC SECTION

1. LEISHMANIOSIS

1.1. Brief history of the disease

Although cutaneous leishmaniosis can be traced back many hundreds of years, one of the first and most important clinical descriptions was made in 1756 by Alexander Russell following an examination of a Turkish patient. The disease, then commonly known as "*Aleppo boil*", was described in terms which are relevant: "After it is cicatrised, it leaves an ugly scar, which remains through life, and for many months has a livid colour. When they are not irritated, they seldom give much pain."

Representations of skin lesions and facial deformities have been found on pre-Inca potteries from Ecuador and Peru dating back to the first century AD (figure 1). They are evidence that cutaneous and mucocutaneous forms of leishmaniosis prevailed in the New World as early as this period.



Figure 1: A pre-inca pottery (left) and Mexican case of mucocutaneous leishmaniosis (right).

Texts from the Inca period in the 15th and 16th centuries, and then during the Spanish colonization, mention the risk run by seasonal agricultural workers who returned from the Andes with skin ulcers which, in those times, were attributed to "*valley sickness*" or "*Andean sickness*"....

Later, disfigurements of the nose and mouth become known as "*white leprosy*" because of their strong resemblance to the lesions caused by leprosy. In the Old World, Indian physicians applied the Sanskrit term *kala azar* (meaning "*black fever*") to an ancient disease later defined as visceral leishmaniosis.

In 1901, Sir Leishman identified certain organisms in smears taken from the spleen of a patient who had died from "*dum-dum fever*". At the time "Dum-dum", a town not far from Calcutta, was considered to be particularly unhealthy. The disease was characterized by general debility, irregular and repetitive bouts of fever, severe anaemia, muscular atrophy and excessive swelling of the spleen. Initially, these organisms were

considered to be trypanosomes, but in 1903 Captain Donovan described them as being new.

The link between these organisms and kala azar was eventually discovered by Major Ross, who named them *Leishmania donovani*. The *Leishmania* [Ross, 1903] genus had been discovered.

1.2. Causative agent and transmission

At least 20 *Leishmania* species exist; they are digenic parasites which are transmitted to various hosts (mainly humans, dogs and rodents)

by bites of sandflies (tiny sand-coloured blood-feeding flies that breed in forest areas, caves, or the burrows of small rodents). About 30 species of sandflies are proven vectors and females become infected by ingesting blood from infected reservoir hosts or from infected people. Old World forms of *Leishmania* are transmitted by sandflies of the genus *Phlebotomus* (figure 2), while New World forms mainly by flies of the genus *Lutzomyia* (Desjeux P., 1996, WHO/TDR, 2004).



Figure 2: *Phlebotomus* spp.

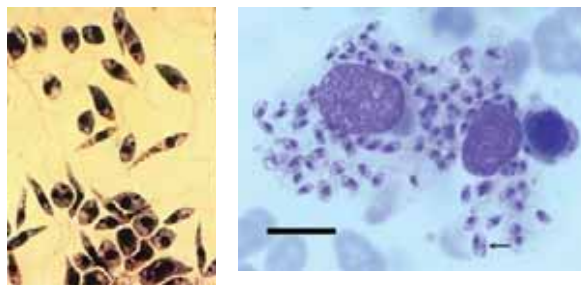


Figure 3: *Leishmania* promastigotes (left) and amastigotes (right) (Ju O. et al., 2004).

Once sandfly vector deposits the infectious promastigotes form of the parasite into the skin of a susceptible mammal, the extracellular flagellated promastigotes attaches to a mononuclear phagocyte, causing phagocytosis through one or more macrophage receptor molecules. Once intracellular, the parasite retracts its flagellum and transforms to the obligate intracellular amastigotes (Wallance R.B., 1998), (figure 3). Parasites invade resting macrophages and reaches cells of the reticuloendothelial system in various organs causing inflammatory processes and immune-mediated lesions (Brugerolle G., 2000a). Life-cycle of *Leishmania* can be seen in figure 4.

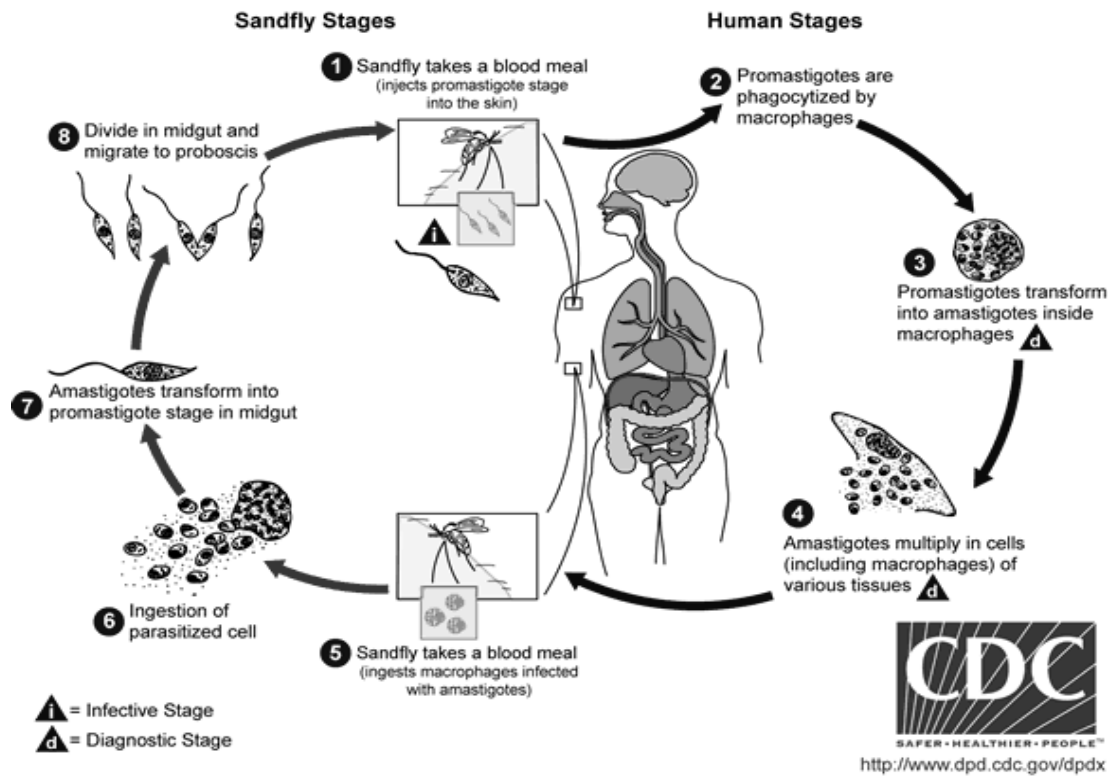


Figure 4: Life cycle of *Leishmania*

Although natural transmission of *Leishmania* occurs principally by the bite of infected sandfly vector other mechanisms may be involved. Last years, the participation of the tick *Rhipicephalus sanguineus* in the epidemiology of canine visceral leishmaniosis has been considered (Coutinho M.T. et al., 2005). For example, in a rural area of Northeast Brazil with a high serological incidence in dogs, the lack of classical vector *Lutzomyia longipalpis*, the cases in human beings and the observation of *Leishmania* in ticks collected in infected dogs suggest that ticks may be responsible for the transmission between dogs (Silva O.A. et al., 2007).

1.3. Disease

Leishmaniosis comprises a variety of syndromes ranging from asymptomatic and self-healing infections to those with a significant morbidity and mortality. The 20 or so infective species and subspecies of parasite cause a range of symptoms, some of which are common (fever, malaise, weight loss, anaemia) and swelling of the spleen, liver, and lymph nodes in the visceral form.

In man the disease occurs in at least four major forms: visceral, cutaneous, mucocutaneous and diffuse cutaneous depending on the specie and the immunological answer.

1. Visceral leishmaniosis (VL), the most serious form (always fatal if left untreated) is characterized by irregular fever, loss of weight, splenomegaly, hepatomegaly and/or lymphadenopathy, and anemia. After recovery, patients may develop a chronic cutaneous leishmaniosis form called “post kala-azar dermal leishmaniosis” (PKDL) which usually requires a long and difficult treatment (e.g. Kala azar due to *L. donovani s.l.*).



Figure 5: VL case.



Figure 6: Typical lesion of CL: Baghdad ulcer, Delhi boil or Bouton d’Orient.

2. Cutaneous leishmaniosis (CL), the most common form, causes 1-200 simple skin lesions which self-heal within a few months but which leave unsightly scars.

3. Mucocutaneous leishmaniosis (MCL) (Espundia), begins with skin ulcers which spread, causing dreadful and massive tissue destruction, especially of the nose and mouth.

4. Diffuse cutaneous leishmaniosis (DCL) produces disseminated and chronic skin lesions resembling those of leprotamous leprosy. It is difficult to treat.



Figure 7: MCL case.

The next table shows the main *Leishmania* species and the kind of Leishmaniosis which they produce.

CUTANEOUS LEISHMANIOSIS	
ANTHROPONOTICS <i>L.tropica</i>	Old World CL (endemic), CL (chronic)
ZOONOTICS <i>L.major</i> <i>L.aethiopica</i> <i>L.infantum</i>	CL (epidemic) CL, MCL, DCL CL
ZOONOTICS <i>L.(L.) Mexicana</i> <i>L.(L.) amazonensis</i> <i>L.(L.) braziliensis</i> <i>L.(L.) panamensis</i> <i>L.(L.) guayanensis</i> <i>L.(L.) peruviana</i>	New World CL, “Chiclero’s ulcer” CL, DCL CL, MCL CL, MCL CL, DCL CL
VISCERAL LEISHMANIOSIS	
ANTHROPONOTICS <i>L.donovani</i>	Old World VL, PKDL
ZOONOTICS <i>L.infantum</i>	VL
ZOONOTICS <i>L.chagasi</i>	New World VL

Table 1: Main *Leishmania* spp (Alvar J., Corachán M., 2004)

Leishmaniosis in dogs (normally named visceral canine leishmaniosis) appears as asymptomatic between 50 % and 60%. Symptoms will be according to infestation grade, immune status of the animal, evolution time of the disease and the affected organs. Initial clinical signs are vague and may be loss of hair, particularly around the eyes, weight loss, fever, anorexia, and exercise intolerance.



Figure 8: Canine leishmaniosis case

Systemic involvement includes non-regenerative anaemia, intermittent pyrexia and generalized or symmetrical lymphadenopathy (the popliteal nodes at the back of the hind legs are the easiest to examine). Cutaneous lesions are very common, and include dry exfoliative dermatitis, nodules, ulcers and onychogryphosis (clawlike curvature of nails) (figure 8). Ocular lesions such as keratoconjunctivitis, uveitis and panophthalmitis may be present. The mucose membrane of the mouth and lips are pale and there may be shallow ulcers there or around the nose. Other signs include intermittent lameness, epistaxis, arthropaties, ascitis and intercurrent diarrhoea. In advanced phases periferical

nervous affection, cachexia and death (Lindsay D.S. et al., 1995, Cordero del Campillo M. et al., 1999).

1.4. Distribution and epidemiology

Leishmaniosis is prevalent on four continents, is widespread in 22 countries in the New World and in 66 nations in the Old World. More than 90 % of the VL cases in the world are reported from Bangladesh, Brazil, India, and Sudan, and more than 90 % of the CL cases occur in Afghanistan, Iran, Saudi Arabia, and Syrian Arab Republic in the Old World and Brazil and Peru in the New World (Desjeux P., 1996).

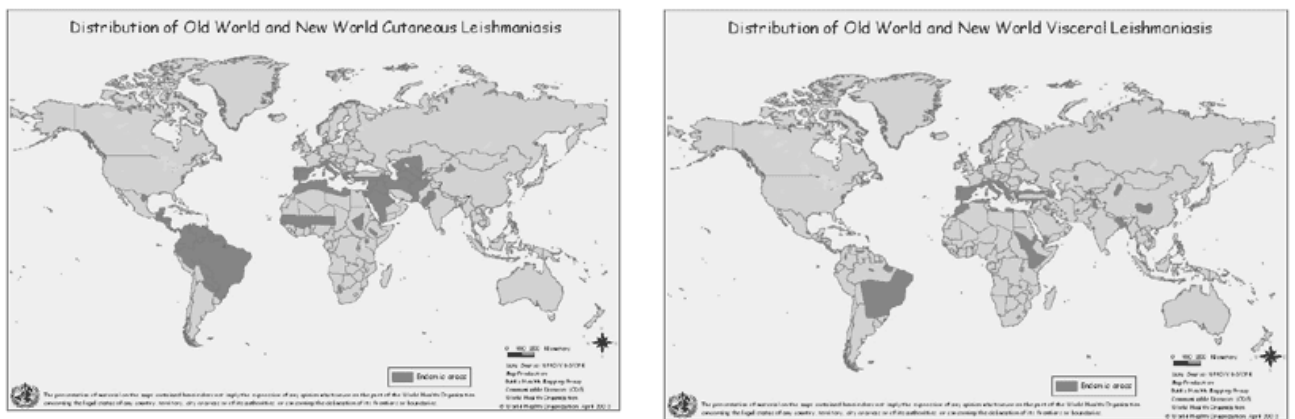


Figure 9: Distribution map of cutaneous leishmaniosis (left) and visceral leishmaniosis (right). Public Health Mapping Group Communicable Diseases (CDS) World Health Organization, October 2003 (www.who.int).

Leishmaniosis is a worldwide health problem that affects more than 12 million people and produces 57.000 deaths annually. The World Health Organization has estimated that 350 million people are at risk for leishmaniosis and is still considered a disease in the category I by the Special Programme for Research and Training in Tropical Diseases (TDR). The annual incidence of cutaneous leishmaniosis is estimated at 1,5 million cases, and the incidence of visceral leishmaniosis is estimated to be 500.000 cases per year (WHO/TDR,2004). However, official data underestimate the reality of the human affliction by these protozoa due to the following:

1. most of the official data obtained are exclusively based on passive detection,
2. numerous cases are not diagnosed,
3. there exists a large number of asymptomatic people, and

4. leishmaniosis require compulsory reporting in only 32 of the 88 endemic countries (Desjeux P., 1996, www.paho.org).

In the Mediterranean region VL and CL are caused by *Leishmania infantum* and transmitted by *Phlebotomus perniciosus*. In Spain, VL incidence is 0,3 cases per 100.000 habitants and has become a frequent co-infection in people infected with human immunodeficiency virus (HIV) so that Spain has a 60 % of coinfection cases of the world. It is estimated that 2-9 % of HIV patients in south of Europe develop VL (Pintado V., 2001). Moreover, dogs are considered to be the main reservoir in this region (Desjeux P., 2003) and it has been reported that between 3 and 5 % of Spanish dog population is seropositive (Alvar J.P.,1997).

1.5. Prevention and control

Sanflies that rest inside buildings (endophilic vectors) can be controlled by spraying houses, chicken houses, stables, etc., with residual insecticides. However, sandflies that rest outside houses (exophilic vectors) cannot be controlled in this way. Where transmission occurs in the wild, individuals should use some form of protection, such as insect repellent or insecticide.

At present there is no a total effective way that healthy or infected dogs can be prevented from infecting sandflies. Soaps, shampoos and pyrethroids sprayed on dogs have not yet been proven to be effective at all. However, deltamethrin-impregnated collar has shown partial clinical protection in dogs (Foglia Manzillo V. et al., 2006). In some endemic areas of human VL is often recommended that all serologically positive dogs are destroyed as they almost certainly carry active infections and may contribute to the spread of human disease (Desjeux P., 1996).

Moreover, vaccines to prevent human leishmaniosis are no ready for use although some investigations are being developed (www.who.int, Machado-Pinto, J. et al. 2002, Khalil E.A. et al. 2000).

Lastly, leishmaniosis has been included in the international program of “Innovative and Intensified Disease Management (IDM)” inside the Program of Control of Neglected Tropical Diases (NTD) which focuses on diseases for which cost-effective control tools do not exist and where large-scale use of existing tools is limited. The group of disease

is composed by: Buruli ulcer, Chagas disease, cholera, human African trypanosomiasis and leishmaniasis and share the following characteristics: lack of awareness, people affected often live in remote rural areas with limited access to diagnosis and treatment, killing or disabling, difficult and costly to manage (diagnosis, treatment and follow up), burden is poorly understood, lack of appropriate control tools and relatively lower investment in research and development.

Some of the disease-specific activities are focused on: increasing awareness and advocacy, supporting affected countries, disease control and management, monitoring epidemiological trends and drug resistance, collaboration with the research community, enhancing access to existing drugs and diagnostics, ensuring drug safety, supporting/promoting the development of new tools and ensuring access to innovation (WHO/NTD, 2007).

1.6. Treatment

The mainstays of therapy for leishmaniosis are the pentavalent antimony (Sb^{V}) compounds **sodium stibogluconate** and **meglumine antimoniate**. They have been the first-line drugs for the treatment of *Leishmania* in humans for 60 years. These must be administered either intravenously or intramuscularly and are associated with considerable gastrointestinal, liver, pancreatic, and cardiac toxicity, sometimes requiring cessation of therapy. Antimony is excreted quickly from the body so multidose program is required. The usual adult dose by intramuscular injection is 20 mg of pentavalent antimony per kg of body weight per day for twenty to twenty-eight days. This regimen may be repeated or continued when necessary. A longer duration may be needed for some forms of leishmaniasis. WHO recommends that treatment should be continued for at least two weeks after anticipated parasitological cure, the exact length of treatment to be determined for each country and for each patient. Some studies have shown that giving pentavalent antimony without an upper limit on the daily dose is more effective and is not substantially more toxic than the regimen with lower daily doses (Herwaldt B.L. et al., 1992). It is believed that about 5 % to 70 % of patients treated with conventional treatment suffer some therapeutic failure due to many causes such as immunological and physiological factors of the patient, deficiencies in pharmacokinetics o drug composition, and the most important, because of the resistances developed. In canine leishmaniosis, the treatment with pentavalent antimony

derivatives does not always provide complete elimination of parasites and in most cases clinical remission (Brugerolle G., 2000b).

Alternative treatments include the polyene antibiotic **amphotericin B** or **liposomal amphotericin B** while **pentamidine** is recommended for mucocutaneous and cutaneous form. Recombinant **interferon gamma** has been used successfully as an adjunct to antimony therapy in cases of treatment failure. Unfortunately, all the treatments mentioned above require repeated doses of parenteral therapy and are not optimal for use in many endemic or epidemic situations. In the search for non-toxic antileishmanials attention has been directed towards used oral antifungal drugs such as **ketoconazole** and **itraconazole**. This is also true for the oral purine (hypoxanthine) analogue, **allopurinol** or parenteral and topical formulations of the aminoglycoside **paromomycin** (Wallance R.B., 1998, Brugerolle G., 2000b). The first oral medication for visceral leishmaniosis, **miltefosine**, has been recently registered. Nevertheless, some problems may limit use of miltefosine. It is an abortifacient and a potential teratogen and is toxic to male gonads in dogs. The long half-life of miltefosine (2-3 weeks) and its narrow therapeutic index might favour the emergence of resistant mutants (Guerin P.J. et al., 2002).

It is noteworthy that some cutaneous lesions can resolve without therapy but symptomatic visceral leishmaniosis is potentially fatal and requires treatment.

1.7. In vitro studies of efficiency of antileishmanial drugs

During the last years, many pharmacological studies of antileishmanial drugs have been reported which most of them are *in vitro* because of the possibility to obtain cultures of the different *leishmania* stages (promastigotes and amastigotes).

In vitro model studies either are an easy tool of work or cheaper than *in vivo* experimental studies. However, there is not existence of a standard methodology to study the *in vitro* susceptibility of antileishmanial drugs. A summary of the investigations reported in the last five years is shown in the table 2.

Authors	Spp.	Stage	Technique	Drugs
Kamau S.W. et al., 2000	<i>L.infantum</i>	Promastigotes	Flux cytometry	Allopurinol
Sereno D. et al., 2000	<i>L.infantum</i>	Amastigotes AALC	Giemsa's dye, manual counting, MTT	Antimony potassium tartrate; amphotericin B; Pentamidina
Smith A.C. et al., 2000	<i>L.donovani</i>	Amastigotes	MTT, Giemsa's dye, manual counting	Tucaresol, Pentostam [®]
Yardley V. and Croft S.L., 2000	<i>L.donovani</i>	Amastigotes	Giemsa's dye, manual counting	Fungizona, Ambisome [®] , Abelcet [®] , Amfocit [®]
Kamau S.W. et al., 2001	<i>L.infantum</i>	Promastigotes	Flux cytometry	Alopurinol, Choralina
Carrió J. et al., 2000a	<i>L.infantum</i>	Promastigotes, AALC	Acid phosphatases- colorimetry	Glucantime [®] , SbV-HCl solution
Proulx M.E. et al., 2001	<i>L.donovani</i>	Promastigotes	Marked timidine	Camptotecina
Kayser O. et al., 2000	<i>L.infantum</i> , <i>L.major</i> , <i>L.donovani</i> , <i>L.enriettii</i>	Promastigotes, amastigotes	MTT	Aphidicolin
Rodríguez-Cabezas M.N. et al., 2001	<i>L.donovani</i>	Promastigotes, amastigotes	Marked timidine	Rodmio (III) complexes
Sereno D. et al., 2001	<i>Leishmania spp.</i>	Amastigotes AALC	Luciferase activity	Glucantime [®] , Pentostam [®] , antimony tartrate, pentamidine.
Piñero J.E. et al., 2002	<i>L.infantum</i>	Promastigotes Amastigotes	Coulter counter ZI Microscope	Abelcet [®] , Fungizone [®]
Kayser et al., 2003a	<i>Leishmania donovani</i> , <i>L. major</i> , <i>L. infantum</i> and <i>L. enriettii</i> <i>L.donovani</i>	Promastigotes Amastigotes	MTT	Gamma-pyrones from Podolepis hieracioides
Ferreira I.C. et al., 2004	<i>L. (L.) amazonensis</i> and <i>L. (V.) braziliensis</i>	Promastigotes	Direct counting	Alkaloid extract of <i>Aspidosperma ramiflorum</i>
Buckners F.B. and Wilson A.J., 2005	<i>Leishmania major</i> and <i>Leishmania amazonensis</i>	Promastigotes, amastigotes	B-lactamase activity- nitrocefin	Pentostam, Amphotericin B
Yardley V., Croft S.L. et al., 2005	<i>L.braziliensis</i> , <i>L.lainsoni</i> , <i>L.mexicana</i> , <i>L.braziliensis</i>	Amastigotes	Giemsa's dye	Miltefosine
Piñero J.E. et al., 2006	<i>L.braziliensis</i> , <i>L.tropica</i> , <i>L.infantum</i> , <i>L.amazonensis</i>	Promastigotes Amastigotes	Coulter counter ZI Microscope	<i>Trans</i> -chalcone
Tasdemir D. et al., 2006	<i>L. donovani</i>	Amastigotes AALC	Alamar blue	Flavonoids and analogues
Cabrera-Serra M.G. et al., 2007	<i>L.braziliensis</i> , <i>L.amazonensis</i> , <i>L.major</i> , <i>L.infantum</i>	Promastigotes	Alamar blue	Perifosine
Ordóñez-Gutiérrez et al., 2007	<i>L.infantum</i>	Promastigotes Amastigotes	Alamar blue Giemsa's dye	Amphotericin B microspheres
Miguel D.C. et al., 2007	<i>L. (L.) amazonensis</i> <i>L. (V.) braziliensis</i> <i>L. (L.) major</i> <i>L. (L.) chagasi</i> <i>L. (L.) donovani</i> <i>L. (L.) amazonensis</i>	Promastigotes Amastigotes	MTT	Tamoxifen

Table 2: Summary of the main studies about *Leishmania in vitro* susceptibility for seven last years.

Pentavalent antimony activity against promastigotes, axenic amastigotes like cells (AALC) and intracellular amastigotes has been reported as related so that there is not a specific susceptibility to each parasitic stage in regulated conditions in cultures (Carrió J. et al., 2000a). For this reason, it is believed that the determination of *Leishmania* growth using extracellular forms by means of acid phosphatases activity is an easy and useful method to screening antileishmanial compounds. In general, phosphatases are responsible for the removal of phosphate groups from a molecule and replacement with a hydroxyl group. The rate of this reaction is easy to follow because the substrate, *p-nitrophenylphosphate*, is colourless and the product, *p-nitrophenol*, is yellow (figure 10). Since the molecules of product are responsible for a colour change, the rate of colour change will be proportional to the rate of reaction. A quantitative measurement of colour change can be done by a spectrophotometer.

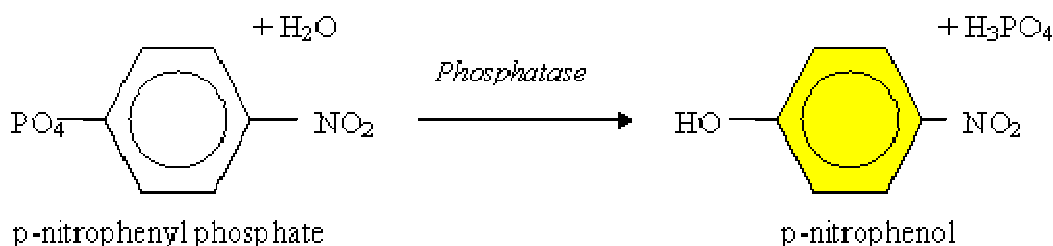


Figure 10: Phosphatases reaction

2. ANTIMONIALS

2.1. Brief history



Figure 11: Alchemical symbol for antimony

Antimony, according to Samuel Johnson's Dictionary of the English Language and popular etymology is *stibium* of the ancients, by the Greeks called *στίβιμ*. The reason of its modern denomination is referred to Basil Valentine, a German monk; who, as the tradition relates, having thrown some of it to the hogs, observed, that, after it had purged them heartily, they immediately fattened; and therefore, he imagined, his fellow monks would be the better for a little dose. The experiment, however, succeeded so ill, that they all died of it; and the medicine was thenceforward called Fr. *antimoine* antimonk. The popular etymology is, as usual in

such cases, supported by an idle tale; however the chemist Basil Valentine is from the end of the 15th century, and the word was already used by Constantinus Africanus of Salerno at the end of the 11th century.

In the period from 1906 to 1908, injections of antimony potassium tartrate (tartar emetic) were successfully used to treat human trypanosomiasis. In 1912, Gaspar de Oliveira Vianna observed its effectiveness in american cutaneous leishmaniosis. They are irritant and very toxic, for this reason the synthesis of less toxic organic antimonials were made. The most important is stibophen, a trivalent antimonial compound which is as effective and much less toxic than antimony potassium tartrate (Einstein R. et al., 1994). Bramachari, in 1920, developed the first pentavalent antimonial compound, urea stibamine and Schmidt, in 1936 introduced the first treatment with sodium stibogluconate (Pentostam[®]) (Rath S. et al., 2003).

Although organic antimonials have been used for the last 60 years, the exact structures of these compounds, and their mechanism of action and toxicity, have not been defined until nowadays.

2.2. Meglumine antimoniate

MGA is commercially available in a parenteral dosage form named Glucantime[®]. It is not available neither U.S.A nor Canada. Two different laboratories supply it in Europe: Merial S.A for the treatment of canine leishmaniosis and Aventis Pharma S.A for humans. Both contain 1.5 grams MGA per 5 ml but only the second case is declared their equivalence to 425 mg of pentavalent antimony.

Moreover, there is a general problem of quality and batch-to-batch variability for both branded and generic drugs; and the poor quality of some generic formulations of the drug in India has led to serious toxicity (Guerin P.J. et al., 2002).

2.2.1. Physical and chemical characteristics

MGA is an amorphous solid susceptible to thermal degradation, readily transforms upon heating into involatile salts, and this property has limited its structural characterization (Demicheli C. et al., 2003).

Mass spectroscopy studies report that MGA consists of a mixture of components of N-methyl-D-glucamine (NMG) coordinated with antimony, with general formulas of (NMG-Sb) $_n$ -NMG (major components; molecular weights

=507, 820, 1132 and 1444) and (NMG-Sb) $_n$ (minor components; molecular weights=314 and 627). Antimony and NMG alternate in these chains, with each antimony co-ordinately linked via two hydroxyl groups from each glucamine that are not in terminal positions are linked to two antimonies (figure 12). These complexes are in equilibrium in aqueous solutions. It has been reported that the extent of polymerization may influence the pharmacokinetic of drug delivery, uptake by reticuloendothelial system, and the intracellular distribution of pentavalent antimony (Roberts W.L. et al., 1998).

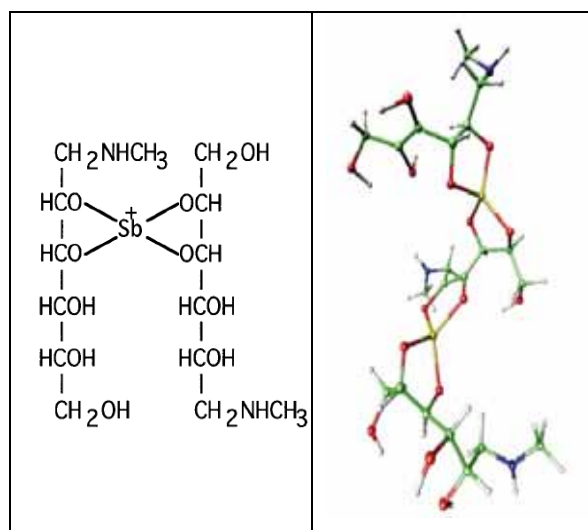


Figure 12: Structure of m/z 507 ion (Sb $_2$ (NMG) $_2$) (left) and three-dimensional structure of an m/z 820 ion (Sb $_2$ (NMG) $_3$) (right) from meglumine antimoniate. H: light gray, C: green, N: blue, O: red, Sb: yellow. (Roberts, W.L., et al., 1998).

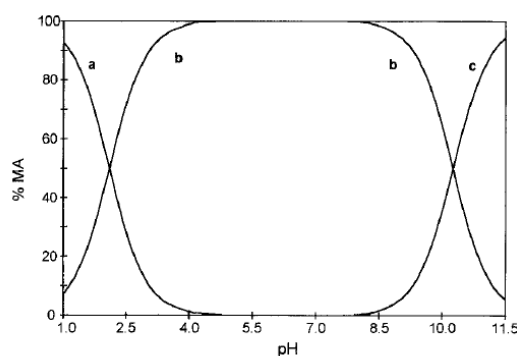


Figure 13: Species distribution curve for MGA as a function of pH, calculated for MGA concentration of 0,04 mol/L. (a) protonated complex, (b) zwitterionic complex, (c) deprotonated complex.

different pH. Protonation constant values for MGA were $10,26 \pm 0.02$ and $12,36 \pm 0.02$. (Demicheli C. et al., 1999). Therefore, MGA contains two dissociable protons which

Determination of MGA ionization state is important to evaluate the possible influence of drug ionization on its passage through *Leishmania* biological membrane and its retention inside the acidic vacuole due to parasites live and replicate within an acidified vacuole of the mammalian macrophage. This localization also implies that the drug, in order to reach the parasite, have to cross distinct compartments of

can be attributed to the amino group ($pK_{a2}=10,26$) and to the antimononic acid group ($pK_{a1}=2,10$). Figure 13 shows that between pH 4.5 and 7.5 the complex exists as 100 % in the zwitterionic form and then MGA ionization state does not depend on pH, in the range of physiological pH (between 5 and 7,5). However, it has been reported that the pH will condition the activity of MGA *in vitro* assays (Carrió J. et al., 2000).

MGA neither appears in European Pharmacopeia, USP, JP nor British Pharmacopeia, however it is included in “Farmacopéia Brasileira IV, 2002” as “Antimoniato de meglumina / *Antienitum megluminum*”. The monography describes MGA as white powder or lightly yellow. It is soluble in water, practically water insoluble in ethanol, ethylic ether and chloroform. It has to contain 32,60 % as minimum and 33,93 % as maximum of pentavalent antimony. One of the purity test described is the determination of pH of a 30 % (w/v) in water which results have to be between 5,5 and 7,5. This pharmacopeia also includes a monography for MGA in injectable solution.

Pharmaceutical manufacturers of MGA consider more specifications than those included in “Farmacopéia Brasileira” when they authorize manufactured batches. Other physical and chemical properties considered are that MGA is odourless, hygroscopic, it has 460 kg/m³ of bulk density and octanol/water partition coefficient ($\log(Pow)$) is - 2,70. Moreover, MGA is described as sensitive to light and it can be decomposed under the effect of heat. It is worth to pointing out that the raw material supplied by Aventis Pharma contain less percentage of pentavalent antimony (from 26,0 % to 28,0 %) than those described in the “Farmacopéia Brasileira”. Furthermore, antimonous antimony percentage is limited under 0,05 % due to its toxicity.

2.2.2. Mode of action

After 60 years of use, the anti-leishmanial mechanism of action of pentavalent antimonials is still not clearly defined. Sb^V is generally considered a pro-drug that first has to be activated by conversion to the trivalent form (Sb^{III}), however, the site of reduction (host macrophage, amastigotes or all) and the mechanism of reduction (enzymatic or nonenzymatic) remain unclear (Outlette M. et al., 2004). It has been reported that trivalent antimony interferes with trypanothione metabolism in drug-sensitive *Leishmania* parasites by two distinct mechanisms. First, Sb^{III} decreases thiol

buffering capacity by inducing rapid efflux of intracellular trypanothione and glutathione in approximately equimolar amounts. Second, Sb^{III} inhibits trypanothione reductase in intact cells resulting in accumulation of the disulfide forms of trypanothione and glutathione. These two mechanisms combine to profoundly compromise the thiol redox potential in both amastigote and promastigote stages of the life cycle (Wyllie S. et al., 2004). In the figure 14, the model for the mode of action of antimonial drugs on *Leishmania* amastigotes is shown.

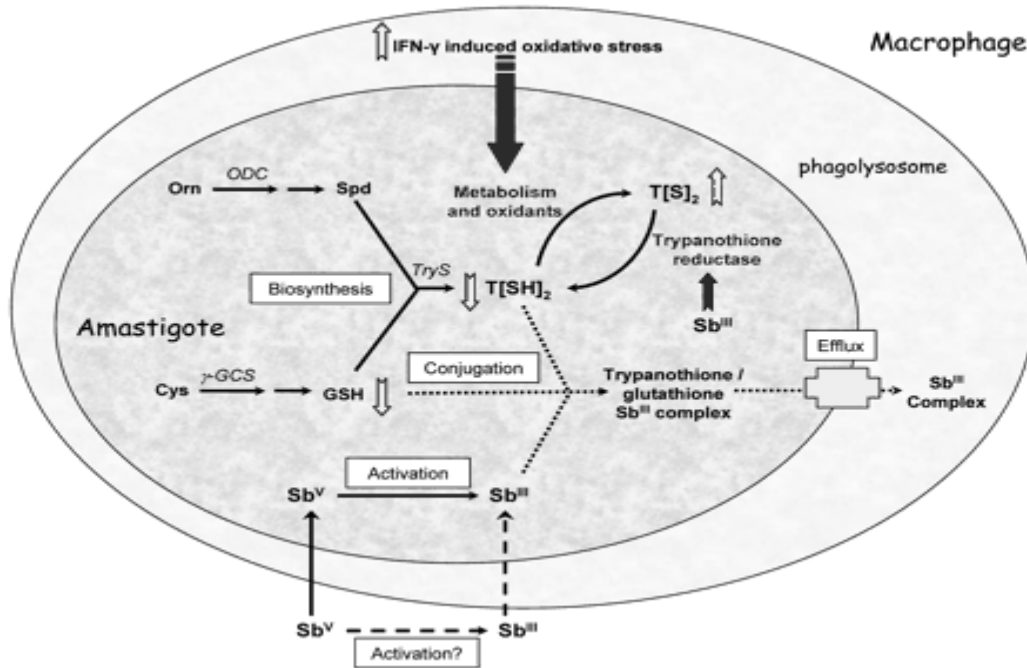


Figure 14: Model for the mode of action of antimonial drugs on *Leishmania* amastigotes. The capacity to replace trypanothione and glutathione effluxed from antimony-sensitive cells is limited by the activity of ornithine decarboxylase (*ODC*) and γ -glutamylcysteine synthetase (γ -*GCS*). In laboratory-derived trivalent antimony-resistant cell lines, these rate-limiting activities are increased severalfold, thereby increasing trypanothione concentrations. Other abbreviations: *Orn*, ornithine; *Spd*, spermidine; *TryS*, trypanothione synthetase (Wyllie S. et al., 2004).

Some studies report that Sb^V accumulates in both stages of the parasite although is higher in axenic amastigotes than in promastigotes (Roberts. W.L. et al., 1995). Despite this, some investigations do not coincide in activity results. Several factors, such as *Leishmania* species, axenization status, medium, pH, and thiol concentration, are likely to influence on drug assays and/or the rate of Sb^V reduction (Carrió J. et al., 2000a).

2.2.3. Pharmacokinetics

In humans antimony compounds are poorly absorbed from the gastrointestinal tract (Ellenhorn M.J., 1997). MGA, a highly water soluble compound, is considered inactive when given enterally. For this reason must be given intralesional or by parenterally route (intramuscular or intravenous). Moreover, the mechanism of permeation of pentavalent antimonials across biological membranes is still poorly understood. Whereas aquaglyceroporins and multidrug-resistance associated proteins (MRP) were found to mediate the passive and active transport, respectively, of Sb^{III} across biological membranes, these transporters do not seem to recognize Sb^{V} . It is likely that pentavalent antimonials cross membranes either by endocytosis or by simple diffusion through the lipid bilayer (Martins P.S. et al., 2006).

Antimony is found in high concentrations in the plasma, liver, and spleen. It has been reported antimony accumulates in hair during MGA therapy of leishmaniasis (Dorea et al. 1987) and small amounts of antimony are retained in tissues during therapy (Dorea et al. 1990). In adults mean total apparent volume of distribution is $0,22 \pm 0,057$ L/kg of body weight (dose 10 mg antimony (Sb) per kg body weight) (Chulay J.D. et al., 1988). Other studies report that $Vd/F(\beta)$, apparent volume of distribution during the β -elimination phase, is $0,30 \pm 0,01$ L/kg in adults and $0,39 \pm 0,03$ L/kg in children (dose 20 mg Sb/Kg) (Cruz A. et al.,2007).

An indeterminate amount of MGA is metabolized to trivalent antimony in the liver. Conversion to trivalent antimony may contribute to toxicity observed with long-term, high-dose therapy (Chulay J.D. et al, 1988).

In adults, the approximate mean half-life following an intramuscular administration of MGA in a dose that provides 10 mg/kg of pentavalent antimony is 51 minutes (initial absorption phase), 2,02 hours (rapid elimination phase) and 76 hours (slow elimination phase). The data were best described by a two compartment with a three term pharmacokinetic model (Chulay J.D. et al., 1988). Other reports do not report similar values respect to the measures of the low antimony concentrations present at later time points and the apparent half-life between 24 and 48 h was 1 day (Cruz A. et al.,2007).

On one side, the time to peak concentration is approximately 2 hours following intramuscular administration of MGA in a dose that provides 10 mg/kg of pentavalent antimony (Chulay J.D., et al., 1988). On the other side, the peak serum concentration is approximately 9 to 12 mg per L following intramuscular administration of MGA in a

dose that provides 10 mg/kg of pentavalent antimony (Chulay J.D. et al., 1988).

Most of the antimony (up to 50%) is rapidly removed unchanged in the urine within 24 hours following a single parenteral administration, primarily by glomerular filtration, with a portion distributing into a deeper compartment, possibly intracellular water. Slow release of antimony from the deeper compartment is complete within 48 hours and may explain the longer apparent half-life noted between 24 and 48 h. (Chulay J.D. et al., 1988, Cruz A. et al., 2007).

A similar disposition profile of antimony was observed in humans with visceral leishmaniosis treated with sodium stibogluconate or MGA for 30 days (Chulay J.D. et al., 1988), healthy dogs after single dose administration of MGA (Valladares J.E. et al., 1996) and in experimental infected dogs with *L. infantum* after multiple dose (Valladares J.E. et al., 1998). Pharmacokinetics parameters are summarised in the table 3.

Parameters	Multiple dose	Single dose	P
	Mean ± S.D.	Mean ± S.D.	
K_{01} (h^{-1})	0,777 ±0,342	0,994±0,270	0,2290
α (h^{-1})	0,496±0,119	0,510±0,123	0,6310
β (h^{-1})	0,083±0,023	0,058±0,025	0,1087
$t_{1/2 K_{01}}$ (h)	1,09±0,52	0,736±0,182	0,3768
$t_{1/2 \alpha}$ (h)	1,41±0,58	1,44±0,39	0,8728
$t_{1/2 \beta}$ (h)	8,76±1,81	13,8±4,51	0,0782
AUC ($\mu g h ml^{-1}$)	149,3±74,9	106,1±18,3	0,4233
C_{max} ($\mu g ml^{-1}$)	30,8±14,1	25,5±4,9	0,6310
$C_{max ss}$ ($\mu g ml^{-1}$)	32,0±13,9	-	
$C_{min ss}$ ($\mu g ml^{-1}$)	1,54±0,47	-	
T_{max} (h)	1,7±0,2	1,4±0,2	0,0247

Table 3: Pharmacokinetic parameters corresponding to analysis of Sb plasma concentration curves obtained both after a single dose of 100 mg kg⁻¹ of MGA to healthy dogs and after a multiple dose administration of 75 mg·kg⁻¹·12 h⁻¹ of MGA for 10 days to dogs with experimentally induced leishmaniosis (Valladares J.E. et al. 1998).

2.2.4. Toxicity

MGA is contraindicated if there is existence of hypersensitivity to MGA, stibogluconate, or other antimony compounds, cardiac disease and/or severe renal disease. It is necessary to be careful in pneumonia and tuberculosis cases, in infants under 18 months of age and if electrocardiogram abnormalities exist.

Treatment with pentavalent antimony compounds is usually well tolerated. However, the general condition of patients with visceral leishmaniosis probably influences the degree to which side effects of the medication may be manifested. Also, malnutrition is

common in these patients and their immune system is often impaired, making them more susceptible to recurrent infections.

The adverse reactions are mainly cardiovascular, hepatic, gastrointestinal, dermatological or renal. One cardiovascular effect described in the bibliography is a polymorphic ventricular tachycardia ("torsades de pointes") which was observed in a 73-year-old male after receiving intramuscular MGA 75 milligrams (mg)/kilogram for treatment of leishmaniosis and amiodarone (Segura I. et al., 1999). Another is the apparition of electrocardiogram (ECG) abnormalities during chronic therapy with MGA, including T-wave inversion and prolongation of the QT interval, and may precede development of ventricular arrhythmias (Chulay J.D. et al., 1985).

Elevations in serum transaminases have been reported during MGA therapy, and hepatitis has occurred in some. For this reason, periodic monitoring of hepatic function is recommended during therapy of leishmaniosis (www.micromedex.com).

Ocasionalmente can appear nauseas, vomiting and anorexia and acute pancreatitis has been identified as a rare side effect. However, hyperamylasemia with or without acute pancreatitis has been observed in HIV patients undergoing antimonial treatment for visceral leishmaniosis (VL) (Delgado J. et al., 1999). Pentavalent antimony could cause generalized rash but the phenomenon is rare even with prolonged courses of the maximum recommended dose. However, high frequency of skin reactions in patients with leishmaniosis treated with MGA produced in Brazil has been reported. The batches used had lower pH and higher concentration of total and trivalent antimony, lead, cadmium and arsenic (Sierra G.A. et al., 2003).

Moreover, septic shock with oliguria has been described occasionally during MGA therapy in a patient with normal renal function so that routine monitoring of renal function tests is recommended during therapy of leishmaniosis (Hantson P. et al., 2000). Among other adverse effects it can be find blood dyscrasias such as anemia and leukopenia, dyspnea, joint stiffness, pain in muscles and joints, headache and malaise peripheral neuritis. Furthermore, a protein-rich diet is recommended throughout treatment with MGA in order to correct beforehand iron depletion and other specific deficiencies (www.micromedex.com).

MGA interact to agents that prolong the QT interval: such as certain antiarrhythmics (types IA, IC, III) or tricyclic antidepressants which may further prolong the QT interval

and may increase the risk of arrhythmia. Moreover, alcohol may potentiate the risk of hepatotoxicity (www.micromedex.com).

2.3. Antimony resistance in *Leishmania*

The use of antimonials is threatened by the emergence of parasite resistance. Although pentavalent antimonials have been used for many years as first-line drugs, numerous treatment failures have been reported (Faraut-Gambarelli F. et al., 1997). These failures can occur from the beginning of the treatment (primary unresponsiveness) or during a relapse (secondary unresponsiveness). The importance of T-cell-mediated immunity in the prevention of relapses may explain the high frequency of relapses observed in HIV patients. However, it has been reported that immunocompetent patients infected with sensitive strains also relapse when the duration of the treatment is too brief (15 days) (Faraut-Gambarelli F. et al., 1997). Moreover, it has been seen that the sensitivity of *L. infantum* strains decrease progressively in relapsing patients treated with MGA (Faraut-Gambarelli F. et al., 1997, Lira R. et al., 1999, Carrió J. et al., 2001). These results are reinforced when Sb^V susceptibility also decreases after dogs treatments with MGA (Gramiccia M. et al., 1992, Carrió J. and Portús M., 2002). The worst situation is found in India where up to 65 % of new patients with visceral leishmaniosis show primary unresponsiveness (Guerin P.J. et al., 2002), which is due to the emergence of antimony-resistant strains of *L. donovani* (Lira R. et al., 1999).

The decreased levels of Sb^{III} in resistant strains seem to be caused either by decreased uptake of Sb^{III} (Gourbal B. et al., 2004) caused by lower expression of the parasite aquaglyceroporin gene (AQP1), which codes the protein responsible for uptake of trivalent metalloid (Marquis N. et al., 2005), or by inhibition of intracellular reductase activity (Shaked-Mishan P. et al., 2001). Once the Sb^{III} is within cell, it would be conjugated to trypanothione, which would be sequestered inside a vacuole by ATP-binding cassette (ABC) transporter MRPA (previously known as p-glycoprotein A; [PGPA]) (Légaré D. et al., 2001). Others transporters of the ABCC family appear to be involved in antimony resistance (Ouellette M. et al., 2004). *Leishmania donovani* clinical isolates not responsive to sodium stibogluconate showed resistance to antimony treatment in both *in vitro* and *in vivo* laboratory conditions. The resistant isolates have increased levels of intracellular thiols. This increase in thiol levels was not mediated by the amplification of glutamylcysteine synthetase, but was accompanied by amplification of trypanothione reductase and an intracellular ATP-binding cassette transporter gene

MRPA. The resistance of parasites to antimony could be reversed by the glutathione biosynthesis-specific inhibitor, buthionine sulfoximine, which resulted in increased drug susceptibility. These results suggest the possible role of thiols and MRPA in antimony resistance in field isolates (El Fadili K. et al., 2005, Mittal M.K. et al., 2007).

2.3.1. P-glycoprotein

P-glycoprotein (PGPA) is a member of the highly conserved super-family of ABC transporters proteins encoded by the MDR1 gene in humans, predominately located in the apical membranes of the epithelia, on the luminal surface of the small intestine, colon, and capillary endothelial cells of the brain and on kidney proximal tubules. It has been linked to multi-drug resistance (MDR) associated with a variety of cancers and can reduce the efficacy of any drug that is among its numerous substrates (Stouch T.R., et al., 2002). Oral bioavailability of drugs is affected by the reduction of their absorption from the small intestine due to the relative role of CYP3a/3A4 and PGPA (Suzuki H. et al., 2000). Even with miltefosine, the first efficient oral treatment against visceral leishmaniosis in India, resistance has been observed due to interaction with PGPA (Rybczynska M. et al., 2001).

The same transporter is encoded by P-glycoprotein gene in the H region of *Leishmania* which confers resistance to heavy metals when present in multiple copies (Callahan H.L. et al., 1991). It is interesting to develop effective agents to reverse PGPA-mediated metal resistance such as Verapamil which can reverse the *in vitro* drug resistance of *L.donovani* clinical isolates to sodium stibogluconate (Valiathan R. et al., 2006). However, high concentrations are required for an efficient and effective inhibition and, in addition, produce undesirable effects. For this reason, the discovery of new, natural products modulators of PGPA is stressed (Osorio E.J. et al., 2005). It has been also reported that 2n-propylquinoline, orally active in the treatment of visceral leishmaniosis in BALB/c mice, inhibits the PGPA activity involved in rhodamine 123 or digoxine transport in Caco-2 cells (Belliard A.M., 2003). These kinds of drugs in combination with current treatment could reverse drug resistance and short the duration of the treatment. Some surfactants/excipients, commonly added to pharmaceutical formulations, have also been reported as inhibitors of PGPA located in the apical membranes of intestinal absorptive cells and enhance the absorption of digoxin and

celiprolol *in vitro* (Zhang H. et al., 2003, Cornaire G. et al., 2004). The advantage is that excipients not have themselves pharmacological activity.

3. CONTROLLED DRUG DELIVERY SYSTEMS

3.1. Introduction

For most of the industry's existence, pharmaceuticals have primarily consisted of simple, fast-acting chemical compounds that are dispensed orally (as solid pills and liquids) or as injectables. During the past three decades, however, formulations that control the rate and period of drug delivery (time-release medications) and target specific areas of the body for treatment have become increasingly common and complex. The current methods of drug delivery exhibit specific problems that scientists are attempting to address. For example, many drugs' potencies and therapeutic effects are limited or otherwise reduced because of the partial degradation that occurs before they reach a desired target in the body. Moreover, in many cases, conventional drug delivery provides sharp increases of drug concentration at potentially toxic levels. Further, injectable medications could be made less expensively and administered more easily if they could simply be dosed orally. However, this improvement cannot happen until methods are developed to safely shepherd drugs through specific areas of the body, such as the stomach, where low pH can destroy a medication, or through an area where some tissue might be adversely affected (Vogelson C., 2001). The goal of all sophisticated drug delivery systems, therefore, is to deploy medications intact to specifically targeted parts of the body through a medium that can control the therapy's administration by means of either a physiological or chemical trigger.

3.2. Macrophage antileishmanial drugs delivery systems

The development of new antiparasitic drugs to market level is rather low. A good strategy to drug development is the optimization of formulations and applications of known antiparasitic drugs, such as MGA. These optimised formulations should enhance the efficiency of the drug and reduce negative side effects at low cost.

The causal agent of leishmaniosis is an intracellular pathogen which reproduces inside macrophages, therefore antileishmanial drugs must gain access to the host cell and resist intracellular degradation and metabolism.

Targeting of drug directly to the macrophages can be enhanced by giving the drug in a particulate form. Particulate drug delivery systems like liposomes, polymeric nano- and microparticles or nanosuspensions may be very efficient (Basu M.K. and Lala S., 2004). They could increase the uptake and accumulation of drugs in macrophages as different studies have reported for *Leishmania* infections (table 4). In some cases, if appropriate ligands are attached to particles, so that they could be easily recognized by the macrophage receptor, then these modified particles could possibly be used very efficiently as vehicles for site-specific delivery.

Drug delivery system	Drug	Parasite	Reference
Liposomes			
	Amphotericin B (Ambisome®)	<i>L.donovani</i>	Croft SL. et al., 1991.
	Meglumine antimoniate	<i>L.chagasi</i>	Frézard F. et al., 2000.
	Meglumine antimoniate	<i>L.chagasi</i>	Schettini D.A. et al., 2003.
Tufts-bearing liposomes	Sodium stibogluconate	<i>L.donovani</i>	Guru PY. et al., 1989.
Sugar grafted liposomes	Pentamidines	<i>L.donovani</i>	Banerjee G. et al., 1996.
Phosphatidyl-serine liposome	Antimony	<i>L.chagasi</i>	Tempone A.G. et al., 2004.
Polymeric particles			
Poly (d,l-lactide) nanoparticles	Primaquine	<i>L.donovani</i>	Rodrigues J.M Jr. et al., 1994.
Polymethacrylate nanoparticles	Pentamidine	<i>L.major</i>	Fusai T. et al., 1997.
Albumin microspheres	Amphotericin B	<i>L.infantum</i>	Sánchez-Brunete J. A. et al., 2007.
Polymeric drug conjugates			
Mannose-substituted poly-L-lysine conjugates	Allopurinol riboside	<i>L.donovani</i>	Nègre É. et al., 1992.
Mannose-grafted phospholipid microspheres (polylactic-co-glycolic acid (PLGA) and phosphatidyl ethanol amine in the molar ratio 1:71).	Dihydroindolo [2,3-a] indolizine	<i>L.donovani</i>	Medda S. et al., 2003.
N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugate containing N-acetylmannosamine (ManN)	NPC1161, 8-aminoquinoleina	<i>L.donovani</i>	Nan A. et al., 2004
Yeast mannan complexes	Antimony	<i>L.amazonensis</i>	Cantos G. et al., 1993, Roberts WL. et al., 1996
Nanosuspensions			
	Aphidicolin	<i>L.donovani</i>	Kayser O., 2000
	Amphotericin B	<i>L.donovani</i>	Kayser O., 2003

Table 4: The main drug delivery systems for antileishmanics

Some studies are based in the selective delivery of antileishmanial drugs by using mannose-grafted carriers. The premise to design these carriers is that one of the routes of phagocytosis of *Leishmania* is dependent on the interaction between the mannose-containing lipopolysaccharides on the parasite cell surface and the macrophage mannose

receptors. Consequently, these systems can maximize the potential of the drug to destroy the parasite at the site where it resides by mimicking the invasion process (Nan A et al., 2004).

The most commonly antileishmanial formulations under study are liposomes and microspheres, curiously, in one of these investigations, when tested for efficacy in lowering parasite load in the spleen, as well as in reducing the hepatic and renal changes associated with infection, the drug intercalated mannose-grafted microspheres were found to be the most active in comparison to drug intercalated liposomes or to the free drug (Medda S. et al., 2003). It is noteworthy that liposomes are made of natural phospholipids which are well tolerated with minimal toxic effects and have inherent tendency to be trapped within the mononuclear phagocyte system (MPS). However, they show low shelf life stability with increasing particle size and quick release of the drug into de solvent in consequence. Liposomes can not be administered orally and from an industrial point of view, scaling up is a major problem because of the requirement for homogeneous particle size and distribution. Safety and quality requirements lead to high production costs which make not affordable for patients in the low income countries where it is more needed (Kayser O. et al., 2002). Because of all these disadvantages, it is considered a good alternative to resort to biopolymers and prepare other particulate drug delivery systems such as nano-microspheres using scalable preparation techniques.

3.3. Macrophage uptake of nano-microspheres

The phagocytic uptake of colloidal drug carrier systems is the major obstacle to the efficient delivery to target sites, however it suppose an avantage to the treatment of leishmaniosis. Many studies have been reported about uptake by phagocytic active cells to examine the role of physicochemical properties of particulate carriers on the phagocytosis, concretely of nanoparticles and microspheres. Size, surface property composition, concentration, and hydrophilicity or lipophilicity of these carriers plays a significant role in the uptake by macrophages. Hydrophobic and relatively large microspheres are more susceptible to phagocytosis than their hydrophilic counterparts (Tabata Y. and Ikada Y., 1988, Roser M. et al., 1998, Ahsan F. et al., 2002, Yoshida A. et al. 2006). Even though the existence of so many uptake studies, it is difficult to

generalize the physicochemical properties of nano-microspheres to enhance phagocytosis.

Different techniques have been reported to quantify phagocytosis capacity of nano-microparticles by phagocytic cells; a) labelling particles with commercially available dyes, normally fluorescein isothiocyanate (FITC) (Privitera N. et al., 1995, Roser M. et al., 1998), b) labelling microparticles with biotin and incubation with a fluorescent streptavidine conjugate (Fischer S. et al., 2004), c) staining cells by dyes like Mayer's hematoxylin solution (Yoshida A. et al., 2006) or Giemsa (Prior S. et al., 2002) and then counting phagocytic cells by confocal, fluorescence or light microscopy. However, organic fluorophores are not ideal labels since they rapidly undergo photobleaching (within seconds to a few hours), which renders them unsuitable for long-term imaging studies. They are also not good for multicolour imaging because of two inherent properties: a) organic dyes have relatively broad emission spectra and hence result in signal overlap from different dyes; and b) one organic dye can only be excited by the lights within a certain narrow wavelength range and it thus needs nearly the same number of excitation light sources as the dyes used (Yu.W.W. et al., 2006). In recent years, the use of semiconductor quantum dots (QDs) has attracted the attention in different fields like microelectronics, optoelectronics and cellular imaging (Hasegawa U. et al., 2005). This new alternative kind of label for long-term imaging will be explained in more detail in the section 3.4.

Macrophages might have a recognition system specific for different molecules, because of which they bind with different carriers to different extents (Ahsan F. et al., 2002). For this reason, the extent of phagocytosis can be improved by coating the particle surface with opsonic materials and activating macrophages with various activating factors. Due to macrophage possess different receptors such as mannose receptors and they help in the process of recognition and endocytosis of particulate carriers and it is the route of phagocytosis of *leishmania*, it to be of interest to find some carrier which interact with this receptor. One of these carriers is the biopolymer chitosan which an extensive description will be shown in the experimental section.

3.4. Macrophage uptake studies using quantum dots

3.4.1. Brief history and definition of quantum dots

In 1932, H.P.Rocksby discovered that the red or yellow colour of some silicate glasses could be linked to microscopic inclusions of CdSe and CdS. It was not until 1985 when these changes in colour were linked to the energy states determined by quantum confinement in these CdSe or CdS “quantum dots” (Borovskaya E. and Shur MS., 2002). More recently, a rapid progress in nanofabrication techniques has lead to create artificial quantum dots.

QDs can be as small as 2 to 10 nanometers and contains a small integer number (of the order of 1-100) of conduction band electrons, valence band holes or excitons, i.e., an integer number of elementary electric charges. Many people refer to QDs as “artificial atoms”. This comparison highlights two properties of QDs, a relatively small numbers of electrons in the dot and many body effects by which the properties of the dot could be dramatically changed by adding just one electron. This analogy can be extended by saying that 2 or more QDs might form an “artificial molecule” (Borovskaya E. and Shur MS., 2002).

3.4.2. Quantum dots features

A quantum dot is a semiconductor nanostructure considered ideal candidate as fluorescent probe for long-term imaging to track whole cells or intracellular biomolecules due to their properties. QDs properties of interest to biologists include high quantum yield, high molar extinction coefficients (~10-100 x that of organic dyes), broad absorption with narrow symmetric photoluminescence spectra from the UV to near-infrared (figure 15), large effective Stokes shifts (figure 16), high resistance to photobleaching and exceptional resistance to photo- and chemical degradation (Medintz I.L. et al., 2005, Gao X. et al., 2005). The fact to have larger molar extinction coefficients, the QDs absorption rates will be 10-50 times faster at the same excitation photon flux and then QDs have been found to be 10-20 times brighter than organic dyes (Gao X. et al., 2005).

Compared with molecular dyes, two properties in particular stand out: the unparalleled ability to size-tune fluorescent emission as a function of core size (it means that QDs of the same material but with different sizes can emit light of different colours), and the broad excitation spectra, which allow excitation of mixed QDs population at a single wavelength far removed (>100 nm) from their respective emissions (figure 16) (Medintz I.L., et al., 2005).

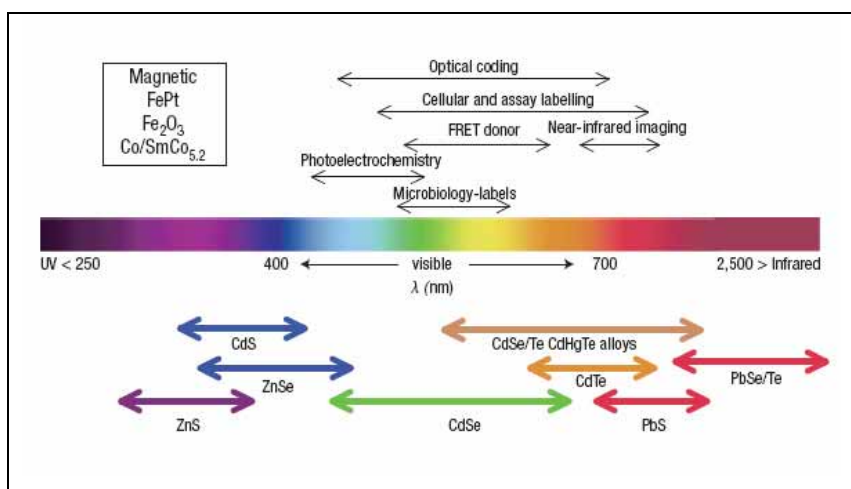


Figure 15: Representative QD core materials scaled as a function of their emission wavelength superimposed over the spectrum. Representative areas of biological interest are also presented (Medintz I.L., et al., 2005)

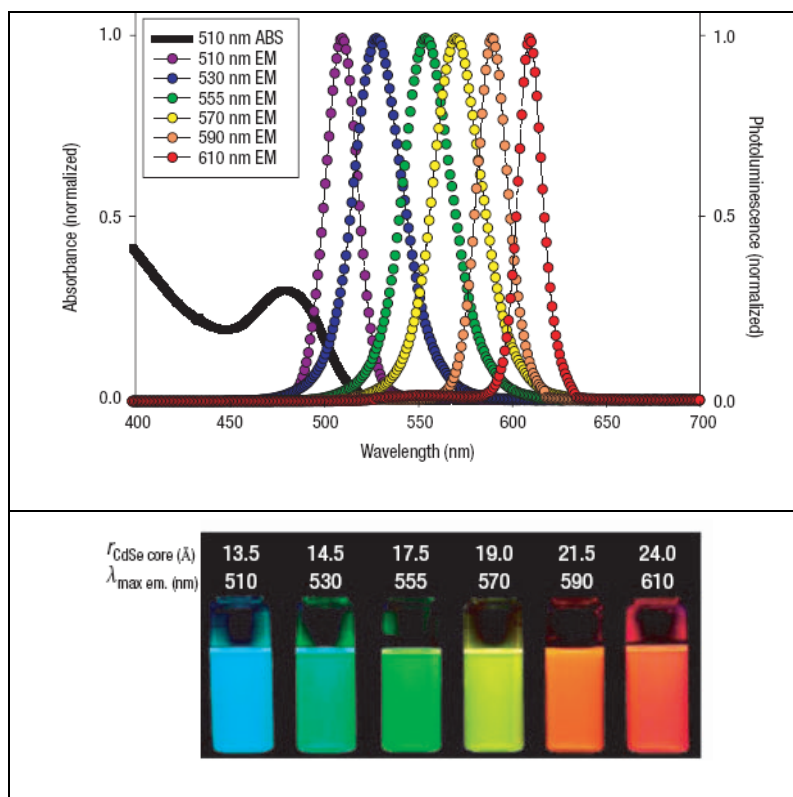


Figure 16: Upper illustration shows the absorption and emission of six different QD dispersions. The black line shows the absorption of the 510 nm, emitting QDs. Lower illustration demonstrates the size-tunable fluorescence properties and spectral range of the six QD dispersions plotted above versus CdSe core size (Medintz I.L. et al., 2005).

3.4.3. Quantum dots conjugates

The best available QD fluorophores for biological applications are made of CdSe cores overcoated with a layer of ZnS because this chemistry is the most refined. The ZnS layer passivates the core surface, protects it from oxidation, prevents that the Cd/Se goes into the surrounding

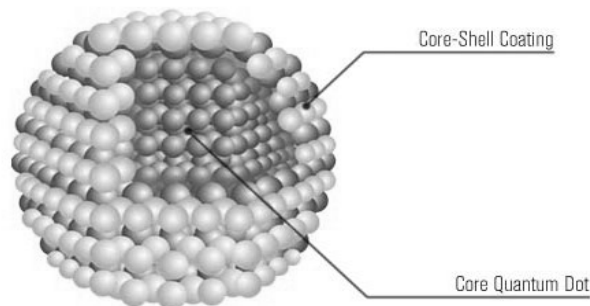


Figure 17: Illustration of QD (www.evidenttech.com)

solution and also produces a substantial improvement in the fluorescence yield.

There have been many reports using QDs for labeling cells, live embryos, tumor cells, antibodies, proteins or DNA (Srinivasan C. et al., 2006), however, their potential toxic effects have recently become a topic of considerable important and discussion (Gao X. et al., 2005, Chang E. et al., 2006). For this reason, some studies have modified the surface of QDs with polymers like chitosan enhancing biocompatibility over their nonencapsulated counterparts (Tan B.W. et al. 2005). Other studies have reported the possibility to incorporate QDs in different kinds of microspheres for both fundamental studies on light and biological tags (Lee J. et al., 2003, Sheng W. et al., 2006, Chu M. et al., 2006 and Artemyev M. et al. 2001).

3.5. Oral particulate delivery

Oral delivery is by far the easiest and most convenient way for drug delivery, especially when repeated administration is necessary. Despite these advantages many drugs, such MGA are not administered orally due to their low bioavailability.

Absorption of particulates in the intestine following oral administration is currently thought to occur with three possible mechanisms: a) by paracelular passage for particles in the micron size range, b) by endocytosis for particles in the nano size range, and c) by transcytosis at the intestinal lymphatic tissues (Peyers'path M cells) where larger particles (several microns) are absorbed exclusively. Aside the particle size, the nature and surface characteristics of the particles affect particle uptake as well (Chen H. and Langer R., 1998).

To improve the particle absorption efficiency are used generally two strategies, first, target delivery systems using specific intestinal ligands and second, mucoadhesive delivery systems constituted by polymers (Vasir J.K. et al., 2003). Some examples of mucoadhesive delivery systems are the elaboration of alginate microparticles of polymyxin able to be taken up by Peyers' path M cells and improve oral bioavailability (Coppi G. et al., 2004) or lipid nanoparticles coated with chitosan for the oral administration of peptide drugs (Garcia-Fuentes M., 2005).

It is noteworthy, the possibility to associate drugs with carrier systems to improve oral absorption such as cyclodextrins, which are cyclic oligosaccharides composed of glucose units joined through α -1,4 glucosidic bonds. Topologically this molecule can be represented by a toroid (in mathematics, a toroid is a doughnut-shaped object and its surface as a torus). No hydroxyl group is present within the toroid cavity which, accordingly, has a pronounced hydrophobic character (figure 18). As a consequence, the ability of the cyclodextrin to form inclusion complexes in aqueous solution derives from its cavity, the interior of which is less polar than water.

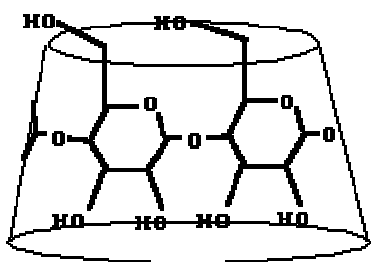


Figure 18: schematic illustration of β -cyclodextrin.

It has been reported that β -cyclodextrin (seven sugar ring molecules) forms a complex with MGA (through hydrogen bonds with the hydrophilic outer surface of the cyclodextrine molecule) which shows effectiveness in an experimental model of cutaneous leishmaniosis if it is administered orally (Demicheli C. et al., 2004). When MGA or its complex with β -cyclodextrin were orally administered to mice at 100 mg of Sb/kg, the antimony concentrations were found to be about three times higher for the association compound than for MGA. Moreover, when the lesions in mice are controlled it can be seen as the effectiveness of the complex by the oral route was equivalent to that of MGA given parenterally at a twofold-higher antimony dose. The same complex, given orally as daily doses of 32 mg of Sb/kg, reduced significantly the number of parasites in the lesions compared to saline ($p < 0,001$) (Demicheli C. et al., 2004). Next studies have observed that during the preparation of the complex MGA- β -cyclodextrine, the heating of the MGA at 55 °C was found to promote the dissociation of MGA into 1:1 Sb-MGA complex and this dissociation improve the oral absorption of the drug (figure 19) (Martins P.S. et al.,

2006). Some suggestions exist that the heating of the MGA solution before administration may be an effective means to improve the oral bioavailability of Sb.

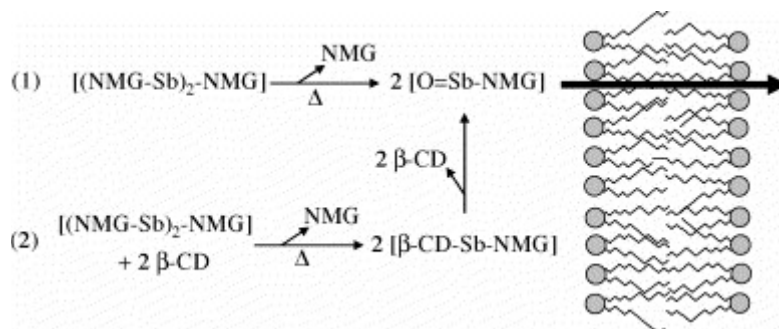


Figure 19: Proposed model for the effect of heating of MGA, in the absence (1) or presence (2) of β -CD, and its impact on the permeation of Sb(V) across biological membranes (Martins P.S. et al., 2006).

The last recent study related to this complex indicates that the freeze-drying process (second step of preparation of MGA/ β -cyclodextrine composition) is required for achieving a high absorption of Sb by oral route because the process promotes the formation of supramolecular nanoassemblies (Frézard F. et al., 2007).

4. MICROENCAPSULATION OF DRUGS

4.1. Introduction

Microencapsulation of drugs, from a technological point of view, is a process in which drugs, under molecular form, solid particles or liquid drops, are surrounded or enveloped by a coating to give particles in micron size range. The product resulted of this process is named “microparticles”, “microcapsules” or “microspheres” according to their morphology and internal structure. In contrast to microspheres, nanoparticles are in the size ranging between 10 and 1000 nm.

Historically, carbonless copy paper was the first marketable product to employ microcapsules. A coating of microencapsulated colourless ink is applied to the top sheet of paper, and a developer is applied to the subsequent sheet. When pressure is applied by writing, the capsules break and the ink reacts with the developer to produce the dark colour of the copy.

The first drug microencapsulated was aspirine around the fifties with the intention to achieve a sustained release of the drug and avoidance of irritation of stomach.

Asajo Kondo asserts in *Microcapsule Processing and Technology* in 1979 that this procedure is something of an art:

“Microencapsulation is like the work of a clothing designer. He selects the pattern, cuts the cloth, and sews the garment in due consideration of the desires and age of his customer, plus the locale and climate where the garment is to be worn. By analogy, in microencapsulation, capsules are designed and prepared to meet all the requirements in due consideration of the properties of the core material, intended use of the product, and the environment of storage...”

4.2. Applications

There are almost limitless applications for microencapsulated material. Microencapsulated materials are utilized in agriculture, foods, cosmetics and fragrances, textiles, paper, paints, coatings and adhesives, printing applications, pharmaceuticals, and many other industries.

The main applications of microcapsules in routine manufacture are summarized in table 5. However, the potential applications of the microencapsulation of drugs can be grouped under the following major categories.

The first type is for **delayed release**. Delayed action is achieved by incorporation of special coating, such as an enteric coating. Other purposes of such treatment are the prevention of side effects related to the presence of the drug in the stomach and protection of the drug from degradation in the highly acidic environment of the stomach (figure 20).

A second application is **sustained release**. Such microparticles provide gradual release of drug in amounts sufficient to maintain therapeutic response for a specific extended period of time. The major advantage is the reduction in frequency of administration and avoidance of peak and valley effects in drug blood level (figure 20).

A third category is for obtaining **control release**. As has been mentioned previously, this application has become increasingly important in the development of methods of

targeting microencapsulated drugs to particular body sites or organs (Donbrow M., 1992). Some of the controlled release microsphere formulations approved by USA FDA are : Lupron Depot[®] (leuprolide acetate for depot suspension), Sandostatin[®] LAR Depot (octreotide acetate for injectable suspension), Nutropin Depot[®] (somatotropin for injectable suspension) (Burgess D.J. et al., 2002).

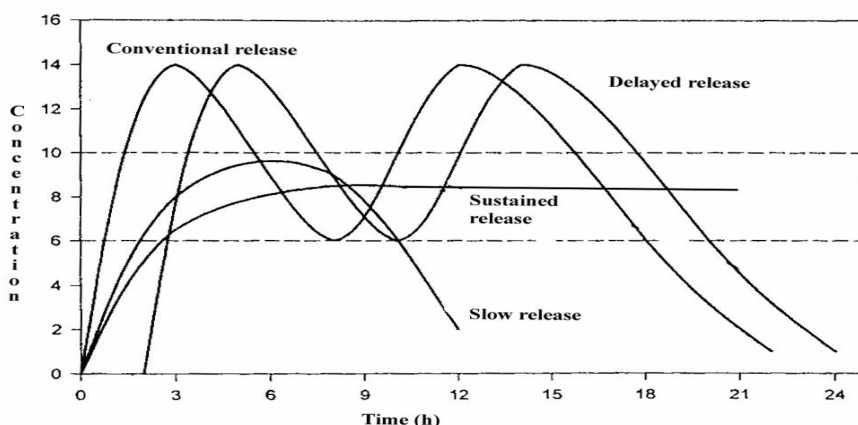


Figure 20: Schematic illustration of plasmatic levels obtained by different release systems.

Purpose	Applications
Taste masking	Fish oils, salts, alkaloids, clofibrate, sulfa-drugs
<u>Drug instability for:</u>	
Storage	Sensitivity to O ₂ , H ₂ O, volatility (vitamins, aspirin, volatile flavours)
Formula components	Isolation from excipients, buffers, other drugs
Digestive juices	Degradables (proteins, enzymes, esters, erythromycin)
Body defenses	Artificial cells (proteins, peptides, enzymes, charcoal)
Isolation from tissues	Irritants, ulcerants (aspirin, KCl)
Dry handling (better mixing and flow)	Liquids; soft, sticky solids (oils, flavours, vitamin A, perfumes)
Sustained and controlled release	Many drugs and agents (coatings: inert, pH-dependent, degradable, permeable or impermeable to ions and buffer agents)
Targeted delivery	Drugs of low therapeutic index or high systemic toxicity (e.g. cytotoxic drugs) in small microcapsules and nanoparticles.
Biotechnology	Diagnosis aids (thermography, radioimmunoassays, biosynthesis (insulin, monoclonal antibodies)

Table 5: Applications of microencapsulation (Donbrow M., 1992).

4.3. Routes and modes of administration

It is noteworthy that microspheres can be for themselves a pharmaceutical dosage form or be included in a secondary pharmaceutical dosage form. Normally, targeted products are delivered parenterally or by infusion or implantation, and hence require sterilization. They are prepared by adapting standard pharmaceutical procedures for sterilizing

solutions, suspensions, semisolids, or solid products according to the stability of the medium used. Careful particle-size control, however, is needed. Larger particles can cause capillary blockage when injected intravenously (Burgess D.J., 2002). Where the final product can not be sterilized by thermal, chemical, or radiation methods, or where these introduce toxic materials, aseptic conditions are needed during microspheres manufacturing and raw materials must be sterile, which can impose severe problems (Donbrow M., 1992).

Intranasal (Martinac A. et al., 2005), intraocular (Gavini E. et al., 2004), and inhalation routes (Yang M. et al., 2007) are also of interest with smaller microparticles and nanoparticles.

For oral products, microencapsulated drugs can be administered in hard gelatine capsules, which may also be enteric-coated, or alternatively as stabilized suspensions in liquids or soft capsules. Another possibility would be make tableted microcapsules (Hansen T. et al., 2004).

4.4. Methods of microencapsulation

Nowadays, more than hundred of microencapsulation processes exist which are usually categorized into two groups: chemical processes and mechanical or physical processes, some of them can be see in the table 6.

Chemical processes	Coacervation (separation of phases)	In aqueous phase (lipophilic drugs and hydrophilic polymers): -Simple coacervation -Complex coacervation In organic phase (hydrophilic drugs and polymers soluble in organic solvents): -by change of temperature -by addition of “no solvent” -by incompatibility of polymer
	Polymerization methods	Interfacial polymerization (IFP) In situ polymerization
Mechanical processes	Solvent evaporation technique Fluid bed coating Pan coating Supercritical fluid (SCF) Spray drying Spray-Freeze-drying (SFD)	

Table 6: Methods of microencapsulation

“**Coacervation**“ term was introduced by Bungeberg de Jong and Kruyt in 1929 to describe macromolecular aggregates or separation of liquid phases in aqueous solutions where, at least one of the phases contained a hydrocolloide.

If one starts with a solution of a colloid in an appropriate solvent (**a**), then according to the nature of the colloid, various changes can bring about a reduction of the solubility of the colloid. Coacervation may be initiated in a number of different ways. Examples are changing the temperature, changing the pH or adding a second substance such as a concentrated aqueous ionic salt solution, other polymer or a non-solvent.

As a result of this reduction a large part of the colloid can be separated out into a new phase. The original one phase system becomes two phases (**b**). One is rich and the other is poor in colloid concentration. The colloid-rich phase in a dispersed state appears as amorphous liquid droplets called coacervate droplets. Upon standing these coalesce into one clear homogenous colloid-rich liquid layer, known as the coacervate layer which can be deposited so as to produce the wall material of the resultant capsules (**c**).

As the coacervate forms, it must wet the suspended core particles or core droplets and coalesce into a continuous coating for the process of microencapsulation to occur (**d**). The final step for microencapsulation is the hardening of the coacervate wall and the isolation of the microcapsules, usually the most difficult step in the total process (**e**) (figure 21).

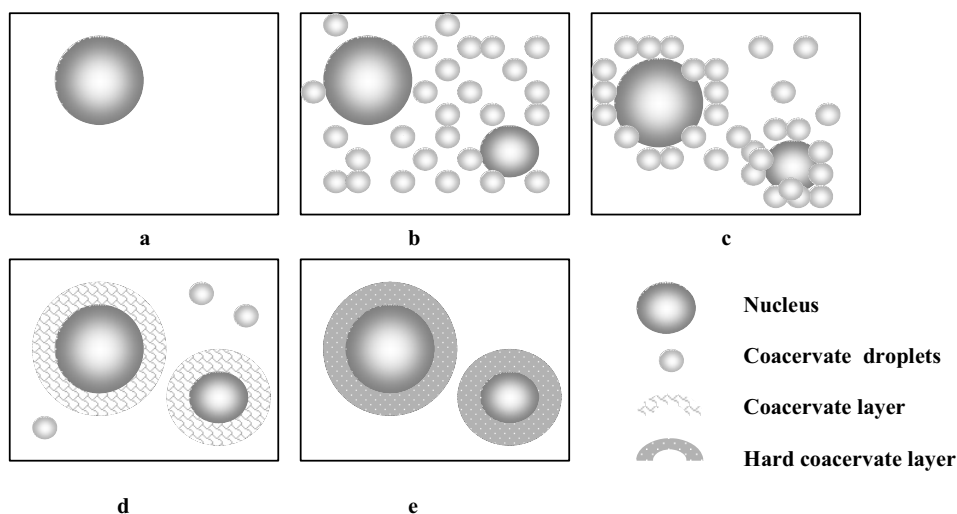


Figure 21: Schematic illustration of microencapsulation by coacervation process.

Simple coacervation only involves the ionization of a polymer, generally gelatine. Coacervation may be initiated adding a non solvent such as ethanol, acetone, dioxane, isopropanol and propanol or an electrolyte such as an inorganic salt.

Complex coacervation can be induced in systems having two dispersed hydrophilic colloids of opposite electric charges. Neutralization of the overall positive charges on one of the colloids by the negative charge on the other is used to bring about separation of the polymer-rich complex coacervate phase. The gelatin-gum arabic (gum acacia) system is the most studied complex coacervation system. Complex coacervation is possible only at pH values below the isoelectric point of gelatin. It is at these pH values that gelatin becomes positively charged, but gum arabic continues to be negatively charged (Donbrow M., 1992, Faulí C., 1993, Thomasin C. et al., 1998).

Interfacial polymerization (IFP) is another chemical method of microencapsulation. This technique is characterized by wall formation via the rapid polymerization of monomers at the surface of the droplets or particles of dispersed core material. A multifunctional monomer is dissolved in the core material, and this solution is dispersed in an aqueous phase. A reactant to the monomer is added to the aqueous phase, and a quickly polymerization is produced at the surfaces of the core droplets, forming the capsule walls (figure 22). The distinguishing characteristic of **in situ polymerization** is that no reactants are included in the core material (Freiberg S. and Zhu X., 2004).

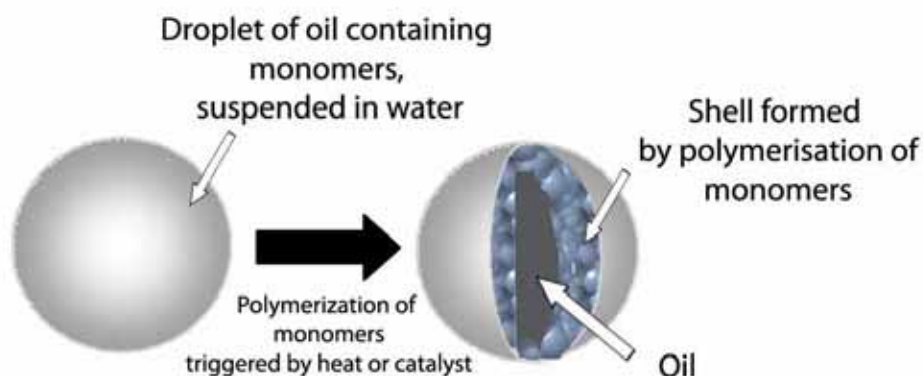


Figure 22: Schematic illustration of microencapsulation of oil by interfacial polymerization. Monomers are added into the oil, which is then stirred in water to form an emulsion of oil micro-droplets. The monomers are then made to join together into a solid polymer by heating, or by adding a catalyst, and they do so at the droplet's surface to form a microscopic shell enclosing the oil.

Solvent evaporation technique (or double emulsion technique) is the most extensively used method of microencapsulation, first described by Ogawa in 1988. It is based on the evaporation of the internal phase of an emulsion by agitation. A buffered or plain aqueous solution of the drug (may contain a viscosity building or stabilising agent) is added to an organic phase consisting of the polymer solution in solvents like dichloromethane (or ethyl acetate or chloroform) with vigorous stirring to form the primary water in oil emulsion. This emulsion is then added to a large volume of water containing an esurfactant like PVA or PVP to form the multiple emulsion (w/o/w). The double emulsion, so formed, is then subjected to stirring until most of the organic solvent evaporates, leaving solid microspheres in suspension in the continuous phase (figure 23). The microspheres can be recovered by filtration or centrifugation, washed and dried (Benita S., 1996, Lamprecht A., et al., 2000, Freiberg S. and Zhu X., 2004,).

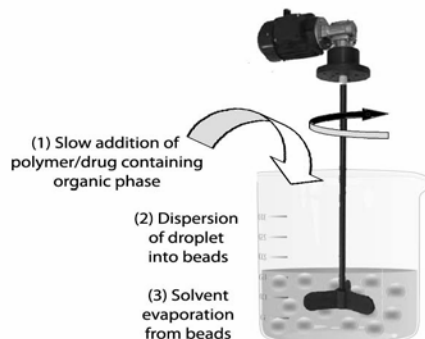


Figure 23: Depiction of sphere formation by solvent evaporation. A solvent-polymer droplet disperses inside the continuous phase forming solvent-polymer spheres; the sphere hardens as the organic solvent evaporates.

Fluid bed coating is a mechanical encapsulation method restricted to encapsulation of solid core materials, including liquids absorbed into porous solids. Solid particles to be encapsulated are suspended on a jet of air and then covered by a spray of liquid coating material. The capsules are then moved to an area where their shells are solidified by cooling or solvent vaporization. The process of suspending, spraying, and cooling is repeated until the capsules' walls are of the desired thickness. This process is known as the **Wurster process** when the spray nozzle is located at the bottom of the fluidized bed of particles (Knezevic Z., et al., 1998) and **top spray fluid bed coating** when the coating liquid is sprayed down on to the particles from above.

In **pan coating**, solid particles are mixed with a dry coating material and the temperature is raised so that the coating material melts and encloses the core particles, and then is solidified by cooling; or, the coating material can be gradually applied to

core particles tumbling in a vessel rather than being wholly mixed with the core particles from the start of encapsulation (Woodard et al., 1994).

Microencapsulation process using **supercritical fluids** comprises a method for microencapsulating a core material comprising the steps of a) mixing a core material with an encapsulating polymer, b) supplying a supercritical fluid capable of swelling the polymer to the mixture under a temperature and a pressure sufficient to maintain the fluid in a supercritical state, c) allowing the supercritical fluid to penetrate and liquefy the polymer while maintaining temperature and pressure sufficient to maintain the fluid in a supercritical state, and d) rapidly releasing the pressure to solidify the polymer around the core material to form a microcapsule. This method requires neither that the polymer nor core materials to be soluble in the supercritical fluid and can be used to rapidly and efficiently microencapsulate a variety of materials for a variety of applications (figure 24) (Gelb J. Jr., 1998, Kompella U.B. and Koushik K., 2001).

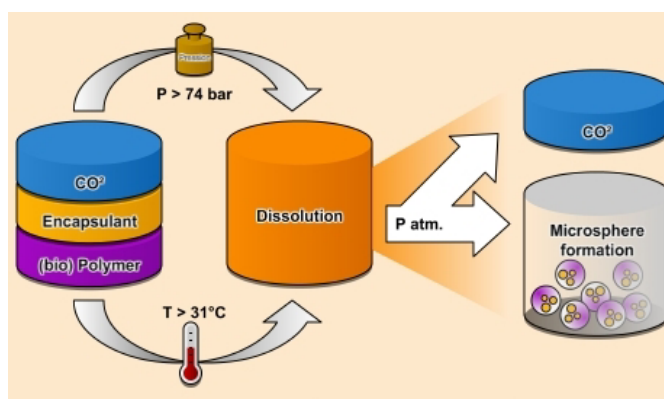


Figure 24: Microsphere formation and encapsulation using supercritical fluids by “Rapid Expansion of Supercritical Solutions (RESS)” (www.gate2tech.com/).

Microencapsulation by **spray drying** is based in the evaporation of moisture from an atomised feed (aqueous or organic solutions, emulsion, or suspension) by mixing the spray and the drying medium. The drying medium is typically air. The drying proceeds until the desired moisture content is reached in the sprayed particles and the product is then separated from the air. This technique will be explained in detail in the experimental section.

Spray-freeze-drying method (SFD) combines processing steps common to freeze-drying and spray drying. It consists in the dissolution of the drug and nebulised it into a cryogenic medium (e.g. liquid nitrogen) which generates a dispersion of shocked-frozen

droplets. The dispersion is then dried in a lyophilizer (Maa Y.F. et al., 1999, Heinzelmann K., et al., 1999).

4.5. Materials used in microencapsulation

The materials used in microencapsulation can be mainly classified in three categories: waxes, proteins and polymers. Some examples are shown in the table 7. Polymers are the most utilized in microencapsulation and their variety is almost infinite. They started to be used for controlled release in 1960s through the employment of silicone rubber by Folkman and Long in 1964 and polyethylene by Desay in 1965. These polymers were not degradable in the systems so that they had to be surgical removed and it limited their applicability. For this reason, in 1970s biodegradable polymers started to be considered a good alternative for drug delivery.

Materials	Examples	
Waxes	Carnauba wax Stearilic alcohol Stearic acid Gelucires®	
Proteins	Gelatine Albumine Collagen	
Polymers	Naturals	Polysaccharides (animal or vegetal): -Agar -Alginates -Dextrane -Arabic gum -Chitosan -Starch
	Semisynthetic	Cellulose derivatives -insolubles: ethylcellulose, cellulose acetobutyrate -pH dependent: cellulose acetophthalate -hydroxipropilmetilcellulose (HPMC)
	Synthetic	Acrylic derivatives Polyvinylpyrrolidone (PVP) N-vinyl-2-pyrrolidone (Crospovidone) Polyethylene glycol (PEG) Polyesters: polycaprolactone, polylactic acid, poly(lactic-co-glycolic acid).

Table 7: Some examples of materials utilized in microencapsulation.

4.6. In vitro release testing of microsphere drug delivery systems

4.6.1. Introduction

Although several microsphere formulations are in market, there is no standardized and validated *in vitro* release technique that would predict the *in vivo* behaviour of the formulation. Various methods are being used for *in vitro* analysis of microspheres but in most cases *in vitro* release data does not correlate with the *in vivo* results. An appropriate *in vitro* method is very important for: (1) quality control of microspheres i.e. to monitor batch to batch variability, (2) assessment of *in vivo* performance of the formulation (3) preliminary stages of product development. For designing an appropriate *in vitro* analysis method, selection of temperature and media is important. Temperature and pH are usually maintained at physiological values (i.e 37°C and pH 7,4). Another important parameter is maintenance of sink conditions, if not might underestimate the release from any drug delivery system considerably (D'Souza S.S. and De Luca P., 2006). Typically, sink conditions are considered to exist if, at the dissolution of 100 % of the highest strength of the product to be tested, a concentration of not more than $\frac{1}{3}$ of saturation will be achieved.

4.6.2. In vitro release methods for microparticulates

The most commonly used methods for microparticulate systems can be grouped into three categories: (1) sample and separate method; (2) flow through cells; and (3) dialysis.

With the **sample and separate technique**, drug-loaded microparticles are introduced into a vessel, and release is monitored over time by analysis of supernatant or drug remaining in the microspheres. This is the most widely used method for *in vitro* release testing of microspheres but aggregation of microspheres and loss during sampling results in lower release rates (D'Souza S.S. and De Luca P., 2006).

In the **flow-through cell** technique, media is continuously circulated through a column containing drug-loaded microparticles followed by analysis of the eluent. Most of the studies reported are developed using in-house modifications of the USP apparatus 4,

however automated equipment is commercially available (Sotax AG (Basel, Switzerland)). A schematic diagram of USP apparatus 4 is shown in the figure 25. This method attempts to simulate the *in vivo* environment by constantly circulating a small volume of media through immobilized microparticles to hydrate the particles and cause dissolution and diffusion of the drug. A major advantage of this method is that samples can be continuously and conveniently sampled and analyzed along with buffer replacement because of the automated process. Disadvantages with this procedure include variation in the flow rate due to clogging of the filter (because of polymer degradation) leading to high-pressure buildup in the system. Also, low flow rates are achieved with the types of filters used (membrane and ultrafilters) and seem to be responsible for low rate and extent of drug release from microsphere formulations. Lastly, rapid replacement of the buffer is difficult to achieve in practice (D'Souza S.S. and De Luca P., 2006). The 2003 AAPS-EUFEPS workshop report recommended the use of USP Apparatus 4 for *in vitro* release testing of microspheres (Zolnik B.S. et al., 2005).

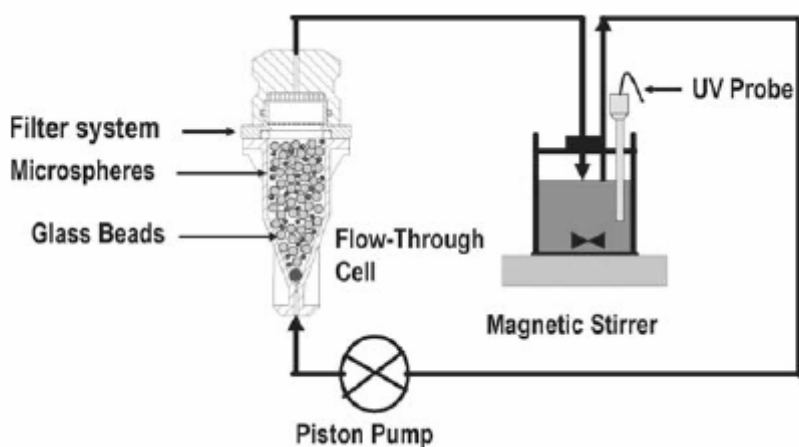


Figure 25: Schematic diagram of USP apparatus 4 showing the placement of the fibre optic probe in the reservoir vessel (Zolnik B.S. et al., 2005).

The **dialysis** method achieves a physical separation of the drug-loaded microparticles from the release media by use of a membrane, which allows for sampling without interference of the microspheres. The membrane should have molecular weight cut off that allows drug molecules to permeate. Most commonly, a dialysis bag containing the suspension of microspheres is used and placed in a vessel containing buffer. Drug diffusion from the dialysis bag into the outer sink may be increased by agitating the

vessel contents, thereby minimizing unstirred water layer effects. Common modes of agitation include a horizontal shaker or using the USP paddle apparatus (BP Apparatus II) under agitation. Other setup includes a tube with dialysing membrane at one end (figure 26) or two chambers separated by a dialysis membrane. Most studies comparing the dialysis technique with the tube method show differences in release rate and profile due to the total volume of media in the dialysis method was much larger than with the tube method so that dialysis bags simulated the *in vivo* environment and retained sink conditions better than the tube method (D'Souza S.S. and De Luca P., 2006). Although dialysis technique (without stirring and if membrane surface is small) has been criticized because of its low *in vivo* predictability in the case of intravenous or oral administration of microparticles, it mimics *in vivo* conditions where the microparticles are immobilized upon administration (subcutaneously or intramuscularly) and surrounded by a stagnant layer causing slow diffusion of drug because sink conditions are not maintained. Achievement of equilibrium with the outer media is slow and would limit and accurate analysis of initial drug levels in formulations where the burst effect is high. Nevertheless, this issue is addressed by using dialysis bags (more surface area) and agitating the outer media to minimize unstirred water layer effects and to prevent accumulation of polymer degradation products. However, because of the ease of sampling and the possibility of total buffer replacement, this method seems to be an attractive option to study drug release from microparticles (D'Souza S.S. and De Luca P., 2006).

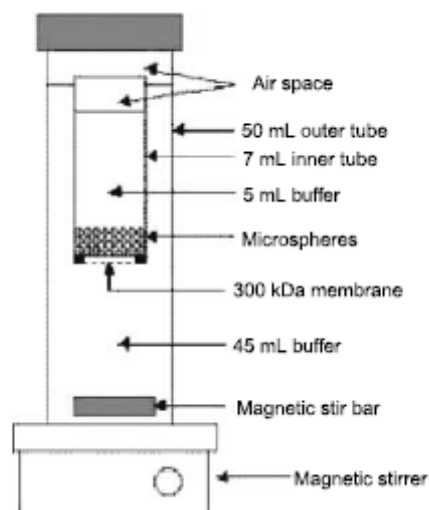


Figure 26: Dialysis method utilizing membrane at one end (D'Souza S.S. and De Luca P., 2006)

Among some *in vitro* drug release studies of chitosan microspheres reported, various *in vitro* release methods have been used. The rate of release of the model drugs from chitosan microspheres was determined in a dissolution apparatus with the dissolution paddle assembly (BP Apparatus II) where 30-50 mg of microspheres were suspended in 200-300 ml of phosphate buffer saline (PBS), pH=7,4 at 37 °C and at 50 rpm agitation rate (He P. et al., 1999a, Dini E. et al., 2003), or with the basket assembly (BP

Apparatus I) where microspheres were sealed in hard gelatine capsules and released in same conditions (He P. et al., 1999b). The content of betamethasone is determined dispersing microspheres in methanol by vortex and analysing the surface drug content in the supernatant by HPLC. Following, the residual of microspheres was dissolved in 0,1% (w/v) acetic acid solution and neutralized for measuring inner core drug content (Huang Y.C. et al., 2002). Dissolution study of chitosan microspheres of diclofenac sodium and acetaminophen was carried out taking microspheres in dialysis bag and attached to the paddles of the dissolution tester at 100 rpm, taking 900 ml of 7,4 pH PBS at 37 °C (Kumbar S.G. et al., 2002, Desai K.G. and Park H.J., 2006). Similarly, release of vitamin C is studied but at 25 °C (Desai K.G. and Park H.J., 2006). A simple method is used to study the release of insulin which consists in the incubation of microspheres in 5 ml of PBS with horizontal shaking at 37 °C (Grenha A. et al., 2005).

4.6.3. Drug release mechanism from microparticulates

In majority of cases, drug release from microparticulates follows more than one type of mechanism. In case of the release of drugs from chitosan particulate systems, it involves three different mechanisms: a) release from the surface of particles (this type of drug release leads to “burst effect”), b) diffusion through the swollen rubbery matrix and c) release due to polymer erosion. These mechanisms are shown schematically in figure 27 (Agnihotri S.A. et al., 2004).

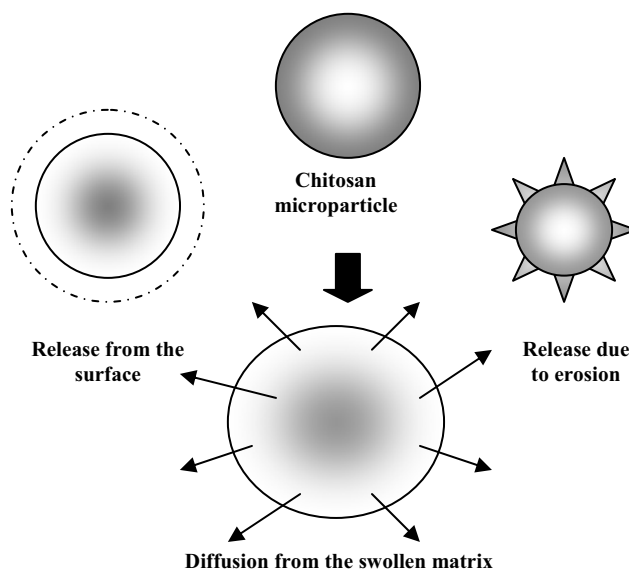


Figure 27: Drug release mechanisms of chitosan microparticles

The modeling of drug release from delivery systems is important for the understanding and the elucidation of the transport mechanisms. Different mathematical models may be applied for describing the kinetics of the drug-release process from microspheres, the most suited being the one which best fits the experimental results. Basically, the mathematical expressions used to describe the kinetics of drug release and the discernment of the release mechanisms are the Higuchi law (Higuchi T., 1961, 1963) and the Korsmeyer-Peppas equation (Korsmeyer R.W. et al., 1983, Ritger P.L. and Peppas N.A., 1987a,b).

The first approach relies on equation (1), which indicates that the fraction of drug released is proportional to the square root of time:

$$Mt/M_{\infty} = K_H t^{1/2} \quad (1)$$

where k_H is the Higuchi's rate constant, and Mt and M_{∞} are cumulative amounts of drug released at time t and infinite time, respectively. If a plot of square root of time versus cumulative amount of drug released yields a straight line, and the slope is one or more than one, then the particular dosage form is considered to follow Higuchi kinetics of drug release.

Under some experimental situations the release mechanism deviates from the Fick's equation, following an anomalous behavior (non-Fickian release). In these cases a more generic equation (second approach), based on the semi-empirical equation, can be used (2):

$$Mt/M_{\infty} = K_K t^n \quad (2)$$

where M_t/M_{∞} is the fraction of drug released at time t ; K_K is a constant comprising the structural and geometric characteristics of the microsphere; and n , the release exponent, is a parameter that depends on the release mechanism and is thus used to characterize it.

Peppas used this n value in order to characterize different release mechanisms. If the n value is 0.5 or less, the release mechanism follows Fickian diffusion (so that equation 2 collapses to equation 1), and higher values $0.5 < n < 1$ for mass transfer follow a non-fickian model (anomalous transport) (Mathew S.T. et al., 2007).

The drug release follows zero-order drug release and case-II transport if the n value is 1 (the drug release rate is independent of time).

For the values of n higher than 1, the mechanism of drug release is regarded as super case-II transport. This model is used to analyze the release of pharmaceutical polymeric dosage forms when the release mechanism is not well known or when more than one type of release phenomena was involved.

Both equations (1) and (2) are short time approximations of complex exact relationships and therefore their use is confined for the description of the first 60% of the release curve.

Another alternative for the description of release profiles is based on the empirical use of the Weibull function (3).

$$M_t = M_\infty \cdot [1 - e^{-(t-t_0)/td}^\beta] \quad (3)$$

where td and β are constants. Although the Weibull function has been used empirically for the analysis of release, a link between the values of β and the diffusional mechanisms of the release has been reported (Papadopoulou V. et al., 2005). For values of β lower than 0,75 the release follows Fickian diffusion either in Euclidian ($0,69 < \beta < 0,75$) or fractal space ($\beta < 0,69$). Values of β in the range 0,75–1,0 indicate a combined mechanism. The specific case of $\beta = 1$ is compatible with first-order release, whereas the concentration gradient in the dissolution medium drives the rate of release. Finally, when $\beta > 1$ the sigmoid shape of the Weibull function indicates that a complex mechanism governs the release process (Papadopoulou V. et al., 2005).

